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The long-term objective of this application was to determine the cellular and molecular mechanisms responsible for increased aromatase expression in breast adipose fibroblasts proximal to malignant epithelial cells. Our results are supportive of the following hypothesis: Regional differences in relative proportions of histological components of the breast adipose tissue (*e.g.*, fibroblasts vs. mature adipocytes) may be the primary cause of estrogenic concentration gradients, since regions containing higher numbers of fibroblasts are the sites of increased aromatase expression and increased rate of tumor development. Although the initiating events are unknown, malignant cells in the regions displaying higher aromatase expression are more likely to proliferate. Secretory products of the tumor stimulated by estrogen may in turn further increase aromatase expression in the surrounding adipose tissue. These products will additionally stimulate proliferation of aromatase-expressing fibroblasts to generate a fibrous capsule around the tumor, *i.e.*, desmoplastic reaction. Estrogens will continue to positively influence neoplastic growth by increasing the expression of secretory products and their receptors in the tumor tissue. Thus, a positive feed-back loop is established in which locally-produced estrogen and tumor-derived factors (*e.g.*, cytokines, growth factors and prostaglandins) act by paracrine and autocrine mechanisms to sustain the growth and development of the tumor.

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Serdar E. Bulun 10/8/98

PI - Signature Date

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5. INTRODUCTION

This Final Report was prepared as a requirement of the Career Development Award entitled "Breast Cancer and Estrogen Biosynthesis in Adipose Tissue" funded by the US Army Medical Research and Materiel Command Breast Cancer Research Program. This report covers research for the period 9/12/94-9/11/98. The long term goal of this Application is to characterize the molecular and cellular mechanisms responsible for estrogen synthesis in adipose tissue surrounding a breast tumor. Since aromatase P450 (P450arom) catalyzes the conversion of C_{19} steroids to estrogens, our studies focus on the expression of P450arom in adipose fibroblasts in relation to a tumor. During preliminary studies, we have detected the highest levels of adipose tissue P450arom transcripts in breast quadrants bearing a tumor compared with tumor-free quadrants.

The grant proposal includes four specific aims: The **first aim** is to quantify adipose tissue P450arom transcript levels in an additional number of mastectomy specimens at various distances from the tumor using a novel quantitative RT-PCR method. We also proposed to quantify these transcripts in breast samples of women undergoing reduction mammoplasty to ascertain the distribution of aromatase expression. This aim also included determining the ratio of fibroblasts, the aromatase-expressing cell type, to mature adipocytes in these tissue samples. This specific aim has been fully accomplished now and will be detailed in **6. BODY**. The **second specific aim** included determination of the promoters that are used to express aromatase in adipose tissue samples proximal to a tumor. This involved, initially, development of a novel quantitative RT-PCR method to simultaneously determine untranslated 5'-ends of P450arom transcripts in adipose tissue samples using 4 different internal standard complementary (c) RNAs. First, we developed this technology and published it in full detail. We then applied this novel method to breast adipose tissue samples from patients with cancer and disease-free women and found that two distinct aromatase promoters (II and I.3) are preferentially used in the breast bearing a tumor in a specific fashion under the control of a cAMP-dependent signaling pathway, whereas another promoter (I.4) that is induced by glucocorticoids and cytokines is primarily used for aromatase expression in the breast fat of disease-free women. Thus, this specific aim has also been accomplished, and the results are detailed in **6. BODY**. **Specific Aim 3** involved characterization of novel 5'-ends of P450arom transcripts in breast cancer tissues. This required using rapid amplification of 5'-cDNA ends (5'-RACE) and sequencing to identify novel P450arom promoter regions, which have not been previously described. We identified a novel untranslated exon in total RNA from four tumor samples and also in adipose tissue samples from the breast, abdomen and buttock. We are still in the process of characterizing this potential promoter region. These data are strongly suggestive of another promoter region responsible for aromatase expression in the breast cancer and adipose tissue. Our progress is detailed **6. BODY**. Finally, the **fourth specific aim** is to determine whether secretory factors of breast cancer cells induce aromatase expression in the surrounding adipose tissue and to characterize such factors. In this regard, we have demonstrated that interleukin (IL)-6, IL-11 oncostatin M (OSM) and leukemia inhibitory factor (LIF) induce aromatase expression in breast adipose fibroblasts in primary culture in the presence of glucocorticoids. Then, the expression of these cytokines was

characterized in breast cancer tissues and cell lines. This specific aim has been accomplished, and our results are presented in **6. BODY**. By further studies not included in this Application, we realized that the primary role of these cytokines is not inducing aromatase expression in breast tumors but to mediate the accumulation of adipose-derived fibroblasts in and around the tumor, the so-called "desmoplastic reaction". These studies regarding the cancer effect on adipocyte differentiation are in progress.

6. BODY

I. We accomplished the goals proposed in **Specific Aim 1**. In addition to 15 mastectomy and 9 reduction mammoplasty samples used for obtaining the preliminary results, we determined the levels of P450arom transcripts in the quadrants of 18 more mastectomy specimens bearing carcinomas and 11 more reduction mammoplasty specimens from disease-free patients (1,2). We had originally proposed to obtain samples from arbitrarily 40 patients in each group. Levels of statistical significance achieved by 18 mastectomy and 11 mammoplasty samples, however, were satisfactory; thus, this obviated testing more samples. Additionally, we performed morphometry to determine the histological composition of adipose tissue, in particular, the ratio of adipose fibroblasts to mature adipocytes in the breast in comparison with adipose tissue from other body sites and published these results in two separate papers (2,3). These studies in reference to **Specific Aim 1** are detailed as follows:

The highest levels of P450arom transcripts were found in the tumor tissues and adipose tissue samples from tumor-bearing quadrants (4). From 18 patients, breast adipose tissue was sampled from four quadrants. Tumor tissue was also obtained. Thus 5 samples were obtained from each patient. Total RNA was extracted, and levels of P450arom transcripts were determined by amplifying a sequence in the common coding region using a quantitative RT-PCR method. In adipose tissue from the tumor-bearing quadrant, P450arom transcript levels were significantly higher ($p=0.018$) in comparison with levels found in tissue distal to the tumor (average of three nontumor-bearing quadrants). The levels of P450arom transcripts in the tumor *per se* were similar to those found in the adipose tissue from the tumor-bearing quadrant. Moreover, total transcript levels were higher in breast adipose tissue of cancer patients in comparison with those of cancer-free individuals, even when the adipose tissue from the cancer patient was taken from a quadrant with no detectable tumor.

***In disease-free breasts, the highest fibroblast-to-adipocyte ratios and P450arom transcript levels were found in the outer regions* (2).** We determined distribution of P450arom mRNA levels and fibroblast to adipocyte ratios in disease-free breasts of premenopausal women between the ages of 19 and 42. Adipose tissue samples were collected from 3 regions of both breasts in 13 women undergoing reduction mammoplasty. Samples were taken from midportions of outer and inner regions, as well as from the midline above the nipple (designated as upper). Mode of sampling was dictated by the technique of surgery. Total RNA was isolated ($n=11$), and an hematoxylin and eosin-stained section was prepared ($n=12$) from the same sample from each region of both breasts. Overall, 67% of the highest fibroblast to adipocyte ratios and 64% of the highest P450arom transcript levels were detected in an outer breast region, whereas in only one patient, the highest values were detected in an inner region. Parametric analysis of variance (ANOVA) showed significant differences between the fibroblast content of the regions [$p(F)=0.037$]. This distribution pattern directly correlates with the most common or the least common sites of carcinoma in the breast in large series, which are the outer and inner regions, respectively. (In 11 of 15 patients of our previous study (5) and 10 of 12 patients in O'Neill's study (6), the tumors were found

in a lateral quadrant.) Moreover, a direct relationship was demonstrated between adipose fibroblasts and P450arom transcripts within the breast, in that regions with the highest fibroblast to adipocyte ratios contained the highest P450arom transcript levels (ANOVA of contrast variables, p=0.0009). This suggests that, similar to our previous findings in the breast bearing a tumor, adipose tissue aromatase expression in the disease-free breast is also determined by the local ratio of fibroblasts to adipocytes. We further conclude that since breast cancer occurs in regions of the breast with the highest levels of aromatase expression, the presence of high fibroblast content and P450arom transcript levels in the outer region of the disease-free breast may be of pathophysiologic significance in the development of breast cancer.

Histological characterization of adipose tissue from various body sites of women using morphometry. Adipose tissue is the primary site of estrogen biosynthesis in postmenopausal women. The two main histologic components of adipose tissue are mature adipocytes and fibroblasts. Aromatase P450 (P450arom) expressed in the fibroblast component of adipose tissue is responsible for catalyzing conversion of C_{19} steroids to estrogens. We have previously demonstrated that, in women, aromatase expression in adipose tissue of various body sites increases with age and that aromatase expression in the hip is markedly higher than in the abdomen. In order to determine whether this age- and regional-dependent variation in aromatase expression is due to an alteration in the ratio of fibroblasts to mature adipocytes, we collected subcutaneous adipose tissue biopsy samples from 19 women (age range: 21-93) at the time of autopsy. Using a computerized image analysis system, we determined by morphometry the proportions of adipocytes, fibroblasts and vascular endothelial cells within histological sections of adipose tissue from mid-abdomen, both breasts and both hips. The percentage of each cell component at each body site was expressed as the mean of triplicate replicates. Statistical analysis of our results did not indicate any correlation between advancing age and fibroblast to adipocyte ratios in the breast, abdomen or hip. Fibroblast to adipocyte ratios were found to be significantly higher in the breast and abdomen compared with the hip ($p<0.05$). No statistical differences were found between the breast and abdomen. These findings suggest that the increase in aromatase expression with advancing age and the higher aromatase expression in the hip compared with the abdomen in women may be due to alterations in specific signal transduction mechanisms rather than a simple increase in local adipose fibroblast numbers.

II. To accomplish the studies proposed in **Specific Aim 2**, we first developed a novel competitive RT-PCR/SDS-PAGE method to quantify promoter-specific P450arom transcripts with unique 5'-ends (7). We then applied this novel technology to human breast adipose tissue samples from mastectomy specimens bearing tumors and reduction mammoplasty samples from disease-free individuals. Thus, we have completed all of the goals in **Specific Aim 2** by determining the distribution of promoter-specific transcripts in adipose samples from tumor-bearing breast specimens, as well as in tissues of cancer-free women as controls and published these results (4). A summary is included below:

Quantification of alternatively spliced transcripts of the P450arom gene in aromatase-expressing human cells (7). Here, we describe a competitive RT-PCR/PAGE

method to quantify three major 5'-termini of P450arom transcripts, *i.e.* promoter II-specific (PII), exon I.3- and exon I.4-specific. Since placental promoter specific exon I.1 is not present in other tissues, we did not attempt to amplify exon I.1-containing transcripts in adipose tissue samples. Using this method, we were able to quantitatively detect multiple transcripts present in primary cultures of human adipose fibroblasts, adipose tissues and ovarian granulosa cells grown in different culture conditions. Most importantly, this method is applicable to quantitative analysis of alternatively spliced transcripts of other genes in which this form of regulation of expression is utilized. First, we have generated 4 internal standard cRNAs specific for 3 untranslated 5'-ends and a coding region using the "looped oligo" technique. The generated cRNAs differed from the wild type mRNA sequence by an extra 27 bp-long AT-rich region. A known copy number of these internal standards was added to each RT-PCR reaction containing target RNA from various tissues, *e.g.*, the adipose tissue. [^{32}P]-labelled dCTP was used in amplification reaction and the products were analyzed on 4% non-denaturing polyacrylamide gels. Radioactivity of bands were quantified by a PhosphorImager. Ratio of radioactivity of the smaller-size band (target mRNA) to that of the larger-size band (internal standard cRNA) was expressed as an arbitrary unit for the level of a P450arom transcript population with a promoter-specific 5'-end. Total P450arom mRNA level in each sample was also quantified using an internal standard cRNA corresponding to the coding region only. In a tissue sample, we sought to demonstrate that the levels of PII-, I.3- and I.4-specific transcripts approximately added up to the total transcript levels determined by amplifying the coding region. Therefore, we accomplished the optimization of internal standard quantities and PCR conditions.

P450arom promoters I.3 and II are primarily used in adipose tissue of the breast bearing a tumor, whereas promoter I.4 use is favored in the breast of cancer-free women (4). Three promoters (II, I.3, and I.4) are used for aromatase expression in human adipose tissue. Using the novel RT-PCR assay described in the previous paragraph, we determined whether particular alternative promoters are preferentially used for aromatase expression in relation to the presence of a breast tumor. We quantified promoter-specific P450arom transcripts using separate cRNA internal standards. In 18 mastectomy specimens, promoter use in adipose tissue samples was evaluated (4). Regardless of proximity to tumor, promoters I.3 and II (the cyclic AMP-inducible promoters) were found to be used in the breast bearing a tumor. This was observed in both adipose tissue samples, whether proximal or distal to a tumor, and in tumor tissues. On the other hand, in reduction mammoplasty samples from 9 cancer-free patients, promoter I.4 (the glucocorticoid plus cytokine-inducible promoter) was found to be primarily used for aromatase expression. We conclude that increased aromatase expression in breast adipose tissue bearing a carcinoma results from transcriptional activation of promoters I.3 and II. These two promoters are known to be used in adipose fibroblasts in response to treatment with a cAMP analogue. Therefore, it is conceivable that breast tumors produce factors that stimulate aromatase expression in stromal fibroblasts through a cAMP-dependent signaling pathway. Thus, **Specific Aim 2** was accomplished after completion of this proposed study.

III. Under **Specific Aim 3,** it was proposed to determine previously unidentified novel promoters of the P450arom gene in breast cancer tissues. To this end, we used the 5′-RACE procedure (rapid amplification of 5′-cDNA ends) and cloned a novel untranslated first exon upstream of the common splice junction of P450arom transcripts (containing the identical common coding region as in other transcripts) from a breast cancer tissue. We designed oligonucleotides to amplify this new exon in 4 breast cancer and surrounding adipose tissue samples. Upon southern hybridization of the amplified products, we estimated that the expression of this new transcript was high in cancer tissues and surrounding fat but relatively lower in breast fat distant to the tumor. Additionally, this novel exon was found in samples of abdominal and buttock fat of cancer-free subjects in levels comparable to those found in breast tumors. From a human genomic library, we recently isolated a 10-kb clone that contains this 100 bp sequence. The upstream genomic region of this novel exon was sequenced and a TATA box was identified 56 bp upstream of the transcription start site. Several regulatory elements were identified within the 600 bp region upstream of this TATA box. These include c-Myb, SP-1, AP-1, v-Myb and Ef-1 sites. We will definitively determine the transcription start site by primer extension and S1 nuclease assays and prepare deletion constructs for the transcriptional regulation studies. We will also attempt to overlap the new genomic clone with this existing genomic clones of the P450arom gene to estimate the location of the novel first exon and upstream promoter region in reference to the common splice junction. These studies will be carried out as previously proposed under Specific Aim 3.

IV. The following studies were completed and published. As indicated in **5. INTRODUCTION**, the goals included in the broad category **Specific Aim 4** have been accomplished. However, we are following our scientific instincts to continue our studies in the direction of further characterizing the regulation cytokine expression in the breast cancer. We will continue to explore the roles of cytokines (*e.g.*, IL-11 and TNF-α) in local estrogen production and peri- and intra-tumoral fibroblast proliferation, *i.e.*, the desmoplastic reaction.

Serum-free conditioned media from human breast cancer cell lines dramatically stimulate aromatase activity of glucocorticoid-treated adipose fibroblasts in monolayer culture (8). In our laboratory, dexamethasone (DEX) was previously found to increase aromatase activity in adipose fibroblasts in culture in a concentration- and time-dependent fashion (9). The presence of serum in the culture medium was mandatory for this effect of glucocorticoids to be manifest, since under serum-free conditions, the stimulatory effect of DEX was greatly reduced. We designed an experiment to determine whether serum can be replaced by secretory products of breast cancer cells in stimulating aromatase activity of DEX-treated adipose fibroblasts: the T47D breast cancer cell line was used for conditioning of serum-free, phenol red-free Waymouth's media. Separately, adipose fibroblasts in culture were maintained in serum-free Waymouth's media for 24 h. Thereafter, adipose fibroblasts were incubated with conditioned serum-free media from T47D, MCF-7, SSC-202, SSC-78 and SSC-30 breast cancer cell lines and DEX ($2.5X10^{-7}M$). Aromatase activity was measured after 24 h. Serum-free conditioned medium from breast cancer cells mimicked fetal bovine serum

(10%) to induce aromatase activity of DEX-treated adipose fibroblasts. Treatment of T47D cell with estradiol potentiated this effect in a dose-dependent fashion. Heat inactivation destroyed the stimulating ability of conditioned medium. The majority of P450arom 5′-termini expressed by adipose fibroblasts incubated with conditioned medium plus dexamethasone contained promoter I.4-specific sequence. We also observed that adding anti-IL-11 antibodies to T47D-conditioned medium abolished its stimulatory effect on aromatase expression in adipose fibroblasts (our unpublished observations). Additionally, we found that conditioned medium from adipose fibroblasts themselves was also efficacious in substituting for serum to stimulate aromatase expression.

Aromatase activity of glucocorticoid-treated adipose fibroblasts in a monolayer co-culture model was markedly stimulated after insertion of T47D cells into this system (our unpublished observations). Adipose fibroblasts and T47D breast cancer cells were co-cultured using cell culture inserts containing membranes with 0.45 μm pores. T47D cells introduced in culture inserts into the serum-free, phenol red-free co-culture system stimulated aromatase activity of glucocorticoid-treated adipose fibroblasts. In this regard, presence of T47D cells mimicked the stimulatory effect of serum on glucocorticoid-treated adipose fibroblasts. Estradiol treatment of cancer cells potentiated this stimulatory effect.

Role of the IL-6, IL-11, LIF, OSM cytokine family and a Jak/STAT pathway in regulation of the adipose-specific P450arom promoter, I.4 (10). We recently discovered that a Jak/STAT signalling pathway mediates the stimulation of expression of the P450arom gene in human adipose tissue. P450arom expression in adipose fibroblasts maintained in the presence of serum and glucocorticoids is regulated by promoter I.4 which lies at least 20 kb upstream of the translation start site. I.4 is a TATA-less promoter, which contains a GRE, an SP-1 binding site and a GAS (interferon-γ Activation Site) element. Furthermore, the stimulatory action of serum or of conditioned media can be mimicked by IL-11, LIF and OSM, as well as by IL-6, provided the IL-6 soluble receptor is also present. Stimulation of the adipose fibroblasts by these factors led to rapid phosphorylation of Jak1, but not Jak2 or Jak3, on tyrosine residues. STAT3, but not STAT1 or ISGF3, was also phosphorylated and bound to the GAS element in the I.4 promoter region. When regions of this promoter were fused upstream of the CAT reporter gene and transfected into adipose fibroblasts, mutagenesis or deletion of the GAS element led to complete loss of reporter gene expression. We conclude that stimulation of aromatase expression by members of the IL-6 cytokine subfamily through this Jak/STAT pathway appears to be a major mechanism regulating estrogen biosynthesis in postmenopausal women.

Expression of transcripts of IL-6 and related cytokines by human breast tumors, breast cancer cells, and adipose fibroblasts (11). The expression of transcripts of cytokines of the IL-6 family has been examined in human breast tumors, breast cancer cell lines, and adipose fibroblasts, by means of RT-PCR amplification. Of the six breast tumor samples examined, all expressed transcripts encoding IL-6 and LIF. Four of the samples also expressed transcripts for OSM and IL-11, and three expressed the IL-6

receptor. Adipose fibroblasts expressed IL-6, IL-11 and LIF, but not the IL-6 receptor, consistent with previous conclusions that IL-6 activity in these cells required addition of IL-6 soluble receptor. In the case of T47D cells, expression of IL-11 protein was confirmed by immunotitration. Moreover, in these cells, expression of IL-11 transcripts was induced 3-fold by addition of E_2 to the culture medium. These results add credence to our previous proposal that breast cancer development is regulated in part by local autocrine and paracrine mechanisms via epithelial/mesenchymal interactions, in which estrogen produced by stromal cells surrounding the tumor acts to stimulate the production of growth factors and cytokines by the tumor cells. Some of these may act to stimulate further the growth and development of the tumor, while these or other factors may act on the surrounding mesenchymal cells in a paracrine fashion to stimulate aromatase expression. Thus a positive feedback loop is established which leads to the development and growth of the tumor.

V. Related Studies Not Included Under the Technical Objectives of the Application:

Determination of promoter usage for aromatase expression in feminizing tumors of the adrenal and liver (12,13). Promoter II was found to be primarily used for extremely high levels of aromatase expression in a rare adrenal tumor from an adult man (12); and a feminizing hepatocellular cancer was demonstrated to use both promoters II and I.3 for extremely high levels of aromatase expression in an adolescent boy (13).

Aromatase Expression in Endometriotic Tissues (14,15). Endometriosis is an estrogen responsive disease. Extremely high levels of aromatase activity and transcript levels were demonstrated in endometriotic stromal cells under the control of promoter II. Prostaglandin E_2 was found to be a very potent inducer of aromatase activity in endometriosis via a cAMP-dependent signaling pathway.

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7. CONCLUSIONS

The studies summarized above supported our working hypothesis. Regional differences in relative proportions of histological components of the breast adipose tissue (*e.g.*, fibroblasts vs. mature adipocytes) may be the primary cause of estrogenic concentration gradients, since regions containing higher numbers of fibroblasts are the sites of increased aromatase expression and increased rate of tumor development. Although the initiating events are unknown, malignant cells in the regions displaying higher P450arom expression are more likely to proliferate. Secretory products of the tumor stimulated by estrogen may in turn further increase aromatase expression in the surrounding adipose tissue. These products will additionally stimulate proliferation of aromatase-expressing fibroblasts to generate a fibrous capsule around the tumor, *i.e.*, desmoplastic reaction. Estrogens will continue to positively influence neoplastic growth by increasing the expression of secretory products and their receptors in the tumor tissue. Thus a positive feed-back loop is established in which locally-produced estrogen and tumor-derived factors (*e.g.*, cytokines, growth factors and prostaglandins) act by paracrine and autocrine mechanisms to sustain the growth and development of the tumor.

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9. APPENDIX

Copies of the publications that are cited in the text or that are relevant to this research effort are attached.

10. PERSONNEL

Serdar E. Bulun, MD

Effects of Conditioned Medium From Different Cultured Cell Types on Aromatase Expression in Adipose Stromal Cells

John E. Nichols, MD, Serdar E. Bulun, MD, and Evan R. Simpson, PhD

OBJECTIVE: *To determine whether serum-free (SF) conditioned media (CM) from several human breast cancer cell lines and primary stromal cell cultures contain factor(s) that mimic the marked stimulatory effects of serum on aromatase activity and aromatase P450 (P450arom) gene expression in adipose stromal cells in culture (ASC) in the presence of dexamethasone (DEX).*

METHODS: *Adipose stromal cells, harvested from fresh adipose specimens, were grown to confluence, switched to SF media, and then incubated in the presence or absence of DEX with CM from T47-D breast cancer cells, pre-treated with or without 17β-estradiol (E2), and with CM from stromal cell cultures. Aromatase activity of the ASC was determined by the [^{3}H]water release assay. Total RNA was isolated, and reverse transcription-polymerase chain reaction was performed to determine the expression of various 5'-termini.*

RESULTS: *T47-D CM stimulated aromatase activity in a concentration-dependent manner, similar to that of serum, in ASC incubated with DEX. Estrogen potentiated this in a dose-dependent fashion. The ASC CM and endometrial stromal cell CM also markedly induced aromatase activity in ASC. Heat inactivation destroyed the stimulating ability of CM. The majority of P450arom 5'-termini expressed by ASC incubated with CM plus DEX contained the promoter I.4-specific sequence.*

CONCLUSIONS: *Conditioned media from several breast cancer cell lines and primary stromal cell cultures can mimic the effects of serum in the presence of DEX to stimulate aromatase activity in ASC. These results suggest that undefined, heat-labile and proteinaceous factors are present in CM that stimulate P450arom expression in a fashion similar to that of serum.* (J Soc Gynecol Invest 1995;2: 45–50)

KEY WORDS: Aromatase, adipose, conditioned medium, breast cancer.

In humans, estrogen biosynthesis occurs in a number of cells and tissues, including the granulosa cells of the ovary,[1] Leydig cells of the testis,[1] syncytiotrophoblast of the placenta,[2] various sites in the brain,[3,4] and in adipose tissue.[5] Conversion of C-19 steroids to estrogens is catalyzed by aromatase P450 (P450arom, the product of the CYP19 gene) in conjunction with the ubiquitous flavoprotein, NADPH-P450 reductase.[6,7] Adipose tissue is the major extragonadal site of estrogen production in both men and women.[8] Adipose tissue estrogen biosynthesis increases not only as a function of body weight and age,[9–11] but there also appears to be a marked increase in the ratio of breast adipose to breast glandular tissue with aging. Thus, in postmenopausal women, adipose tissue is the principal site of estrogen biosynthesis and has been correlated with the incidence of both endometrial and breast cancer.[12,13] Indeed, women with central obesity have up to three times the risk of developing breast cancer.[14] O'Neill et al[15] and Bulun et al[13] independently presented data indicating higher aromatase activity and expression in tumor-containing breast quadrants as compared to non-tumor-containing breast quadrants. It is well known that certain breast tumors can undergo marked cellular proliferation and production of local growth factors in the presence of estrogens, and, in turn, these tumor-secreted growth factors may induce surrounding adipose tissue to increase aromatase activity further by increased expression of the P450arom gene.[16]

Recent studies have indicated that tissue-specific expression of the aromatase CYP19 gene is regulated, at

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least in part, through use of tissue-specific promoters by means of alternative splicing mechanisms.[17–20] Aromatase expression in the ovaries and testes is driven by a proximal promoter (PII), which is located just upstream from the start site of translation. On the other hand, expression in the placenta is driven primarily from a distal promoter, I.1, located at least 40 kb upstream from the start of transcription. Furthermore, expression in adipose tissue appears to be regulated by use of yet another promoter, I.4, located 20 kb downstream from the placental promoter I.1.[17] Untranslated exons containing the starts of transcription from each of these tissue-specific promoters are spliced into a common 3′ splice junction just upstream from the start of translation in exon II of the P450arom gene. Thus, transcripts in these different tissue-specific sites of expression contain unique 5′ termini; however, the coding region and, therefore, the protein products are identical in each tissue site of expression.

As a consequence of the use of alternative promoters in this fashion, regulatory mechanisms controlling the expression of aromatase in each of the tissue sites of expression are quite different. In adipose stromal cells (ASC), aromatase expression is stimulated modestly by glucocorticoids, giving rise to P450arom transcripts with 5′ ends specific for promoter I.4.[17] However, with the addition of serum to glucocorticoid-treated ASC, aromatase activity is markedly increased.[21] This is because flanking sequences upstream from promoter I.4 of the CYP19 gene contain elements that respond to both glucocorticoids and factor(s) in serum.[22] To begin to understand which factor(s) might be responsible for mediating the stimulation of aromatase expression in ASC, we sought to determine whether the effect of serum can be mimicked by conditioned medium (CM) from a number of cells in culture, including several epithelial breast cancer cell lines such as MCF-7 and T47-D cells, endometrial stromal cells in culture, as well as CM from ASC themselves. We found that CM from each of these cells in culture was able to mimic the effect of serum to stimulate aromatase expression by ASC.

MATERIALS AND METHODS

Tissue Acquisition and Processing

Subcutaneous adipose tissue was obtained at the time of surgery from women undergoing breast reduction mammoplasty, abdominoplasty, or liposuction for either symptomatic macromastia or obesity. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center, Dallas. Adipose tissue was minced finely and incubated in Krebs bicarbonate buffer containing bovine serum albumin (4% wt/vol) (Pentax fraction V; Miles Laboratories, Elkhart, IN) and collagenase (0.6 mg/mL) (type 1A; Sigma, St. Louis, MO) for 45 minutes in a 37°C shaking water bath. After incubation, the undigested tissue was removed by filtering through a gauze mesh, and the resulting preparation was centrifuged at 400 × *g* for 7 minutes. The floating adipocyte and lipid layer was removed, and the remaining stromal cell pellet was washed and centrifuged twice more to remove any remaining collagenase.

Cell Cultures

Adipose stromal cells were suspended in Waymouth's MB 752/1 enriched media (Gibco/BRL, Grand Island, NY) containing 10% NuSerum (Collaborative Research, Bedford, MA), and the number of stromal cells was determined by counting in a hemocytometer. Fresh suspensions of stromal cells were plated in six-well, 35-mm culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 10^5 cells per dish and kept in an incubator in a humidified atmosphere with 5% CO_2 at 37°C. Media were changed after 48 hours and thereafter at intervals until the cells became confluent, which usually occurred in 5–7 days. Confluent ASC, which assume a fibroblast-like appearance, were then placed in serum-free (SF) Waymouth's media, and the media were changed at least three times over the next 24 hours to remove any remaining serum factors. We obtained CM from MCF-7 and T47-D breast cancer cells (American Tissue Culture Collection, Rockville, MD) as well as SSC 202, SSC 78, and SSC 30 breast cancer cells (kindly provided by Dr. Ali Gadzar, Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas), and primary cultures of ASC and endometrial stromal cells (kindly provided by Dr. Ann Word, University of Texas Southwestern Medical Center, Dallas). These CM were used to treat ASC in the presence or absence of dexamethasone (DEX, 250 nmol/L) (Sigma) for 24 hours. We obtained CM from breast cancer cells and primary cell cultures after incubation in SF media for 24 hours, to allow accumulation of secreted growth factors from these cells, after a washout period of 24 hours with SF media similar to the protocol for ASC.

T47-D breast cancer cells were grown to confluence in phenol red-free RPMI 1640 medium (Gibco/BRL) and incubated with or without 17β-estradiol (E2, 10^{-7} to 10^{-9} mol/L) or a steroidal estrogen antagonist, ICI 182,780 (Zeneca Pharmaceuticals, Cheshire, UK), for 24 hours, after a 24-hour washout period with SF media. This CM was then used to treat ASC. Alternatively, heat inactivation of CM was performed by placing the CM in a 100°C shaking water bath for 15 minutes and then cooling to room temperature before use.

Aromatase Activity Measurement

Aromatase activity was assayed in intact ASC after the addition of [1β-^{3}H]androstenedione (150 nmol/L) (Dupont, Boston, MA) to the medium. At the end of a 2-hour incubation period, medium was removed, and the incorporation of tritium from [1β-^{3}H]androstenedione

into [^{3}H]water was assayed as described previously in detail.[5] The cells were then scraped from the dishes, homogenized, and assayed for protein using the BCA Protein Assay (Pierce, Rockford, IL). Results are expressed as pmole/mg protein per 2 hours. Each bar represents the mean of results (±standard error of the mean [SEM]) using triplicate sets of dishes.

Isolation of RNA

From ASC maintained in the presence or absence of DEX and in the presence or absence of serum or CM, total RNA was isolated by a single-step method using TRI-SOLV (Biotecx, Houston, TX), following the protocol suggested by the supplier. The RNA concentration was determined by spectrophotometric absorption at 260 nm.

Oligonucleotide Sequences

Synthesized oligonucleotides (oligos) were used as primers and radiolabeled probes.[17] The sequence of the 20-mer 3′ oligo used for initial primer extension was 5′ATTCCCATGGAGTAGCCAGG3′ (AROM III, complementary to coding exon III of the P450arom gene), whereas those of the 5′ oligos used during amplifications were as follows: exon I.3-specific primer, 5′GATAAGGTTCTATCAGACC3′; exon I.4-specific primer, 5′GTAGAACGTCACCAACTGG3′; promoter II-specific primer, 5′GCAACAGGAGCTATAGAT3′; and coding exon II-specific primer, 5′TCTGAGGTCAAGGAACAC3′. The sequences of the different oligos used as [^{32}P]-labeled probes for each of the exon-specific amplification products were as follows: for the exon I.3-specific sequence, 5′GCAGCATTTCTGACCTTGG3′; for the exon I-4-specific sequence, 5′GGTTTGATGGGCTGACCAG3′; for the promoter II-specific sequence, 5′TGTGGAAATCAAAGGGACAGA3′; and for the coding exon III-specific sequence, 5′CAGGCACGATGCTGGTGATG3′.

Reverse Transcription-Polymerase Chain Reaction Amplification (RT-PCR) and Hybridization

Amplification of the 5′ ends of the P450arom transcripts from the various treated and untreated ASC was accomplished with the exon-specific oligo pairs as listed above. Initial primer extension, using 10 μg total RNA, was performed at 37°C for 60 minutes using 100 pmol of the 3′ oligo AROM III, 1 mmol/L final concentration of each dNTP, 5× first strand buffer (Gibco/BRL), 10 mmol/L dithiothreitol, 400 units MMLV-RT (BRL, Gaithersburg, MD), and 40 units of RNAsin (Promega, Madison, WI) in a final volume of 50 μL. Each amplification master mix contained 50 pmol of the 3′ oligo AROM III, 100 pmol of a specific 5′ oligo, 500 μmol/L final concentration of each dNTP, and PCR buffer (Perkin Elmer Cetus, Norwalk, CT), in a final volume of 50 μL (39.5 μL of amplification master mix, 0.5 μL [2.5 units] of AmpliTaq DNA polymerase [Perkin Elmer Cetus], and 10 μL of initial primer extension reaction mix). The mix was amplified for 25 cycles at 93°C for 1 minute, 41°C for 1 minute, and 72°C for 1 minute in a thermocycler (Perkin Elmer Cetus). The amplified products were then treated with DNase-free RNase (Boehringer, Indianapolis, IN) for 30 minutes at 37°C and then size-fractionated (to confirm appropriate size of the amplified products) by electrophoresis on a 1.8% agarose gel and transferred to a blotting nylon membrane (Hybond N+; Amersham, Amersham, UK) by capillary elution in 0.4 mol/L NaOH solution. Southern hybridization with the 5′ termini-specific oligonucleotide probes end-labeled with [^{32}P] was continued overnight at Tm − 5°C for each oligo. Autoradiographs were exposed to blotting membranes for 4–16 hours. Samples containing no RNA were included to preclude product carry-over contamination.

RESULTS

As can be seen in Figure 1, and as documented previously, DEX alone had a minimal effect on aromatase activity of ASC. However, addition of serum created a tenfold increase in aromatase activity, suggesting the presence of a serum-derived aromatase stimulating factor(s). From the results presented here, it is apparent that SF CM from T47-D cells can mimic the effects of serum, as shown by a sevenfold increase in aromatase activity when ASC were incubated in the presence of 100% CM and DEX. Moreover, dilution of the CM with SF medium resulted in a decrease of stimulation in a concentra-

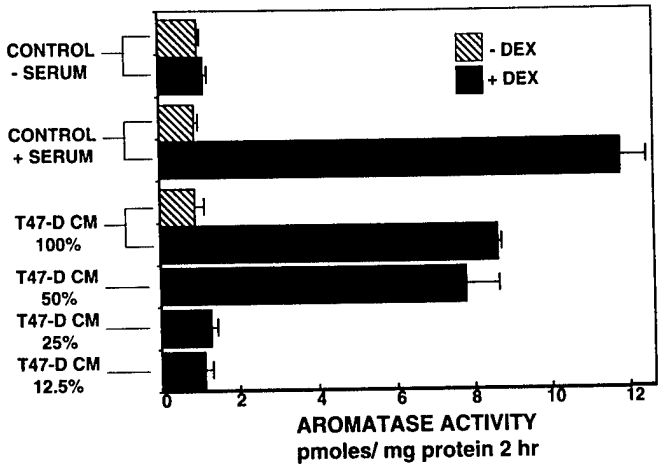

Figure 1. Aromatase activity of human ASC incubated with conditioned medium (CM) from T47-D cells in various dilutions. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM in various dilutions (100% to 12.5%) for a further 24 hours. Aromatase activity was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

tion-dependent fashion. In addition, aromatase activity of ASC in the presence of CM from T47-D cells but in the absence of DEX was similar to that in the presence of SF media alone (without DEX). Similar effects were seen using CM from MCF-7 cells and SSC 30, SSC 78, and SSC 202 breast cancer cell lines (data not shown).

Figure 2 depicts the effects of CM from T47-D cells treated previously with or without E2 for 24 hours, in concentrations of 10^{-7} to 10^{-9} mol/L, on aromatase activity of ASC in the presence of DEX. As shown, DEX alone caused a modest stimulation of aromatase activity, whereas serum alone was inhibitory. There are consistent observations, but are particularly apparent in this experiment. Pre-treatment of T47-D cells with E2 resulted in a dose-dependent effect, such that CM from E2-treated T47-D cells caused a greater stimulation of aromatase activity of DEX-treated ASC cells than did CM from cells not treated with E2. At 10^{-7} mol/L concentration, E2 led to more than a 1.5-fold stimulation of aromatase activity as compared to no E2 treatment. Furthermore, a steroidal anti-estrogen compound (ICI 182,780) blocked this action of E2 to enhance the aromatase-stimulating activity of CM from T47-D cells. These results are consistent with the hypothesis that estrogen can stimulate T47-D breast cancer cells, which are known to possess estrogen receptors, to secrete factors that can stimulate aromatase activity of ASC.

In addition to CM from several breast cancer cell lines, we also examined the effects of CM from adipose stromal cells per se as well as endometrial stromal cells, both in primary culture. As seen in Figure 3, aromatase activity of ASC, in the presence of DEX, also was markedly stimulated by CM from these nonmalignant cells. This suggests that whatever factor is responsible for this increase in aromatase activity appears to be ubiquitous, or else there are a number of factors that can cause this increase, which may be expressed differentially in different cell types.

Because the stimulatory factor(s) present in both CM and serum most likely represents a peptide growth factor(s), we examined whether it could be destroyed by heating. Figure 4 shows the effects of heat inactivation of CM, which resulted in a complete loss of its ability to stimulate aromatase activity of ASC treated with DEX. Thus, it is likely that the factor(s) in CM responsible for the stimulation of aromatase activity is heat-labile and probably proteinaceous in nature.

We also sought to determine whether the stimulation of aromatase expression by CM in the presence of DEX resulted from the activation of expression of promoter I.4 of the CYP19 gene, similar to the action of DEX in the presence of serum.[17] Total RNA from ASC treated with or without DEX and CM or serum was isolated, and then RT-PCR and Southern hybridization were performed. In Figure 5, the blot probed with the exon I.4-specific probe was exposed for only 4 hours because of its much greater intensity, whereas the blot hybridized with the exon I.3-specific probe was exposed for 16 hours. As can be seen, DEX in the absence of serum stimulated only expression of I.4-specific transcripts. Transcripts presented in untreated cells contained primarily I.3- and PII-specific se-

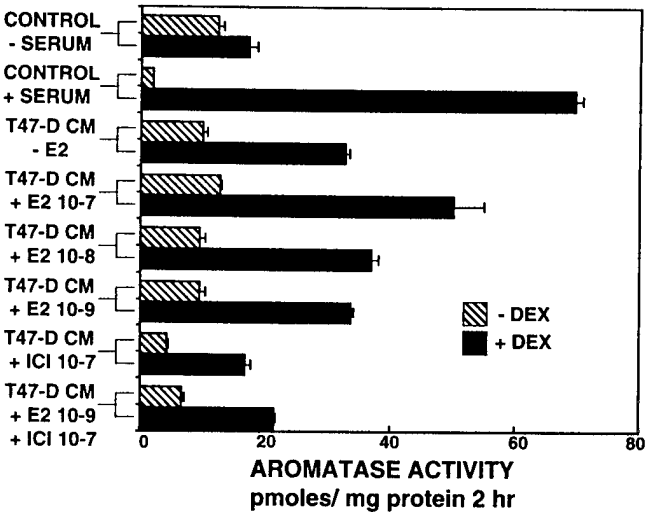

Figure 2. Effect of estrogen on the ability of T47-D conditioned medium (CM) to stimulate aromatase activity of ASC. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM for a further 24 hours. The CM was prepared from T47-D cells treated with estradiol (E2) in various concentrations (0–10^{-7} mol/L) in the presence or absence of ICI 182,780 (10^{-7} mol/L). Aromatase activity of the ASC was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

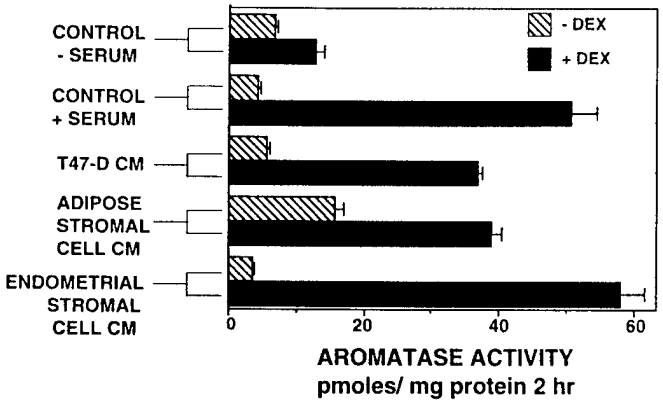

Figure 3. Aromatase activity of ASC incubated with conditioned medium (CM) from various cell types. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM from T47-D cells, ASC, or endometrial stromal cells for a further 24 hours. Aromatase activity was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

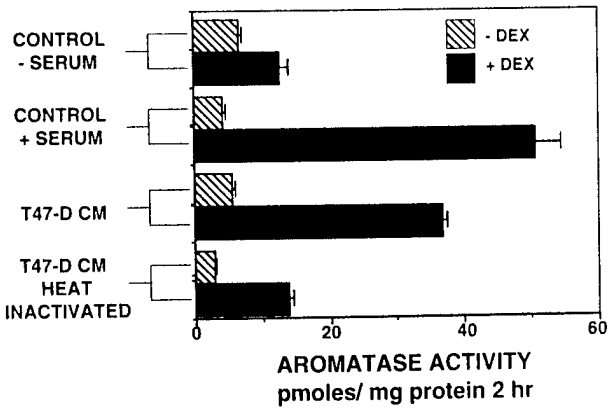

Figure 4. Effect of heat-inactivated conditioned medium (CM) on aromatase activity of ASC. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM from T47-D cells for a further 24 hours. Heat-inactivated CM was prepared as described in Materials and Methods. Aromatase activity was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

quences (not shown). However, in the presence of DEX and serum, expression of both I.4- and I.3-specific transcripts was noted; by far, the majority of transcripts contained I.4-specific sequences, as expected.[17] In a similar fashion, I.4-specific transcripts predominated in cells treated with CM from T47-D cells.

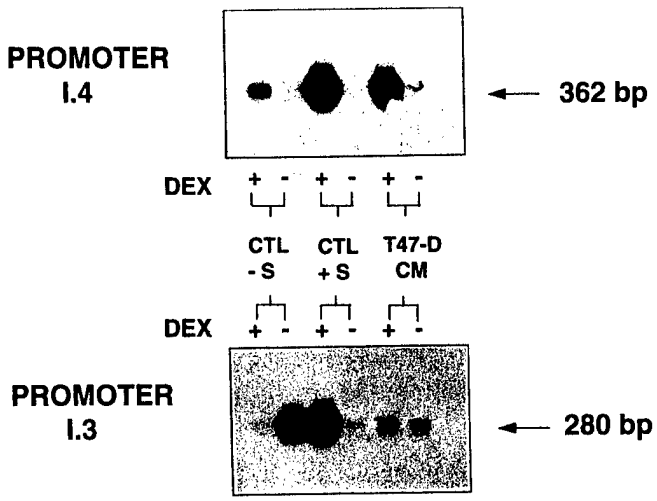

Figure 5. RT-PCR amplification of specific 5′ termini of P450arom transcript in RNA from ASC. ASC were maintained in SF medium for 24 hours and then maintained in the presence or absence of dexamethasone (DEX) (250 nmol/L), NuSerum (10%), or CM from T47-D cells for a further 24 hours. RNA was then isolated and used for RT-PCR of P450arom transcripts with 5′ termini, as described in Materials and Methods. Following electrophoresis and Southern blotting, the filters were subjected to autoradiography. The blot in the upper panel (exon I.4-specific probe) was exposed for 4 hours, whereas that in the lower panel (exon I.3-specific probe) was exposed for 16 hours. CTL = control; S = serum.

DISCUSSION

The results of this study indicate that CM from a variety of cell types and cell lines of human origin are capable of mimicking the effect of serum to stimulate aromatase expression by ASC maintained in the presence of glucocorticoids. Moreover, this activity is likely to be the consequence of a proteinaceous factor or factors secreted by these cells. Cells producing such a factor or factors include MCF-7, T47-D, and SSC-30, -78, and -202 breast cancer cell lines, as well as endometrial stromal cells and ASC themselves, in primary culture. It has been reported previously that another breast cancer cell line, MDA-MB231, also produces a factor(s) with similar properties.[23] Cells producing such a factor(s) are both mesenchymal and epithelial in origin. The stimulatory effect of such factors appears to be due to use of the promoter I.4, whose activity is confined primarily to adipose tissue and fetal liver.[17,24] Thus, the use of RT-PCR indicates that the expression of promoter I-4-specific transcripts is greatly enhanced in the presence of glucocorticoids plus CM, just as it is in the presence of glucocorticoids plus serum.

In previous studies, we and others have shown that aromatase expression in breast adipose tissue proximal to a tumor is greater than at sites distal to the tumor,[13,15] which suggests that cross-talk occurs between a breast tumor and the surrounding adipose tissue in terms of the ability of the latter to synthesize estrogens. These results suggest the possible existence of local paracrine and autocrine loops mediating stromal-epithelial cell interactions, whereby estrogens produced by ASC proximal to a tumor stimulate the tumor cells to produce growth factors. These growth factors in turn stimulate the growth and development of the tumor, but also may act in a paracrine fashion to stimulate the surrounding ASC to produce more estrogens, thus resulting in a positive feedback loop which further supports development of the tumor.[16]

The results of the present experiments support this concept by showing that a number of cell lines, including breast cancer cell lines and ASC themselves, do indeed secrete proteinaceous factors that can stimulate ASC to increase aromatase expression. That several cell types have this ability suggests that a number of factors may stimulate ASC to produce estrogens. Further studies must determine the identities of the factors produced by these various cell types that can stimulate estrogen biosynthesis by ASC via activation of promoter I.4 of the CYP19 gene.

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Aromatase P450 Gene Expression in Human Adipose Tissue

ROLE OF A Jak/STAT PATHWAY IN REGULATION OF THE ADIPOSE-SPECIFIC PROMOTER*

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In the present report we describe a heretofore unrecognized role for a Jak/STAT signaling pathway, namely the stimulation of expression of the aromatase P450 (CYP19) gene, and hence of estrogen biosynthesis, in human adipose tissue. Expression of this gene in adipose tissue as well as in adipose stromal cells maintained in the presence of serum and glucocorticoids is regulated by a distal TATA-less promoter, I.4, which contains a glucocorticoid response element, an Sp1 binding site, and an interferon-γ activation site (GAS) element. The stimulatory action of serum (in the presence of dexamethasone) can be replaced by interleukin (IL)-11, leukemia inhibitory factor, and oncostatin-M, as well as by IL-6, providing the IL-6 soluble receptor is also present. Stimulation of the cells by these factors led to rapid phosphorylation of Jak1, but not Jak2 or Jak3, on tyrosine residues. STAT3 but not STAT1 was also phosphorylated and bound to the GAS element in the I.4 promoter region. When regions of this promoter were fused upstream of the chloramphenicol acetyltransferase reporter gene and transfected into the cells, mutagenesis or deletion of the GAS element led to complete loss of reporter gene expression. Since adipose tissue is the major site of estrogen biosynthesis in men and in postmenopausal women, this pathway involving a Jak/STAT signaling mechanism acting together with glucocorticoids and Sp1 appears to be the principal means whereby estrogen biosynthesis is regulated in the elderly.

Estrogen biosynthesis in humans occurs in a number of tissue sites of expression including the granulosa cells and corpus luteum of the ovary (1, 2), the Leydig cells of the testis (3), the syncytiotrophoblast of the placenta, various sites in the brain including the hypothalamus, amygdala, and hippocampus (4, 5), as well as in adipose tissue (6). The significance of adipose tissue as a source of estrogens was first recognized some 20 years ago by MacDonald, Siiteri, and their colleagues (7–10) who determined the fractional conversion of circulating androstenedione to estrone in male and female human subjects. They found that not only was there a striking increase with obesity, suggesting that most of the extragonadal conversion occurred in adipose tissue, but also that there was an equally striking increase with age for any given body weight.

Estrogen biosynthesis is catalyzed by an enzyme known as aromatase P450 (11–14) (P450arom, the product of the CYP19 gene (15)). CYP19 is a member of the P450 superfamily of genes, which currently contains over 300 members in some 36 gene families (15). Typically, these enzymes catalyze the insertion of oxygen atoms derived from molecular oxygen into organic molecules to form hydroxyl groups. In the case of P450arom, three such attacks by molecular oxygen give rise to loss of the C19 angular methyl group of the steroid substrate as formic acid, with concomitant aromatization of the A-ring to give the phenolic A-ring characteristic of estrogens (16).

A few years ago we and others cloned and characterized the CYP19 gene, which encodes human P450arom (17–19). The coding region spans 9 exons beginning with exon II. Sequencing of rapid amplification of cDNA ends-generated cDNA clones derived from P450arom transcripts present in the various tissue sites of expression revealed that the 5′-termini of these transcripts differ from one another in a tissue-specific fashion upstream of a common site in the 5′-untranslated region (20–22). Using these sequences as probes to screen genomic libraries, it was found that these 5′-termini correspond to untranslated exons that are spliced into the P450arom transcripts in a tissue-specific fashion, due to the use of tissue-specific promoters. Placental transcripts contain at their 5′-ends untranslated exon I.1, which is located at least 40 kilobases upstream from the start of translation in exon II (20, 23). This is because placental expression is driven from a powerful distal placental promoter, I.1, upstream of untranslated exon I.1. On the other hand, transcripts in the ovary contain sequence at their 5′-ends that is immediately upstream of the start of translation. This is because expression of the gene in the ovary utilizes a proximal promoter, promoter II. By contrast, transcripts in adipose tissue contain yet another distal untranslated exon, I.4, which is located in the gene 20 kilobases downstream from exon I.1 (24–26). A number of other untranslated exons have been characterized by ourselves and others (22, 27), including one specific for brain (28). Splicing of these untranslated exons to form the mature transcripts occurs at a common 3′-splice junction, which is upstream of the start of translation. This means that although transcripts in different tissues have different 5′-termini, the protein encoded by these transcripts is always the same, regardless of the tissue site of expression; thus, there is only one human P450arom enzyme encoded by a single copy gene.

Using reverse transcription polymerase chain reaction with an internal standard, we have studied aromatase expression in samples of adipose tissue obtained from women of various ages and have found a marked increase in the specific content of P450arom transcripts in adipose tissue with increasing age,

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thus providing a molecular basis for the previous observation that the fractional conversion of circulating androstenedione to estrone increases with age (29). Furthermore, there are marked regional variations in aromatase expression, with the highest values being found in adipose from buttocks and thighs as compared with abdomen and breast (29, 30).

We also used this reverse transcription polymerase chain reaction technique to examine regional variations in aromatase expression in breast adipose tissue and have found that highest expression occurs in adipose tissue proximal to a tumor, as compared with that distal to a tumor (31, 32). This is in agreement with previous observations regarding the regional distribution of aromatase activity within breast adipose (33, 34) as well as an immunocytochemical study (35). These results suggest there is cross-talk between a breast tumor and the surrounding adipose cells in terms of the ability of the latter to synthesize estrogens and that factors produced by developing breast tumors may set up local gradients of estrogen biosynthesis in the surrounding fat via paracrine mechanisms (36).

We also found that aromatase expression does not occur in adipocytes but rather in the stromal cells that surround the adipocytes, and that may themselves be preadipocytes (37). These stromal cells grow in culture as fibroblasts. Consequently we have employed these cells in primary culture as a model system to study the regulation of estrogen biosynthesis in adipose tissue (38). When serum is present in the culture medium, expression is stimulated by glucocorticoids including dexamethasone (39). Under these conditions P450arom transcripts contain primarily untranslated exon I.4 at their 5′-ends (25). We subsequently have characterized the region of the CYP19 gene upstream of exon I.4 and have found it to contain a TATA-less promoter as well as an upstream glucocorticoid response element and an Sp1 sequence within the untranslated exon, both of which are required for expression of reporter gene constructs in the presence of serum and glucocorticoids (40). Additionally, we found this region to contain an interferon-γ activating sequence (GAS)[1] element. Such sequences are known to bind transcription factors of the signal transducers and activators of transcription (STAT) family (41–43).

In the present study we have observed for the first time that the effect of serum to stimulate aromatase expression in human adipose stromal cells (in the presence of glucocorticoids) can be mimicked by specific factors, namely members of the interleukin-11 (IL-11), oncostatin-M (OSM), IL-6, and leukemia inhibitory factor (LIF) lymphokine family. This stimulation is mediated by a member of the Jak family of tyrosine kinases as well as a STAT transcription factor, which binds to the GAS element within promoter I.4 of the P450arom gene. Thus we have uncovered a regulatory pathway whereby expression of the P450arom gene, via the distal promoter I.4, is stimulated by members of the above cytokine family. Since P450arom transcripts in adipose tissue appear to be derived primarily from expression of promoter I.4 and since adipose tissue is the major site of estrogen biosynthesis in men and in postmenopausal women (44), this pathway composed of a Jak/STAT signaling mechanism acting in conjunction with glucocorticoids and Sp1 appears to be the principal means whereby estrogen biosynthesis is regulated in the elderly.

[1] The abbreviations used are: GAS, interferon-γ activation site; STAT, signal transducers and activators of transcription; IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin-M; RIPA, radioimmune precipitation buffer; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; bp, base pair(s); FCS, fetal calf serum.

EXPERIMENTAL PROCEDURES

Materials—Jak1, Jak2, and Jak3 antisera were the generous gift of Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN). STAT1 (Cat. number S21120) and ISGR3 (Cat. number G16930) antisera were purchased from Transduction Laboratories (Lexington, KY). The latter antiserum is raised against a mixture of STAT1α and STAT1β. STAT3 antiserum was a generous gift of Dr. Christopher Schindler (Columbia University, New York). IL-11, OSM, LIF, IL-6, and IL-6 soluble receptor were purchased from R & D (Minneapolis, MN). INFα and INFγ were purchased from Sigma. Anti-phosphotyrosine antibody (4 G10) was purchased from UBI (Lake Placid, NY). Herbimycin, H7, and cycloheximide were purchased from Sigma.

Cell Culture and Preparation of Nuclear Extracts—Subcutaneous adipose tissue was obtained from women at the time of reduction abdominoplasty or reduction mammoplasty. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were prepared as described (38) and maintained in primary culture in Waymouth's enriched medium containing 10% Nu serum (10% v/v) (Collaborative Research Inc.) and allowed to grow to confluence (5–6 days) before treatment. At this time, serum was removed for 24 h, and the cells were treated with 250 nM dexamethasone for 48 h before recombinant human IL-11, OSM, IL-6, and LIF were added at various concentrations and times depending on the different requirements for each experiment. Aromatase activity was determined by the incorporation of tritium into [^{3}H]water from [1β-^{3}H]androstenedione as described previously (38). Cytoplasmic and nuclear extracts were prepared as described by Cooper *et al.* (45) and Dignam *et al.* (46) with some modifications. Cells were cooled on ice, scraped from the plates, washed 3 times with phosphate-buffered saline, and then lysed in modified RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% aprotinin, 1 mM phenanthroline, 10 mM pepstein, 0.1% leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 15% glycerol) with protease and phosphatase inhibitors for 30 min on ice. Lysates were precleared by centrifugation for 30 min at 4 °C. For nuclear extracts, after cells were swollen in 2 ml of hypotonic buffer (containing protease and phosphatase inhibitors as in RIPA) they were homogenized with 12 strokes of an all glass Dounce homogenizer (B type pestle). Nuclei were centrifuged, and the pellet was then suspended in 500 μl of chilled buffer (20 mM Hepes, pH 7.7, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% dithiothreitol, and 20% glycerol and protease and phosphatase inhibitors as in RIPA). After centrifugation, the supernatant was stored at −70 °C until used.

Immunoprecipitation and Western Blotting Analysis—Immunoprecipitations were performed essentially as described by Harlow and Lane (47) or following instructions of the manufacturers of the respective antibodies. Lysates were incubated with nonimmune serum and Protein A-Sepharose (50 μl of 50% slurry) for 1 h, followed by centrifugation. Antibodies were incubated with lysates (100 μg) overnight at 4 °C. Immunoprecipitates were isolated with Protein A- or Protein G-coupled agarose or Sepharose (Oncogene Sciences or Sigma) and washed carefully 3 times with the same lysis buffer mentioned above. Proteins in the immunoprecipitates were resolved by 8% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad), and incubated with the appropriate antibody followed by anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase and the enhanced chemiluminescence detection system (Amersham International, U.K.).

DNA Reporter Gene Constructs Containing Deletion Mutations, Site-directed Mutagenesis, and Transient Transfections—Chimeric DNA constructs containing the GAS element in the genomic region flanking the 5′-end of promoter I.4 of the human CYP19 gene were prepared using polymerase chain reaction. These constructs were fused upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. For mutagenesis, an 800-bp fragment containing the GAS element was digested by *Hin*dIII and *Eco*RI and gel-purified to generate the template for mutagenesis. The GAS element was mutated from TTCCTGTGAA to TTCGACTGAA by polymerase chain reaction. The mutated fragment also was fused upstream of the CAT reporter gene. The fidelity of mutagenesis was verified by dideoxy sequencing using a Sequenase DNA kit (U.S. Biochemical Corp.). The transfection was performed by means of calcium phosphate coprecipitation with minor modifications as described (40). Nearly confluent adipose stromal cells in primary culture were transfected with 20 μg of cesium chloride-purified plasmid. Glycerol shock was carried out for 1 min. The cells were allowed to recover overnight in serum-containing media, serum-starved for 24 h, and then treated with 250 nM dexamethasone for 48 h and 10 ng/ml IL-11 overnight. The transfected cells were then harvested and lysed by

means of freeze/thaw 3 times. Total protein was estimated with a kit as recommended by the manufacturer (Pierce). The CAT assay was performed using normalized amounts of cell proteins, which were incubated for 6–8 h at 37 °C. The products of CAT reactions were analyzed by silica gel thin layer chromatography followed by autoradiography.

Electrophoretic Mobility Shift Assay and Southwestern Blotting Analysis—A double-stranded oligonucleotide (5′-GGGTGTTTCCTGTGAAAGTT-3′) was Klenow-labeled using [α-^{32}P]dCTP and then incubated (5000 cpm) with nuclear extracts (5 μg) on ice for 10 min. For the competition assay, the unlabeled oligonucleotide used as competitor was added simultaneously with the labeled fragment at various ratios. The resulting DNA-protein complexes were analyzed by electrophoresis using an 8% polyacrylamide gel with 0.5 × Tris borate-EDTA as running buffer. Southwestern blotting analysis was done following the procedure described by Singh *et al.* (48) with minor modification. Nuclear extracts (60 μg) were fractionated on 8% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was processed through a denaturation/renaturation cycle with 6 M guanidine hydrochloride. Then the membrane was hybridized with the Klenow-labeled probe employed in gel shift analysis, followed by washing and autoradiography.

Generally, experiments were performed at least 3 times on separate occasions, and frequently more, to generate consistent results.

RESULTS

IL-11, OSM, and LIF Stimulate Aromatase Activity in Cultured Adipose Stromal Cells in the Presence of Dexamethasone—In previous studies we observed that the stimulatory effect of glucocorticoids on aromatase activity in cultured human adipose stromal cells was manifest only if serum was present in the medium (39). As shown in Fig. 1*A*, a number of lymphokines, namely IL-11, OSM, and LIF can mimic the action of serum to stimulate aromatase activity of adipose stromal cells in the presence of dexamethasone but not in its absence. In the case of IL-11 and OSM, the effect far exceeded that of serum. Following exposure for 24 h, half-maximal stimulation by IL-11 and OSM was achieved at concentrations of ~2–4 ng/ml (~10^{-10} M), although the stimulation by LIF was maximal at 0.5 ng/ml. The time course of stimulation by IL-11 (Fig. 1*B*) was biphasic, with an initial rapid phase lasting 12 h followed by a slower phase extending beyond 48 h of stimulation.

As shown in Fig. 1*C*, IL-6 had no stimulatory activity up to a concentration of 20 ng/ml, either in the presence or absence of dexamethasone. However, a stimulatory action of IL-6 was manifest upon addition of the IL-6 soluble receptor at concentrations ranging from 5 to 20 ng/ml. At concentrations of IL-6 and its receptor of 20 ng/ml each, stimulation was equal to that achieved by adding OSM at a saturating concentration of 10 ng/ml. In other experiments, interferon-α and interferon-γ were found to have no effect to stimulate aromatase activity of these cells (data not shown).

IL-11 Rapidly and Specifically Stimulates Tyrosine Phosphorylation of Jak1 Kinase—To determine whether Jak kinases are involved in the action of IL-11 to activate estrogen biosynthesis in adipose stromal cells, we examined their ability to undergo tyrosine phosphorylation in the presence of IL-11 (Fig. 2). Adipose stromal cells prepared from mammoplasty and abdominoplasty samples and maintained in primary culture were treated with dexamethasone for 48 h with or without IL-11 (10 ng/ml) for 10 min. The cells were lysed, the Jak kinases were immunoprecipitated with the appropriate antibodies, and the immunoprecipitates were resolved on SDS-PAGE. The gels were subsequently blotted to filters and probed with a monoclonal antibody against phosphotyrosine. IL-11 stimulation resulted in the appearance of a band of ~130 kDa, which was immunoprecipitated using antibodies to JAK1 and was undetectable in the absence of IL-11. By contrast, Jak2 kinase was constitutively phosphorylated on tyrosine since the Jak2 band (~130 kDa) was evident prior to the IL-11 treatment, and the level of tyrosine phosphorylation of Jak2 did not change even after IL-11 stimulation. By contrast, tyrosine phosphorylation of Jak3 was not apparent with or without IL-11 treatment. Similar results were obtained using LIF and OSM. Nonimmune serum and an unrelated immune serum failed to precipitate proteins of 130 kDa (data not shown). Additionally, the 130-kDa bands corresponding to Jak1 and Jak2 were not observed when the immunoprecipitations were conducted in the the presence of peptides used to raise these antibodies (data not shown). Probing with a mixture of Jak1, Jak2, and Jak3 antisera showed that no change in the amount of kinase protein occurred within the time frame of the experiment (*lower panel*). It is concluded that in human adipose stromal cells, Jak2 is constitutively phosphorylated on tyrosine residues, and only Jak1 is phosphorylated on tyrosine residues in response to IL-11 treatment under the conditions employed here. A similar situation has been reported to occur in T-lymphocytes stimulated with interferon-α (49).

Characterization of Jak1 Tyrosine Phosphorylation—The kinetics of tyrosine phosphorylation of Jak1 were examined using the same cell lysates as utilized above, and the results are shown in Fig. 3*A*. Phosphorylation was maximal 10 min after IL-11 addition and then subsequently declined. In a study of the concentration dependence of Jak1 phosphorylation (Fig. 3*C*), it was observed that phosphorylation was undetectable employing IL-11 at a concentration of 1 ng/ml. Phosphorylation was detectable when 5 ng/ml IL-11 was used (visible on the original autoradiograph if not on the printed figure) and reached a maximum at a concentration of IL-11 of 10 ng/ml.

Since OSM and LIF also stimulate aromatase activity in adipose stromal cells in primary culture, their action to stimulate tyrosine phosphorylation of Jak1 was also examined. Adipose stromal cells were treated with dexamethasone for 48 h and with FCS (15%), OSM (5 ng/ml), IL-11 (5 ng/ml), and LIF (5 ng/ml) for 10 min. The cells were lysed, the cell lysates were mixed with Jak1 antibody, and the precipitates were resolved by SDS-PAGE and probed with anti-phosphotyrosine monoclonal antibody. The effects of herbimycin A (a tyrosine kinase inhibitor), H7 (a serine/threonine kinase inhibitor), and cycloheximide on Jak1 tyrosine phosphorylation were also examined. As shown in Fig. 4, OSM and LIF could induce tyrosine phosphorylation of Jak1 to about the same extent as IL-11. The tyrosine phosphorylation of Jak1 induced by IL-11 was inhibited by herbimycin A, whereas H7 had no effect. These results further support the concept that the phosphorylation of Jak1 induced by IL-11 is indeed on tyrosine residues. In addition, the rapid phosphorylation of Jak1 was not inhibited by cycloheximide, an inhibitor of protein synthesis, indicative that this is not mediated by the new synthesis of protein factors.

STAT3 Phosphorylation and Binding to the GAS Element in the Upstream Region of Promoter I.4 of the CYP19 Gene—In order to determine whether STAT transcription factors are involved in signal transduction in adipose stromal cells in primary culture in response to IL-11, we examined phosphorylation of STAT1 and STAT3 by immunoprecipitation employing appropriate antisera and subsequent probing with anti-phosphotyrosine monoclonal antibody. Adipose stromal cells prepared from mammoplasty and abdominoplasty samples and maintained in primary culture were treated with dexamethasone for 48 h with or without IL-11 (10 ng/ml) for 10 min. The cells were lysed, and aliquots were mixed with the appropriate antibodies. As can be seen in Fig. 5, a band of 92 kDa was immunoprecipitated by STAT3 antiserum after the cells were treated with IL-11. However, no bands were detectable following immunoprecipitation with antisera raised against STAT1. As

[2] C. Schindler, personal communication.

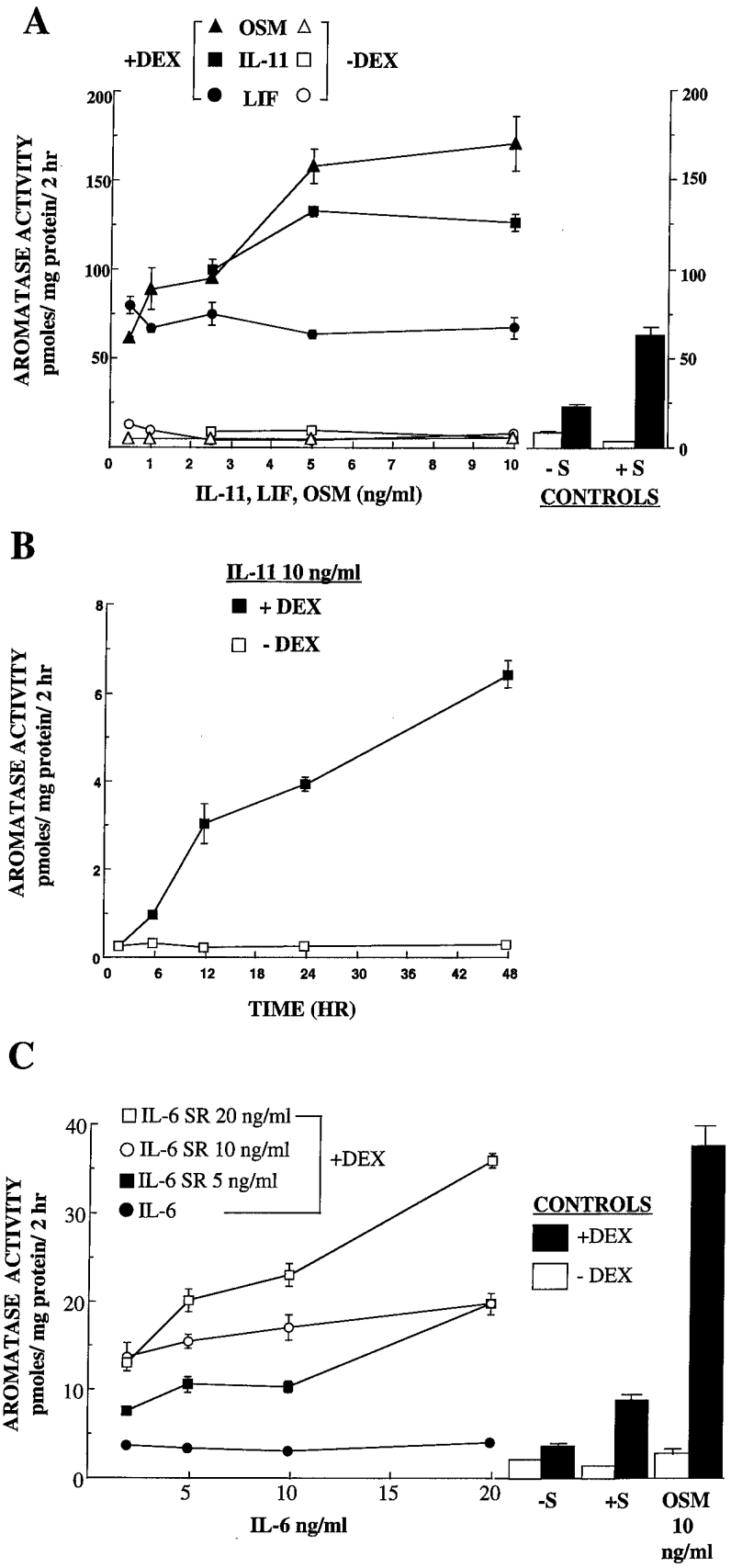

FIG. 1. *A*, concentration dependence of the actions of LIF, IL-11, and OSM to stimulate aromatase activity of adipose stromal cells in the absence (*open symbols*) or presence (*solid symbols*) of dexamethasone (250 nM). Confluent adipose stromal cells in primary culture were maintained for 24 h in the presence or absence of dexamethasone (250 nM) and LIF (*circles*), IL-11 (*squares*), or OSM (*triangles*) in various concentrations. Control dishes incubated in the absence or presence of serum are indicated as ± *S*, with *open bars* indicating the absence and *solid bars* the presence of dexamethasone. *B*, time course of stimulation of aromatase activity of adipose stromal cells by IL-11. Confluent adipose stromal cells in primary culture were maintained for 24 h in the presence (*solid squares*) or absence (*open squares*) of dexamethasone (*DEX*). IL-11 (10 ng/ml) was added to half the dishes, and incubation continued for an additional 48 h. *C*, action of IL-6 and its soluble receptor on aromatase activity of adipose stromal cells. Confluent adipose stromal cells in primary culture were maintained for 24 h in the presence of dexamethasone (250 nM) and in the presence or absence of IL-6 (2–20 ng/ml) and its soluble receptor (*SR*; 5–20 ng/ml). *Solid circles*, no SR; *solid squares*, 5 ng/ml SR; *open circles*, 10 ng/ml SR; *open squares*, 20 ng/ml SR. Control dishes were incubated in the absence or presence of serum (±*S*) or OSM (10 ng/ml) and in the absence (*open bars*) or presence (*solid bars*) of dexamethasone. In each of the experiments shown in *A–C*, aromatase activity was measured at the end of the incubation period as described under "Experimental Procedures." Data are presented as the mean ± S.E. of results from triplicate replicate dishes.

a positive control, we showed by Western blotting that the anti-STAT1 antibody reacted with not only recombinant STAT1 but with STAT1 present in adipose stromal cells treated with dexamethasone in the presence or absence of IL-11 (data not shown). Although the STAT3 antiserum cross-reacts with STAT1α,[2] the use of the commercial STAT1α antiserum ruled out the possibility that the STAT3 antiserum was detecting STAT1.

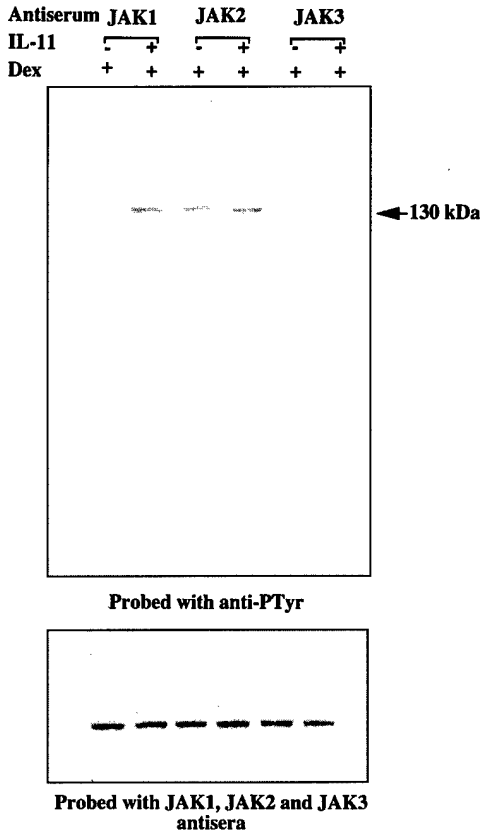

FIG. 2. **Effect of IL-11 on tyrosine phosphorylation of Jak1.** Adipose stromal cells in primary culture were placed in serum-free medium for 24 h. The cells were treated with dexamethasone (*Dex*) for 48 h before addition of IL-11 (10 ng/ml) for 10 min. The cells were collected and washed, and extracts were prepared. Aliquots of extracts (2×10^7 cells) from untreated and treated cells were immunoprecipitated with Jak1, Jak2, or Jak3 antisera as described. The immunoprecipitates were resolved by means of SDS-PAGE and transferred to filters. The filters were probed with the 4G10 anti-phosphotyrosine monoclonal antibody (*top panel*) or with a mixture of Jak1, Jak2, and Jak3 antisera (*lower panel*).

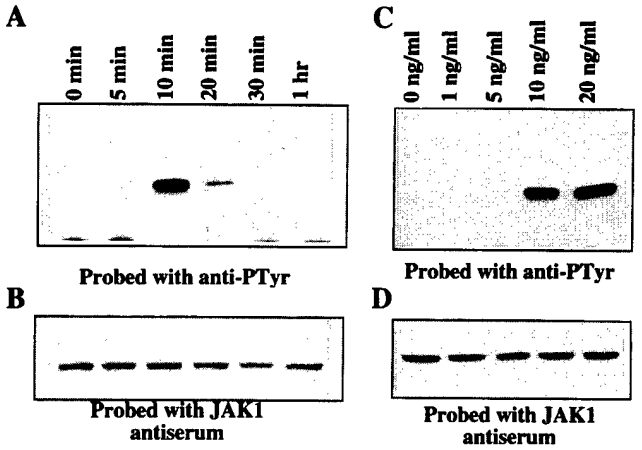

FIG. 3. **Time course and concentration dependence of tyrosine phosphorylation of Jak1.** Adipose stromal cells in primary culture were treated with dexamethasone (250 nM) for 48 h; IL-11 (10 ng/ml) was then added, and incubation continued for 5, 10, 20, 30, and 60 min. Cells were collected at the different time points, and extracts were prepared and then immunoprecipitated with Jak1 antiserum. The immunoprecipitates were fractionated by SDS-PAGE, transferred to filters, and probed with anti-phosphotyrosine monoclonal antibody (*panel A*) or Jak1 antiserum (*panel B*). Cells were treated with dexamethasone for 48 h; IL-11 was added at concentrations of 1, 5, 10, and 20 ng/ml; and incubation was continued for 10 min. Cells were collected, and extracts were prepared. The extracts were precipitated with Jak1 antiserum, and precipitates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride filter. The transferred proteins were probed with anti-phosphotyrosine monoclonal antibody (*C*) or Jak1 antiserum (*D*). Other details are described under "Experimental Procedures."

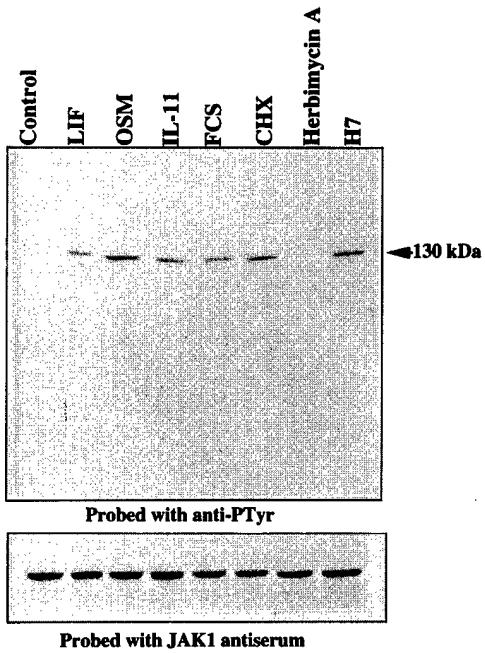

FIG. 4. **Effect of OSM and LIF on tyrosine phosphorylation of Jak1 in adipose stromal cells in primary culture.** Cells were treated with dexamethasone (250 nM) for 48 h; and then OSM (5 ng/ml), LIF (5 ng/ml), IL-11 (10 ng/ml), or FCS (15%) were added, and incubation continued for 10 min. Following treatment with dexamethasone, cells in other dishes were incubated with herbimycin A (5.2 μM) for 14 h, H7 (40 μM) for 30 min, or cycloheximide (*CHX*) (10 μM) for 6 h as indicated. Then IL-11 (10 ng/ml) was added, and incubation continued for a further 10 min. Immunoprecipitation and Western blot analysis were carried out as described under "Experimental Procedures."

To examine the binding activity of nuclear factors to the GAS element of promoter I.4, nuclear extracts were prepared from adipose stromal cells treated with dexamethasone for 48 h and treated with IL-11 for 30 min. Incubation of nuclear proteins from cells treated with IL-11 plus dexamethasone or serum plus dexamethasone with the wild-type promoter I.4 GAS element between −288 and −269 bp as radiolabeled probe (5′-GGGTGTTTCCTGTGAAAGTT-3′) gave rise to a single band (Fig. 6). The band was barely detectable in cells treated with dexamethasone alone. A 100-fold excess of nonradiolabeled consensus GAS sequence resulted in complete competition of the DNA binding (*lane 5*), although use of a mutated sequence (see above) resulted in no displacement (data not shown). In addition, binding of the probe to nuclear protein was displaced when anti-STAT3 serum was added (*lane 7*) but not when anti-STAT1 or control nonimmune sera were employed (data not shown).

Southwestern blot analysis also was performed to further examine the binding of the GAS probe to nuclear proteins prepared from cells treated with IL-11 plus dexamethasone. Nuclear proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The transferred proteins were subjected to a denaturation/renaturation cycle and hybridized with the radiolabeled probe, followed by autoradiography. As shown in Fig. 7, the radiolabeled GAS probe hybridized to a 92-kDa protein; the intensity of the band was greatly increased when nuclear extracts from cells treated with IL-11 or serum plus dexamethasone were employed, as compared with those treated with dexamethasone alone. The apparent

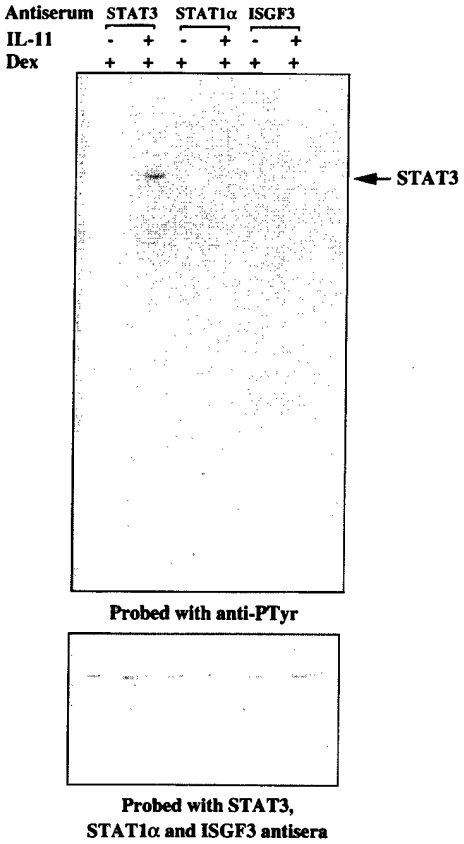

FIG. 5. **Effect of IL-11 on tyrosine phosphorylation of STAT3.** Adipose stromal cells were treated with dexamethasone (*Dex*; 250 nM) for 48 h. IL-11 (10 ng/ml) was then added, and incubation continued for 10 min. Extracts were prepared, and aliquots were immunoprecipitated with appropriate antisera as indicated. Immunoprecipitates were resolved on SDS-PAGE and transferred to a nylon membrane. The transferred proteins were probed with anti-phosphotyrosine monoclonal antibody (*top panel*) or a mixture of appropriate antisera (*lower panel*) as described under "Experimental Procedures."

molecular mass of the nuclear protein that bound to the probe (92 kDa) was similar to that of STAT3 (43). In control experiments, when the mutated GAS sequence was used as probe, no hybridization was detected. Additionally, hybridization to the native probe was also conducted in the presence and absence of a 100-fold excess of native and mutated sequence. Whereas the former resulted in displacement of the radiolabeled probe, the latter did not (data not shown).

The GAS Element Is Essential for Expression of P450arom Fusion Gene Constructs in Adipose Stromal Cells Incubated with IL-11—Genomic constructs containing the wild type GAS, a deletion mutation, and a site-directed mutation fused upstream of the CAT reporter gene were transfected into adipose stromal cells by means of calcium phosphate coprecipitation. Cells were allowed to recover overnight in medium containing 10% serum and then deprived of serum for 24 h followed by dexamethasone treatment for 48 h and IL-11 or serum treatment for 16 h. Cytosolic proteins were prepared, and CAT assays were performed (Fig. 8). CAT reporter activity was undetectable in cells treated with dexamethasone alone, but it was readily apparent in extracts of cells treated with IL-11 or serum plus dexamethasone. CAT reporter gene expression was lost when the GAS element was deleted or mutated to the sequence TTCGACTGAA. These results indicate that the intact GAS element is essential for IL-11- and serum-induced expression driven by promoter I.4.

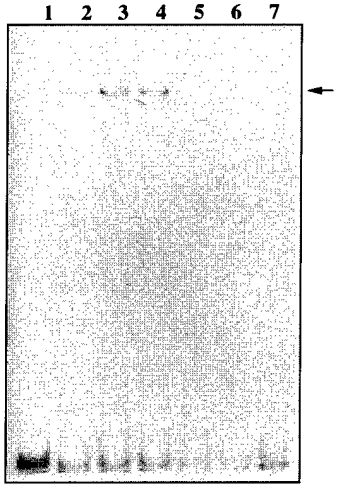

FIG. 6. **Gel mobility shift analysis of proteins binding to the GAS element.** Adipose stromal cells in primary culture were treated with dexamethasone (250 nM) for 48 h and then with IL-11 (10 ng/ml) or serum for 30 min. Nuclear extracts were analyzed by gel shift analysis employing the ^{32}P-labeled −288/−269 bp fragment as probe. Nuclear extracts prepared from cells treated with dexamethasone (250 nM) alone (*lane 2*), IL-11 (10 μg/ml) plus dexamethasone (*lane 3*), or 15% FCS plus dexamethasone (*lane 4*) were incubated with the radiolabeled −288/−269 bp fragment, and the reaction mixtures were subjected to polyacrylamide gel electrophoresis in an 8% gel. For competition, a 100-fold molar excess of the nonradiolabeled −288/−269 bp fragment (*lane 5*) was added to the incubation mixture. To determine whether STAT3 is a component of the protein binding to the GAS, anti-STAT3 serum (2.5 μl) was incubated with the radiolabeled DNA probe in the absence (*lane 6*) or in the presence of nuclear extracts (*lane 7*). *Lane 1*, free probe electrophoresed in the absence of nuclear extract. Other details are described under "Experimental Procedures." The *arrow* indicates the position of the radiolabeled band.

DISCUSSION

The findings of the present study reveal a hitherto unrecognized role for a Jak/STAT signaling pathway, namely the stimulation of expression of the P450arom gene and hence of estrogen biosynthesis in human adipose tissue. The extracellular ligands that initiate this response are members of the IL-11/OSM/LIF family of lymphokines (50, 51). Ligands that have no effect include interferon-α, interferon-γ, and IL-6. However, responsiveness to IL-6 is established upon addition of soluble IL-6 receptor. Members of this lymphokine family employ a receptor system involving two different Jak-associated components, gp130 and LIFRβ, or a related β-component (52). However, the IL-6 receptor complex includes an α component whose cytoplasmic domain is apparently not involved in signaling (52) and which can exist in a soluble form (53). Recently an α-subunit of the IL-11 receptor complex has been cloned (54), although this does not apparently exist in a soluble form. The concentration dependence of the stimulation of aromatase by IL-6, IL-11, LIF, and OSM is indicative of high affinity receptor binding, since half-maximal stimulation was achieved at ligand concentrations of approximately 2 ng/ml (considerably less in the case of LIF), which corresponds to a molar concentration of $\sim 10^{-10}$ M. Of the stimulatory lymphokines, the response to OSM in the presence of dexamethasone was the greatest in terms of aromatase induction and far exceeded the response to serum. Addition of LIF or IL-11 together with OSM resulted in no further increase in stimulation, suggesting that aromatase expression was maximally induced in the presence of OSM and that all of these lymphokines utilized the same signal transduction pathway (data not shown). It should be noted that a variety of other growth factors have no action to stimulate aromatase expression of adipose stromal cells including epidermal growth factor, platelet-derived growth factor, fibroblast

growth factor, growth hormone, prolactin, and IGF-1 (data not shown).

Addition of IL-11 resulted in a rapid phosphorylation of Jak1 kinase in a concentration-dependent fashion, with a maximal effect obtained after 10 min and at a concentration of IL-11 of 10 ng/ml, similar to the concentration of IL-11 required for maximal stimulation of aromatase activity. By contrast, Jak3 kinase was not phosphorylated under these conditions to any significant extent, whereas Jak2 kinase was phosphorylated to an equal extent both in the presence and absence of IL-11. A similar action of interferon-α has been reported in human T-lymphocytes (49). As indicated by blotting with an anti-phosphotyrosine antibody and by inhibition in the presence of herbimycin A, this phosphorylation occurred on tyrosine residues present in the Jak1 kinase. Both gp130 and LIFRβ can associate with and activate at least three members of the Jak family, Jak1, Jak2, and Tyk2, but utilize different combinations of these in different cells (50). From the results presented here, it is apparent that Jak1 is the kinase of choice in human adipose stromal cells. Although there was a rapid phosphorylation of Jak1 on tyrosine residues, Western blot analysis utilizing an antibody against Jak1 indicated that there was no change in the absolute levels of Jak1 throughout this time period of stimulation.

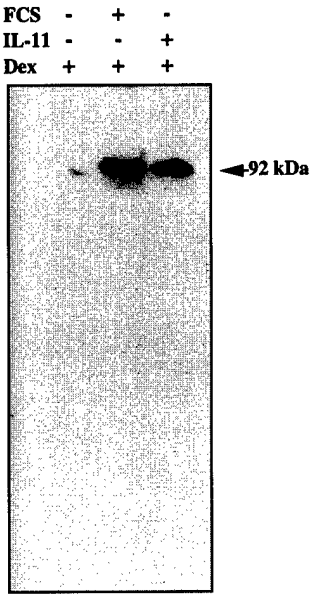

FIG. 7. **Southwestern blot analysis of proteins binding to the GAS element.** Nuclear extracts (60 μg) were separated by SDS-PAGE on an 8% gel, and proteins were transferred to a nitrocellulose membrane. The transferred proteins were subjected to a denaturation/renaturation process and hybridized to the ^{32}P-radiolabeled −288/−269 bp fragment followed by autoradiography. *Lane 1*, nuclear extracts prepared from cells treated with dexamethasone (*Dex*; 250 nM) alone; *lane 2*, nuclear extracts prepared from cells treated with dexamethasone plus IL-11 (10 ng/ml); *lane 3*, nuclear extracts prepared from cells treated with dexamethasone in the presence of serum (15% FCS). Other details are described under "Experimental Procedures."

The action of IL-11 also results in the rapid phosphorylation of STAT3 on tyrosine residues, but this was not the case for STAT1. Recently it has been shown that STAT3 is the substrate of choice for the IL-6/LIF/OSM lymphokine receptor family and that the specificity of STAT phosphorylation is not based upon which Jak kinase is activated (43, 50, 55) but rather is determined by specific tyrosine-based motifs in the receptor components, namely gp130 and LIFRβ, shared by these lymphokines (56). Gel shift analysis, utilizing a double-stranded oligonucleotide corresponding to the wild-type GAS sequence in the promoter I.4 region of the P450arom gene as a probe, indicated binding to a single component. This binding was barely detectable in control cells but was present within 30 min of addition of IL-11 to the cells. This binding was competed by addition of excess nonradiolabeled probe and was also competed upon addition of anti-STAT3 antibody. These results are indicative that STAT3 can interact with the GAS element

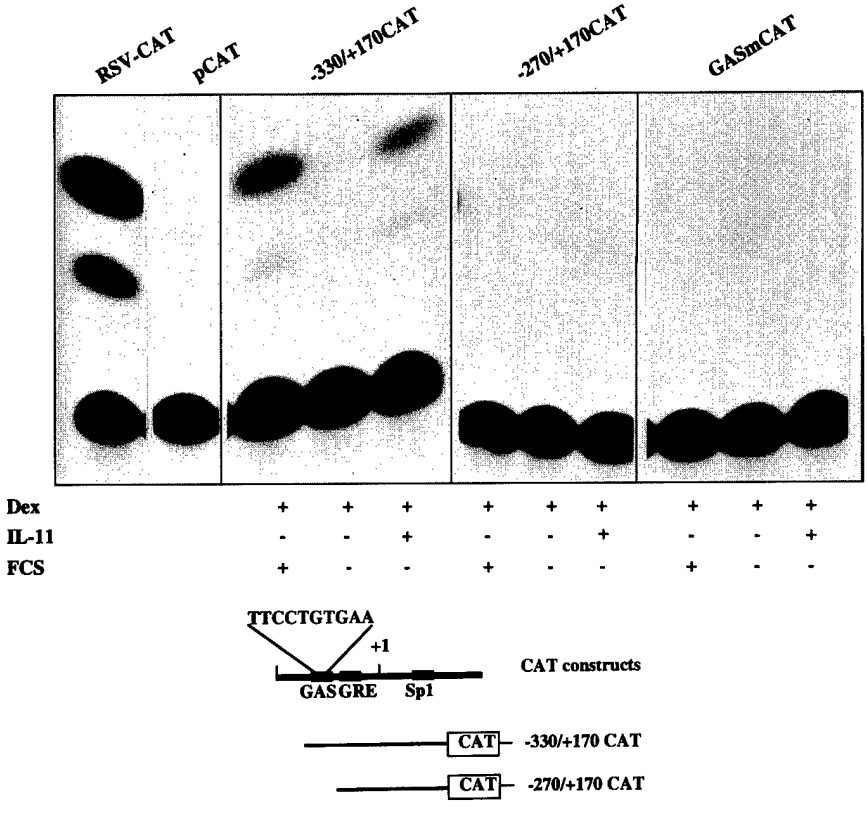

FIG. 8. **Role of the GAS element in transient expression of −330/+170 bp/CAT fusion gene construct.** Fusion gene constructs containing the wild-type −330/+170 bp sequence, the sequence in which the GAS element was deleted (−270/+170 bp), and the mutated GAS sequence (GASmCAT) linked to CAT, were transfected into adipose stromal cells in primary culture. Cells were treated with 250 nM dexamethasone (*Dex*) for 48 h and incubated with or without IL-11 (10 ng/ml) and serum (15% FCS) for 16 h. Cells were then harvested, and lysates were prepared for assay of CAT activity. The products of the CAT reaction were analyzed by TLC followed by autoradiography. RSV-CAT and pCAT are positive and negative vector controls, respectively. Other details are described under "Experimental Procedures."

present in the promoter I.4 region of the P450arom gene upon addition of IL-11 to these cells. This interaction in turn results in activation of expression, as indicated by transfection experiments employing chimeric constructs in which the region −330/+170 bp of the I.4 promoter region was fused upstream of the CAT reporter gene. The results indicate that deletion of the GAS sequence, as well as mutagenesis of this sequence, resulted in complete loss of IL-11- and serum-stimulated expression in the presence of glucocorticoids.

Activation of this pathway of expression by these lymphokines is absolutely dependent on the presence of glucocorticoids. This action of glucocorticoids is mediated by a glucocorticoid response element downstream of the GAS element (40). Additionally, an Sp1-like element present within untranslated exon I.4 also is required, at least for expression of the −330/+170 bp construct (40). These sequences, while present within a 400-bp region of the gene, are not contiguous, and the nature of the interaction among STAT3, the glucocorticoid receptor, and Sp1, to regulate expression of the P450arom gene via the distal promoter I.4 remains to be determined.

Activation of aromatase expression by serum in the presence of glucocorticoids is not confined to cells present in adipose tissue but also has been reported in skin fibroblasts (57) and in hepatocytes derived from fetal liver (58). In each of these cell types the P450arom transcripts contain exon I.4 as their 5′-terminus (27, 59); however, the factors that mimic the action of serum to stimulate aromatase expression in these cell types have as yet to be elucidated. On the other hand, in placenta where the distal promoter I.1 is employed (20) and in ovarian granulosa cells where the proximal promoter II is employed (20, 23), this signaling pathway is not in effect. Thus, aromatase expression in ovarian granulosa cells is driven primarily by cyclic AMP-dependent mechanisms (60).

As indicated previously, adipose tissue is the major site of estrogen biosynthesis in elderly women and men. The fact that this expression is confined to the stromal cells rather than the adipocytes themselves is consistent with the known actions of IL-11 to inhibit the differentiation of 3T3 L1 fibroblasts into adipocytes (61). Since adipose stromal cells are believed to function as preadipocytes and can be converted to adipocytes under appropriate nutritional stimuli, a role of these lymphokines may be to maintain these cells in the preadipocyte state for which aromatase expression is a marker. As indicated previously, aromatase expression in adipose increases dramatically with age (6, 29). There is also a marked regional distribution, with expression being greatest in buttock and thigh regions as compared with abdomen and breast (29, 30). However, within the breast there is also a marked regional variation with expression being highest in sites proximal to a tumor as compared with those distal to a tumor (31, 32).

Based on the results presented here we suggest that aromatase expression in adipose tissue may be under tonic control by circulating glucocorticoids and that regional and age-dependent variations may be the consequence of paracrine and autocrine secretion of lymphokines. Schmidt and Loffler (62), as well as ourselves (64), have shown that conditioned medium from a number of cell types including adipose stromal cells themselves and endometrial stromal cells, as well as breast tumor cells lines, can mimic the actions of serum to stimulate aromatase expression in the presence of glucocorticoids. Thus numerous cell types including breast cancer cells produce factors that are stimulatory of aromatase expression by adipose stromal cells. In preliminary experiments we have shown that a stimulatory factor present in conditioned medium from T47D breast cancer cells can be titrated by an anti-IL-11 antibody (data not shown). Additionally, Reed and colleagues have found that fibroblasts derived from breast tumors secrete IL-6 (34, 63).

Such local paracrine mechanisms could be important in the stimulation of breast cancer growth by estrogens. Commonly, breast tumors produce a desmoplastic reaction whereby there is local proliferation of stromal cells surrounding the tumor, strongly indicative of the production of growth factors by the tumor. These proliferating stromal cells express aromatase, as indicated by immunocytochemistry (35). It is possible then to envision a positive feedback loop whereby adipose stromal cells surrounding a developing tumor produce estrogens, which stimulate the tumor to produce a variety of growth factors and cytokines (64). Some of these act to stimulate the further growth and development of the tumor in a paracrine and autocrine fashion. Additionally, these or other factors act to stimulate proliferation of the surrounding stromal cells and expression of aromatase within these cells. Thus a positive feedback loop is established by paracrine and autocrine mechanisms, which leads to the continuing growth and development of the tumor (32, 36). Further insight into the paracrine mechanisms involved in regulation of estrogen biosynthesis in human adipose tissue will await the characterization of the particular cytokines that are being produced as well as their levels of expression, both of which may vary in a region- and age-dependent fashion.

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Quantitative detection of alternatively spliced transcripts of the aromatase cytochrome P450 (*CYP19*) gene in aromatase-expressing human cells by competitive RT-PCR

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C19 steroids are converted to oestrogens in a number of tissues by a specific form of cytochrome P450, namely aromatase P450 (P450arom; the product of the *CYP19* gene). The human *CYP19* gene comprises nine coding exons, II–X. The tissue-specific expression is determined by the use of tissue-specific promoters, which give rise to P450arom transcripts with unique 5′-untranslated sequences. The majority of the transcripts present in the ovary contain promoter-II-specific sequences, while transcripts in the placenta contain exon I.1. Transcripts in adipose tissue possess exon I.3 and exon I.4. Also, the distribution of alternative transcripts in adipose stromal cells depends on the culture conditions. Therefore, a competitive RT-PCR method was designed to quantitatively detect alternatively spliced transcripts present in various tissues and cells maintained in different culture conditions. Specific synthetic transcripts with different 5′ termini (exon I.3, exon I.4 and promoter-II-specific sequences) and the coding region were used as internal standards. This competitive RT-PCR method was used to quantitatively detect three 5′ termini, i.e. promoter-II-specific sequence, exon I.3 and exon I.4, in transcripts in human adipose stromal cells and ovarian granulosa cells in primary culture. The quantity of total P450arom transcripts was judged by amplifying the coding region. We were also able to quantify rare transcripts which could not be detected previously by Northern analysis.

KEYWORDS: alternatively spliced transcripts, competitive RT-PCR, human, aromatase cytochrome P450 (*CYP19*) gene.

INTRODUCTION

The biosynthesis of oestrogens from androgens is catalysed by an enzyme termed aromatase cytochrome P450 (P450arom; the product of the *CYP19* gene) which is localized in the endoplasmic reticulum of cells in which it is expressed.[1–5] This enzyme is responsible for binding the C_{19} steroid substrate and catalysing the series of reactions leading to the formation of the phenolic A ring characteristic of oestrogens.[6–10]

In most vertebrate species examined, aromatase expression occurs in the gonads and in the brain. In the case of humans, the aromatization reaction occurs

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in a number of cells and tissues including ovarian granulosa cells,[11] testicular Sertoli,[12] Leydig cells,[13,14] placenta[2,3] and adipose tissue of both males and females,[15] and various sites of the brain including the hypothalamus,[16] amygdala and hippocampus.[17] However, the principal sites of oestrogen biosynthesis in the human female are the ovarian granulosa cells in pre-menopausal women, the placenta in pregnant women and adipose tissue in post-menopausal women. The physiological significance of oestrogen biosynthesis in human placenta and adipose tissue is unclear at this time; however, the latter has been implicated in a number of pathophysiologic conditions. Oestrogen biosynthesis by adipose tissue not only increases as a function of body weight but also as a function of age.[18,19] Further, there appears to be a relationship between oestrogen biosynthesis in adipose and several disease states such as post-menopausal endometrial and breast cancer.[20,21]

The human *CYP19* gene encoding aromatase cytochrome P450 has been cloned and spans more than 75 kilobases (kb) in size; however, the coding region is about 35 kb and contains nine exons (II–X). The heme-binding region is located on the last coding exon (X), and the translation initiation site is located in exon II. Upstream of exon II are located a number of untranslated exons which are spliced into the 5′ ends of transcripts in a tissue-specific fashion.[22] For this reason, specific 5′ termini are found in the transcripts encoding P450arom in different tissues. All of the sequences are spliced onto exon II at the same 3′ splice junction, upstream of the start of translation, and thus the coding region and the protein are the same regardless of the tissue-site of expression. The majority of the transcripts present in the ovary contain sequences specific for promoter II, whereas transcripts in the placenta contain sequences specific for exon I.1. Adipose tissue expresses two species of transcripts, containing exon I.3- and I.4-specific sequences.[23] Also, the distribution of alternative transcripts in adipose stromal cells[23] depends on their culture conditions. Thus, the cytochrome P450arom gene encodes a number of alternatively spliced transcripts in various tissues regulated by tissue-specific promoters. In order to comprehend the regulation of the *CYP19* gene in any given tissue, it is very important to have a quantitative estimation of the various transcripts containing the different 5′ termini present in that particular tissue. The RACE (rapid amplification of cDNA ends) procedure was utilized previously to amplify alternative 5′ termini sequences from P450 arom transcripts.[23] However, this method may not amplify different transcripts with equal efficiency, and thus may not yield a true quantitative distribution of the various 5′ termini in *CYP19* gene transcripts in a particular cell or tissue. Northern analysis can detect only abundantly expressed transcripts. Therefore, considering the complexity of the expression of alternatively spliced transcripts of the *CYP19* gene, it became essential to design a sensitive method to detect the 5′ termini of various transcripts.

Here, we describe a competitive RT-PCR method to quantify three major 5′ termini of *CYP19* gene transcripts, i.e. promoter-II-specific and exon I.3- and exon I.4-specific. Using this method we were able to quantitatively detect multiple transcripts present in primary cultures of human adipose stromal cells and ovarian granulosa cells grown in different culture conditions. Also, for the first time, we report analysis of the alternative transcripts present in cultured adipose stromal cells treated with dexamethasone and the cytokine IL-11. Most importantly, this method is also applicable to quantitative analysis of alternatively spliced transcripts of other genes in which this form of regulation of expression is utilized.

MATERIALS AND METHODS

Internal standards

Figure 1 schematically depicts the principle and steps involved in generating internal standards which were used as competitors for RT-PCR of human P450arom transcripts. To prepare internal standards, three PCR products (AP-PII, AP-I.3 and AP-I.4) were amplified from partial cDNA clones (RACE clones[23]) specific for promoter-II-specific sequence, exon I.3 and exon I.4, respectively. RT-1, RT-2, RT-3 (sense oligos specific for each untranslated exon) and RT-4 (antisense oligo for exon II) were used as primers, and their sequences are given in Table 1. Simultaneously, one PCR product (AP-Cod) was amplified from a cytochrome P450arom cDNA clone[24] using the sense primer (RT-5) from the non-coding region of exon II and the antisense primer (RT-6) from exon IV. PCRs were set up as described in the detailed protocol of RT-PCR (see following paragraph), except [α-^{32}P] dCTP was not included, and were performed on a Gene Amp PCR system 9600 (Perkin-Elmer). Reaction conditions were as follows: denaturing temperature 93°C for 1 min, annealing temperature 41°C for 1 min, extension temperature 72°C for 1 min, total cycles 30. By combining one of each of the AP-PII, AP-I.3, AP-I.4 (PCR products) with AP-Cod (PCR product), PCR was used to amplify three different sequences containing alternative 5′ termini, i.e. promoter-II-specific sequence, exon I.3 or exon I.4. The antisense primer (RT-13, Table 2) contains a 27 bp random AT rich

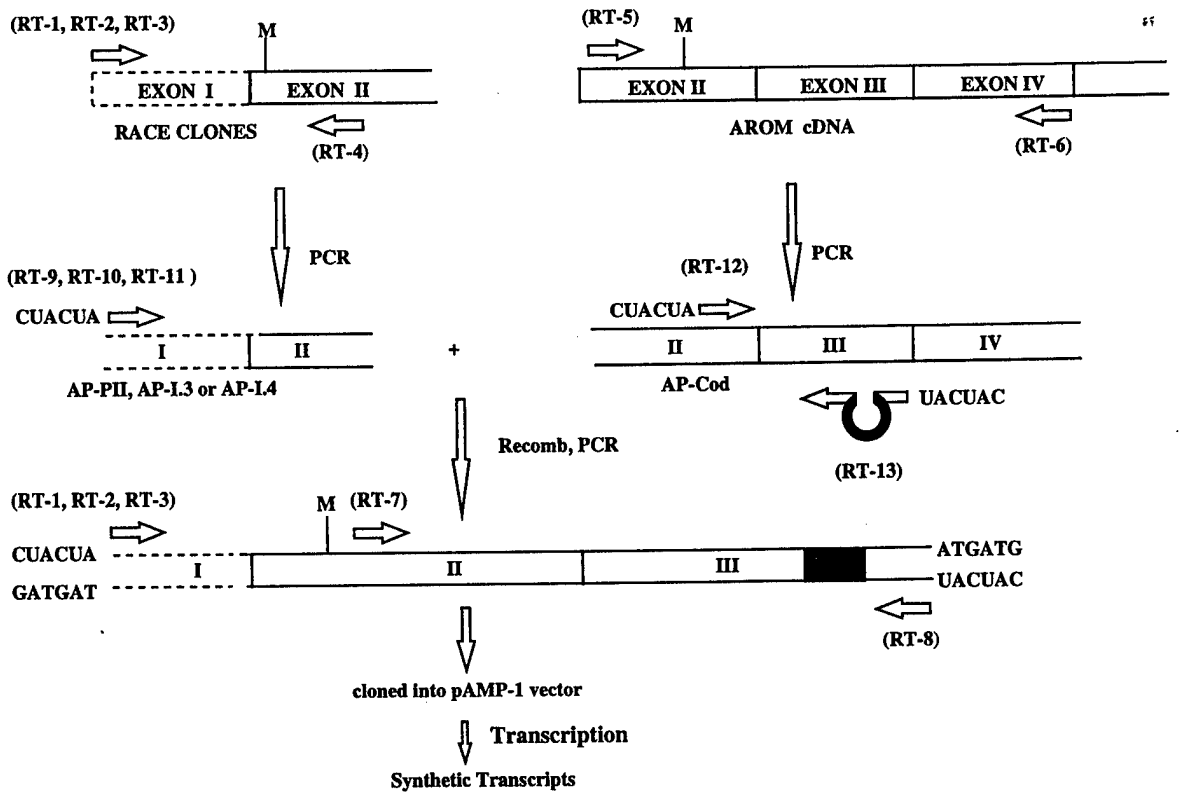

Fig. 1. Schematic diagram of the synthesis of internal standard transcripts. Dashed lines depicts the variable region of clones. All numbers in parentheses represent the oligo number (for sequences see Tables 1 and 2). Black region in oligo RT-13 and final PCR product represents the 27 bp random AT-rich insert sequence (loop).

Table 1. Oligonucleotides used as primers for PCR amplifications

RT-1	5′ end sense oligo from exon I.4 5′ GTG ACC AAC TGG AGC CTG 3′
RT-2	5′ end sense oligo from exon I.3 5′ GAT AAG GTT CTA TCA GAC C 3′
RT-3	5′ end sense oligo from promoter-II-specific sequence 5′ GCA ACA GGA GCT ATA GAT 3′
RT-4	3′ end antisense oligo from coding exon II 5′ CAG GCA CGA TGC TGG TGA TG 3′
RT-5	5′ end sense oligo from untranslated region of exon II 5′ TCT GAG GTC AAG GAA CAC 3′
RT-6	3′ end antisense oligo from exon IV 5′ TTG TTG TTA AAT ATG ATG C 3′
RT-7	5′ end sense oligo from coding exon II 5′ TTG GAA ATG CTG AAC CCG AT 3′
RT-8	3′ end antisense oligo from exon III 5′ CAG GAA TCT GCC GTG GGG AT 3′
GAPDH1	5′ end sense oligo from GAPDH gene 5′ CGG AGT CAA CGG ATT TGG TCG TAT 3′
GAPDH2	3′ end antisense oligo from GAPDH gene 5′ AGC CTT CTC CAT GGT GGT GAA GAC 3′

region (loop[25]) and was designed to include a sequence specific to exon III on either side of the 27 bp loop region (Fig. 1). Sense primers (RT-9, RT-10, RT-11) were 5′ termini specific. Primers used in these reactions are listed in Table 2. A similar reaction was set up for amplifying part of the coding region (Exon II–Exon III) using primers RT-12 and RT-13 (Table 2) and a P450arom cDNA clone[24] as a template. CUA tails in the sense primers (5′ end primers, RT-9, RT-10, RT-11, RT-12) and a CAU tail in the antisense primer (3′ end primer, RT-13) were incorporated according to the CloneAmp System instructions (Gibco-BRL). The PCR products were then cloned into the pAMP-1 vector. These constructs were transcribed using SP6 polymerase according to the Promega Kit (Madison, WI, USA). Thus four internal standard transcripts were synthesized, each with the 27 bp random AT rich loop. Three of them are specific for the three major 5′ termini of the *CYP19* gene, i.e. exon I.3, I.4 and promoter-II-specific sequences and one transcript specific for the coding region between exons II and III. A known copy number of these transcripts was added to each RT-PCR reaction containing target RNA from the various tissues studied.

Table 2. Oligonucleotides used as primers for PCR amplification of specific regions of alternate exons I, which were subsequently cloned into the vector

RT-9	5′ end sense oligo from exon I.4 5′ CUA CUA CUA CUA GTA GAA CGT GAC CAA CTG 3′
RT-10	5′ end sense oligo from exon I.3 5′ CUA CUA CUA CUA GAT AAG GTT CTA TCA GAC 3′
RT-11	5′ end sense oligo from promoter-II-specific sequence 5′ CUA CUA CUA CUA GCA ACA GGA GCT ATA GAT 3′
RT-12	5′ end sense oligo from coding exon II 5′ CUA CUA CUA CUA TTG GAA ATG CTG AAC CCG AT 3′
RT-13	3′ end antisense oligo from exon III containing a 27 bp random AT rich insert 5′ CAU CAU CAU CAU CAC AGG AAT CTG CCG TGG GGA TGA GGG GTC CAA TTC AAA TAT TTA TAT TAA AAT TAT TTT AAA CCA TGC AGT AGC CAG 3′

Underlined nucleotides represent a 27 bp random AT rich sequence inserted in the oligonucleotide.

Table 3. Expected sizes of PCR amplified products

Alternative Exon I	Target	Internal standard
Exon I.4–Exon III	294 bp	321 bp
Exon I.3–Exon III	395 bp	422 bp
Exon I.3 (truncate)–Exon III	289 bp	
Exon II–Exon III (promoter-II-specific)	305 bp	332 bp
Exon II–Exon III (coding region)	194 bp	221 bp
GAPDH	306 bp	

RT-PCR

RNA samples were treated with DNase I (Gibco-BRL) at room temperature for 15 min to remove DNA contamination from the RNA samples. DNase I was inactivated at 90°C for 5 min. Then RNA was denatured at 65°C for 2 min and annealed to random hexamers (Gibco-BRL) by allowing the samples to cool slowly to 37°C. To synthesize the complementary DNA (cDNA), the annealed RNA was reverse-transcribed using Superscript II (Gibco-BRL) at 42°C for 1 h in the presence of 166 μM of each dNTP, 50 mM Tris-HCl (pH 8·3), 75 mM KCL, 3 mM $MgCl_2$, 10 mM DTT and 5 U of RNasin (Promega). cDNA was amplified using the polymerase chain reaction in the presence of 200 μM of each dNTP, 4 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8·3, 0·001% (w/v) gelatin, 10 pmol of each primer, 0·3 μCi of [α-^{32}P] dCTP (Amersham) and 2 U of *Taq* Polymerase (Perkin-Elmer) in a total volume of 10 μl. RT-1, RT-2, RT-3 and RT-7 primers (Table 1), specific for exon I.4, exon I.3, promoter-II-specific sequence and coding exon II respectively, were used as 5′ end antisense primers. RT-8 primer (Table 1) specific for exon III was used as 3′ end antisense primer. PCR conditions were: denaturing at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s, for 25 cycles and were performed on a Gene Amp PCR system 9600 (Perkin-Elmer). One quarter of the PCR products was analysed on 4% non-denaturing polyacrylamide gels. Labelled molecular weight marker (pBR322-Msp1-digested, New England Biolabs) was loaded simultaneously on the gel to assess the sizes of the amplified products. The molecular weight marker was end-labelled using 10 U of T4 polynucleotide kinase (Gibco-BRL) in the presence of 70 mM Tris-HCl (pH 7·6), 10 mM $MgCl_2$, 100 mM KCl, 1 mM of 2-mercaptoethanol and 30 μCi of [γ-^{32}P] ATP (Amersham). Gels were either autoradiographed with Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA) or scanned on a PhosphorImager (Molecular Dynamics, CA, USA) and quantitatively analysed using Image-Quant software. Expected sizes of PCR products are listed in Table 3.

To check the integrity of cDNA, the 'housekeeping' gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous marker. Primers were designed from the sequence deposited in Genbank (Table 1). cDNAs were amplified in the presence of 2 pmol of each primer, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8·3), 1·5 mM $MgCl_2$, 50 mM KCl, 0·001% (w/v) gelatin, 0·3 μCi of [α-^{32}P] dCTP (Amersham) and 2 U of *Taq* polymerase (Perkin-Elmer) at 94°C for 1 min and 72°C for 1 min for 22 cycles.

RNA isolation

Total RNA was isolated from adipose stromal cells according to the described method with minor modifications.[26] Dishes of frozen confluent adipose stromal cells were scraped in guanidinium thiocyanate. Total RNA from granulosa cells was extracted as described.[27] Total RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water and then precipitated with the addition of ethanol and potassium acetate.[26]

Cell culture

Subcutaneous adipose tissue was obtained from women at the time of reduction mammoplasty. Follicles were obtained from women undergoing hysterectomy or bilateral oophorectomy for benign gynecological disease. Written consent was given preoperatively for the use of these tissues using a consent form. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were isolated and maintained as primary cultures in Waymouth's enriched medium containing Nu Serum (15%, v/v) (Collaborative Research Inc.) as described previously.[28] Upon reaching confluence, the cells were placed in serum-free or FCS-containing (15%, v/v) Waymouth's enriched medium for 24 h. The cells were then treated with dexamethasone (250 nM) in medium containing 15% FCS for 48 h to maximally induce P450arom mRNA levels. For treatment with dexamethasone and IL-11, IL-11 (10 ng ml^{-1}) was added to the culture medium after dexamethasone treatment and cells were incubated for 24 h. Media were removed, and the cells were frozen at −70°C until used for RNA isolation. Human granulosa-lutein cells were obtained and cultured as previously described.[29] Cultured granulosa cells were treated with 10 μM forskolin for 48 h.

RESULTS

Herein we describe a sensitive quantitative RT-PCR method to detect three major alternatively spliced transcripts of the *CYP19* gene in human adipose stromal cells and granulosa cells. By using this method, we were also able to amplify the 5′ termini of transcripts which were present in very low copy number in tissues in the presence of other abundantly expressed transcripts. Before discussing these results in detail, we will describe the standardization and validation of our RT-PCR method.

A number of experiments were performed to select one set of PCR conditions and reagent concentrations (oligonucleotides, dNTPs and Mg^{2+}) which efficiently amplified all three 5′ ends of alternatively spliced transcripts (data not shown). Comparative efficiency of PCR amplifications for the 5′ termini, i.e. promoter-II-specific sequence, exon I.3, exon I.4 and the coding region of the P450arom gene, were determined experimentally. For these experiments, we used only the internal standard transcripts (synthetic transcripts), because the only difference between synthetic transcripts and target sequences was the 27 bp random

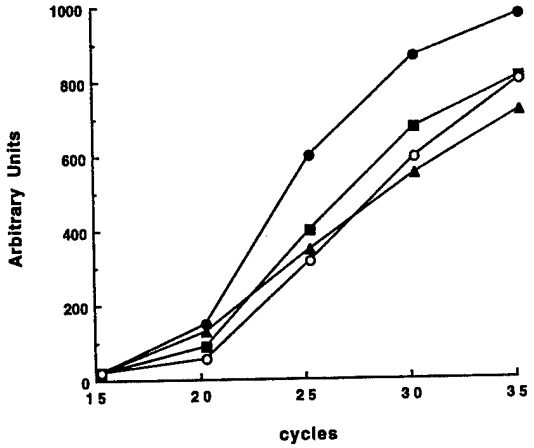

Fig. 2. The effect of the number of PCR cycles on amplification of synthetic transcripts (150 fg). Four reactions were set up, one for each of the synthetic transcripts. Aliquots were collected after the indicated number of cycles and subjected to gel electrophoresis and quantification by means of a phosphorimager. ▲, cod; ●, PII; ■ I.3; ○, I.4.

AT rich sequence inserted into the synthetic transcripts. Our PCR primers do not include that sequence, and are similar for target and synthetic transcripts. We preferred to use synthetic transcripts in comparison to amplified DNA fragments because we wanted to eliminate the differences caused by reverse transcription. Thus we mimicked all conditions for synthetic transcripts (internal standard) as for target transcripts. Therefore, amplification of the synthetic transcripts will reflect the efficiency of amplification of the corresponding unknown target transcripts. Experiments were designed to address each of the following issues:

(1) The quantitative nature of PCR has previously been questioned.[30,31] This conclusion was largely based on techniques which involved a high number of PCR cycles. To introduce more sensitive methods for detecting PCR products, submaximal numbers of cycles have been used which lie in the linear range of amplification. Amplification of the 5′ terminus of each transcript was tested after 15, 20, 25, 30 and 35 cycles by using the appropriate specific primers (Fig. 2). Twenty-five cycles were found to be the most appropriate because amplification under these conditions is within the linear range and is also submaximal. All experiments described hereafter employed amplification for 25 cycles.

(2) The amplification efficiency of transcripts containing three alternate 5′ termini (e.g. exon I.3, I.4 and promoter-II-specific) and the coding region may not be equal. To answer this question, equal quantities of all four synthetic transcripts were reverse transcribed according to the protocol described in

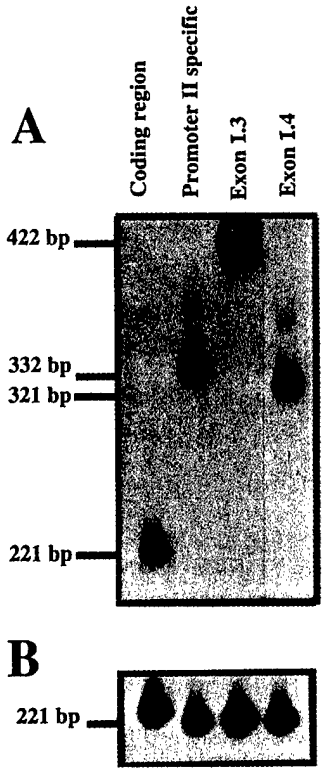

Fig. 3. Amplification of synthetic transcripts. (A) Each transcript was amplified using the appropriate specific primers as indicated. (B) Each transcript was amplified using coding-region-specific primers.

Materials and Methods and amplified by PCR using their corresponding primers (Table 1). Results are shown in Fig. 3. As expected, amplified bands were of expected size and of almost the same intensity (Fig. 3A). Equal quantities of all transcripts used were confirmed by amplification of the coding region (exon II–exon III) specific sequence which was common to all transcripts (Fig. 3B).

(3) Specific amplification of each 5′ terminus from the mixture of P450arom transcripts containing alternate 5′ termini may not be possible. To ensure that the products generated in the RT-PCR method were specific for the desired 5′ termini, we set up four PCRs using the cDNA of one synthetic transcript (internal standard), e.g. exon I.3-specific transcript, and four sets of primers for exon I.3, I.4, promoter-II-specific sequence and the coding region in each reaction (Fig. 4). Similar reactions were also set up for the cDNAs of the other three synthetic transcripts, i.e. for exon I.4, promoter-II-specific and coding region. As was expected, no cross-reaction (amplification) was detected, not even weak amplification after very long exposure. Thus, each synthetic transcript specific for one 5′ terminus was only amplified by primers specific for that sequence and for the coding region (Fig. 4).

(4) The amplification efficiencies of each transcript in the presence of the other alternatively spliced transcripts may not be equal. One picogram of each of the three synthetic transcripts, exon I.3, exon I.4 and promoter-II-specific, were mixed together. To approximate *in vivo* conditions, 50 ng of sheep kidney (a tissue lacking *CYP19* gene expression) RNA was added. The mixture was reverse transcribed, divided into four tubes and amplified by using the four sets of primers (Fig. 5). Amplification efficiencies of exon I.3, exon I.4 and promoter-II-specific sequences were almost similar. As was expected, amplification of the coding region was approximately three times higher than of other regions because the coding region is common in all synthetic transcripts.

(5) The most important question was: when varying quantities of alternatively spliced transcripts are present, will the amplification efficiency of the 5′ terminus of any one transcript be affected? To answer this question, we designed nine experiments (Table 4). Various quantities of each synthetic transcript were mixed with a constant amount of the other two transcripts resulting in nine different combinations. Sheep kidney RNA (50 ng) was added in each experiment to approximate an *in vivo* situation. Results are shown only for two experiments using varying concentrations of promoter-II-specific transcripts (Fig. 6). Similar experiments were performed with various concentrations of exon I.3 and exon I.4-specific transcripts (data not shown). We concluded that the amplification efficiency of the 5′ terminus of one transcript as a function of its concentration is not affected by the presence of different concentrations of other alternate transcripts (Fig. 6).

For standardization purposes, we used synthetic transcripts (internal standard) alone because they reflect the amplification efficiency of target sequences. When these synthetic transcripts were mixed with unknown target RNA, they should compete so that by comparing amplification of known quantities of internal standard, quantities of unknown transcripts can be calculated. Our goal was to quantitatively detect the 5′ termini of the several alternatively spliced transcripts from cells grown under different culture conditions as well as from tissues. Thus, we mixed three different quantities of internal standard transcript (coding-region specific) with the same quantity of RNA from adipose stromal cells grown in the absence of serum. As was expected, two amplified fragments were observed (Fig. 7). Internal standard cDNA amplification (221 bp) was competing with target cDNA amplification (194 bp). Identical experiments were performed using internal standard transcripts specific for exon I.4, exon I.3 and promoter-II-specific sequences, and similar patterns were obtained (results not shown).

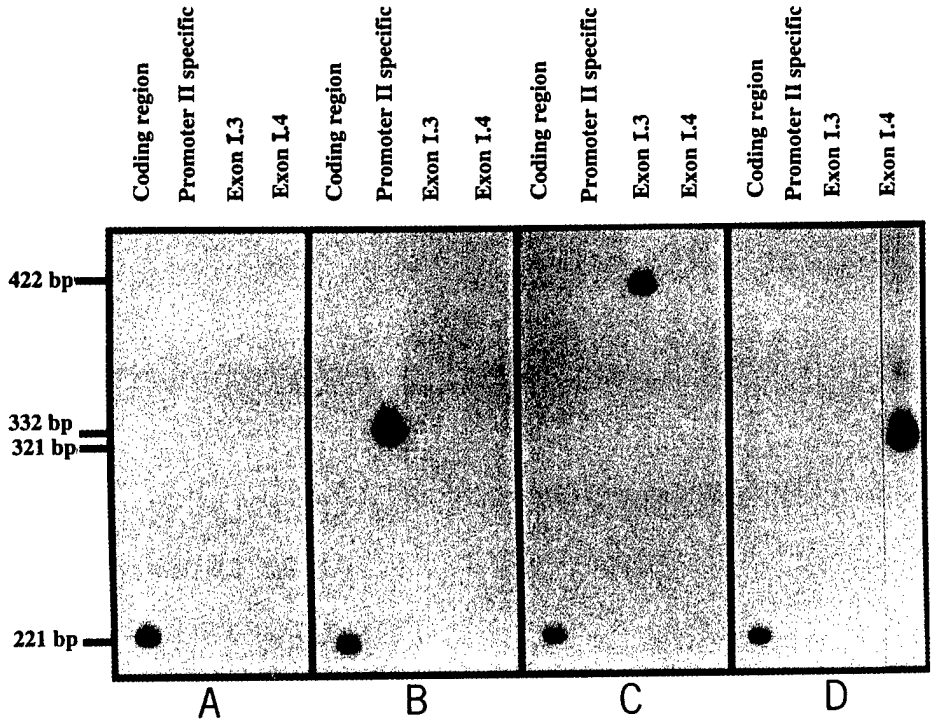

Fig. 4. Cross-amplification reactions. Four reactions were set up for each synthetic transcript by using primers specific for coding region, promoter-II-specific sequence, exon I.3 and exon I.4, as indicated. (A) Coding-region-specific transcript; (B) promoter-II-specific transcripts; (C) exon I.3-specific transcripts; (D) exon I.4-specific transcripts.

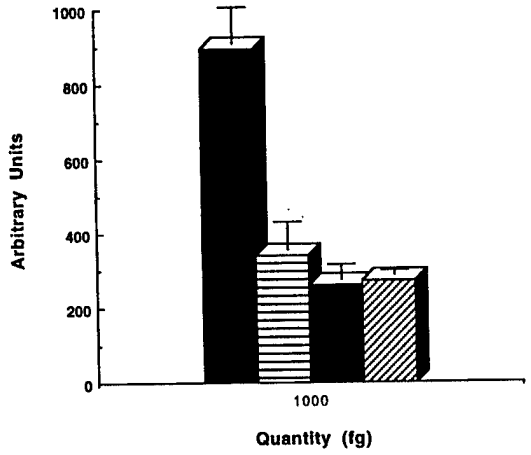

Fig. 5. Amplification of each transcript from the mixture of three synthetic transcripts (1 pg of each), employing specific primers. ■, cod; ▤, PII; ▦, I.3; ▨, I.4. Results shown are the mean ± SD of data from three separate experiments.

Results of the experiments using cultured cells are shown in Fig. 8 and Table 5. Quantification of each transcript in the cells was performed using two or three different concentrations of internal standard. The concentration at which amplified products of target and synthetic transcripts were equal was considered the concentration of target transcripts (corrected for size difference because molar ratio is equal). Because of the complexity of the gel, the data showing amplified products from target and internal standard transcripts for each 5′ terminus is not shown but results are presented in Table 5.

In human adipose stromal cells cultured in the absence of serum, the amplification products of promoter II and exon I.3 (truncate)-specific sequences appeared to be similar in abundance. There was no amplification of exon I.4-specific sequences (Fig. 8A). In adipose stromal cells treated with dexamethasone in the presence of serum, we observed good amplification of exon I.4 and I.3 (truncate)-specific sequences. There was very low expression of promoter-II-specific transcripts (Fig. 8B, Table 5). Transcripts containing exon I.4-specific sequences and exon I.3 (truncate)-specific sequences were almost equal in abundance (Table 5). Total P450arom transcript expression (detected by amplifying the coding region) was about four times higher than in adipose stromal cells grown in the absence of serum (Table 5). When human adipose stromal cells were treated with dexamethasone and the cytokine IL-11, total P450arom transcripts were present in a high copy number, that was similar to the abundance of total P450arom transcripts present in cells treated with dexamethasone plus serum (Table 5). All three 5′ termini were amplified (Table 5). Although transcripts containing exon I.4-specific sequences were the most

Table 4. Conditions used to standardize quantification of internal standard

Standard experiments	Amounts of exon-specific synthetic transcripts			
	Promoter-II-specific	Exon I.3	Exon I.4	Sheep kidney
1	50–1000 fg	50 fg	50 fg	50 ng
2	50–1000 fg	500 fg	500 fg	50 ng
3	50–1000 fg	1000 fg	1000 fg	50 ng
4	50 fg	50–1000 fg	50 fg	50 ng
5	500 fg	50–1000 fg	500 fg	50 ng
6	1000 fg	50–1000 fg	1000 fg	50 ng
7	50 fg	50 fg	50–1000 fg	50 ng
8	500 fg	500 fg	50–1000 fg	50 ng
9	1000 fg	1000 fg	50–1000 fg	50 ng

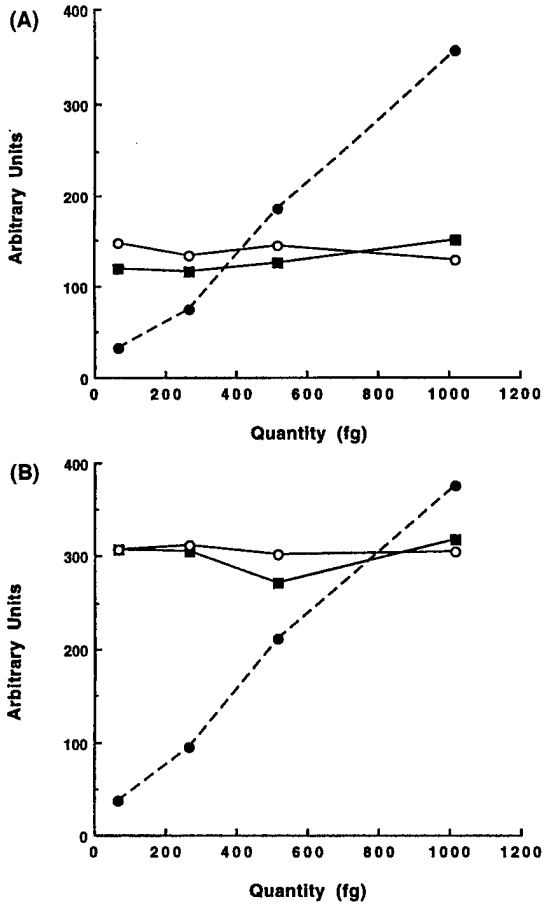

Fig. 6. Amplification of mixtures of three transcripts containing varying quantities of promoter-II-specific transcripts in the presence of a fixed amount [(A) 500 fg; (B) 1000 fg] of the other two transcripts, i.e. exon I.3 and exon I.4 (see Table 4 for details of concentration of each transcript in the mix). ●, PII; ■, I.3; ○, I.4.

abundant, promoter-II- and exon I.3 (truncate)-specific transcripts were also present in higher copy number in comparison to cells treated with dexamethasone alone.

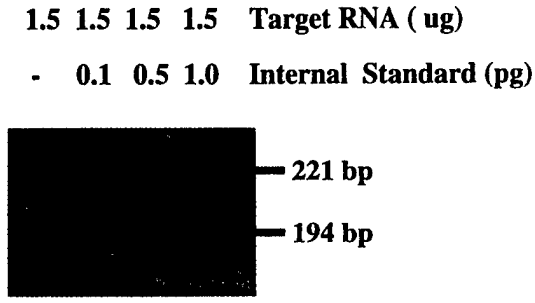

Fig. 7. Competitive RT-PCR: 1·5 μg of adipose stromal cell RNA was mixed with 0–1 pg of internal standard transcript (coding-region-specific) and amplified by using coding-region-specific primers. The size of the internal standard amplified product was 221 bp, and that of the target amplified product was 194 bp.

In primary cultures of human ovarian granulosa cells treated with forskolin for 48 h, promoter-II-specific sequences were those mainly amplified (Fig. 8C, Table 5). Transcripts containing exon I.3 (truncate)-specific sequences were in very low copy number (Table 5). Transcripts containing exon I.4 were undetectable.

DISCUSSION

Tissue-specific expression of the human *CYP19* gene appears to be regulated by tissue-specific promoters in the ovary, placenta and adipose tissue.[22] This conclusion is based on the presence of specific 5′ termini present in transcripts encoding P450arom in each of these tissues. Transcripts specific for proximal promoter-II-specific sequence are found in the ovary, whereas transcripts specific for the distal promoter I.1 are found uniquely in placenta. Adipose tissue contains mainly two species of transcripts with exon I.3 and I.4-specific sequences. On the other hand, when adipose stromal cells are maintained under

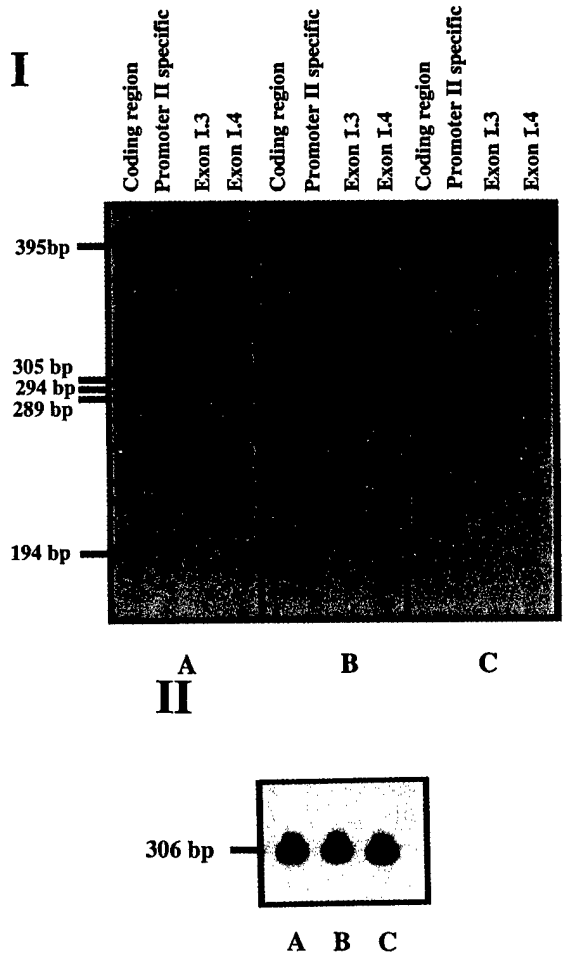

Fig. 8. (I) Amplification of specific 5′ terminal sequences from cDNA of 1 μg of RNA isolated from (A) adipose stromal cells maintained in the absence of dexamethasone and serum; (B) adipose stromal cells in the presence of dexamethasone +serum; (C) ovarian granulosa cells in the presence of forskolin. The specific 5′ terminus being amplified is indicated at the top of each lane. (II) Amplification of GAPDH cDNA from 500 ng RNA isolated from cultured cells. Panels A, B, C are as indicated above.

different culture conditions,[23] different 5′ termini are found. Therefore, it became very important to design a very sensitive, rapid and quantitative method to detect multiple alternatively spliced transcripts present in various human tissues and cells grown in different culture conditions. Thus, we designed a quantitative RT-PCR method in which we used four different internal standards (synthetic transcripts) specific for transcripts containing each 5′ terminus, i.e. promoter-II-specific sequence, exon I.3-, exon I.4-containing transcripts, as well as the coding region. Use of internal standards in quantitative RT-PCR has become common these days, but our method of synthesizing internal standards by inserting a 27 bp random sequence in combination with direct cloning using the CloneAmp System (Gibco-BRL) is very rapid, especially since it was necessary to prepare multiple internal standards. Using trace amounts of [α-^{32}P] dCTP in the PCR reaction, it was possible to resolve the reactions on a 0·3 mm non-denaturing polyacrylamide gel which was dried and exposed to either X-ray film or phosphorimager for quantification. This technique yielded quick and accurate results, and additionally, it was possible to distinguish amplified bands differing by only 5 or 6 bp.

In addition to standardization of these basic techniques, the aim of our research was to amplify multiple 5′ termini of alternate transcripts using the same reaction conditions, since employing separate reaction conditions for each specific 5′ terminus would have proved difficult for two reasons: (1) Setting up separate PCRs could have resulted in pipetting errors which are more likely to occur for smaller volumes, while this can be minimized by making master mixes. (2) Setting up separate PCRs is time consuming, especially when the sample number is very high. Because we have to amplify the 5′ termini of the transcripts of many samples, to simplify the procedure we decided to normalize one set of PCR conditions for amplification of three 5′ termini, i.e. promoter-II-

Table 5. Quantification of multiple alternatively spliced transcripts (copy number $\times 10^6$) present in 1 μg of total RNA from human adipose stromal cells and ovarian granulosa cells maintained in primary culture under different conditions

	Adipose stromal cells			Granulosa cells
Alternative transcripts	−Serum −Dexamethasone	Dexamethasone +serum	Dexamethasone* +IL-11	+Forskolin
Promoter-II-specific sequence	1·2	0·12	2·4	12·0
Exon I.3	1·6	4·4	2·5	0·04
Exon I.4	—	4·8	6·0	—
Coding region	3·6	14·4	12·0	11·6

* This experiment was conducted separately.
These experiments were performed twice yielding similar results. The data presented in the table was obtained from a single experiment.

specific sequences, exon I.3, exon I.4 and the coding region. To check the validity of these reaction conditions, we performed a number of experiments using synthetic transcripts (internal standards). The same primer pairs were able to amplify sequences from both target RNA and synthetic cRNA. The only difference was that products amplified from synthetic RNA were 27 bp larger in size.

Further, when using the same conditions for four sets of primers (specific for each 5′ terminus) in which the antisense oligo was the same, there was a high possibility of cross-reaction. However, no cross-amplification was observed (Fig. 4). Efficiencies of amplifications of the 5′ termini were also checked by mixing equal quantities (1 pg) of synthetic transcripts (Fig. 5). This was very important information, since we were trying to amplify one 5′ terminus in one tube from the mixture of several 5′ termini. We found equal efficiencies of all three 5′ termini amplified. Further, when we amplified coding-region-specific sequences, we observed three times more amplified product as of untranslated exons (Fig. 5). This was the anticipated result, since the coding region amplified product should be the sum of all three transcripts used, as it is common to all synthetic transcripts. The concentration of these transcripts in 1 μg of cultured cells was also in a similar range (Table 5), thus we could conclude that amplification efficiencies of all three untranslated exons were equal in our experiments using human adipose stromal cells and granulosa cells.

Similarly, we performed experiments using various combinations of the three synthetic transcripts containing promoter-II-specific sequence, exon I.3 and exon I.4 (Table 4, Fig. 6). We observed that the efficiency of amplification of one 5′ terminus was not changed in the presence of varying concentrations of the other two 5′ termini. This was a very important point when trying to amplify and quantify multiple 5′ termini from alternatively spliced transcripts present in a tissue. Further, competition experiments were also performed and satisfactory results were obtained (Fig. 7) showing competition between target cDNA and cDNA of internal standard transcripts. Thus, we were able to calculate the amount of unknown target transcript by comparing amplified products with the amplified products of known quantities of internal standard transcripts added in the reaction.

After satisfactory standardization of the PCR conditions, we amplified different 5′ termini of transcripts present in human adipose stromal cells and ovarian granulosa cells in primary culture. Previously we observed that the distribution of the various 5′ termini in adipose stromal cells appeared to be a function of the culture conditions under which the cells were maintained.[23] By Northern analysis, we could detect 5′ termini of only abundantly expressed transcripts. We also amplified 5′ terminal sequences by means of the RACE method,[23] but the observed distribution may not reflect the true quantitative distribution of these termini in the *CYP19* gene transcripts in a particular cell or tissue.

Our present results employing adipose stromal cells grown in the absence of serum or dexamethasone revealed the presence of equal concentrations of transcripts containing exon I.3 (truncate)- and promoter-II-specific sequences. There was no amplification of exon I.4-specific sequences. Results obtained from adipose stromal cells treated with dexamethasone (250 nM) in medium containing 15% FCS are in agreement with our earlier study.[23] Exon I.3 and exon I.4-specific sequences were amplified, but exon I.4-specific sequences were more abundant in dexamethasone-treated cells (Fig. 8). Promoter-II-specific sequences were present in very low copy number. Total levels of P450arom transcripts (see coding region, Fig. 8, Table 5) were elevated relative to control cells, which is in accordance with our earlier observation that, in the presence of serum, glucocorticoids stimulate aromatase activity.[32] Recently, we have shown that the effect of glucocorticoids in the presence of serum is regulated by promoter I.4.[33] More importantly, we found that the stimulatory action of serum (in the presence of dexamethasone) can be replaced by the cytokine IL-11. Moreover, we have shown that this operates via a Jak/STAT signaling pathway to regulate aromatase P450 gene expression by using promoter I.4.[33] Therefore, we decided to amplify different transcripts from adipose stromal cells treated with dexamethasone and IL-11. As expected, we observed a high abundance of exon I.4-specific sequences (Table 5). Interestingly, we also observed amplification of promoter-II-specific and exon I.3 (truncate)-specific sequences (Table 5).

In ovarian granulosa cells treated with forskolin, only promoter-II-specific sequences were amplified (Fig. 8). Exon I.3 (truncate)-specific sequences were present in very low copy number. The presence of promoter-II-specific sequences in cells treated with forskolin is in agreement with our earlier studies based on Northern analysis.[34] Presence of exon I.3 (truncate)-specific sequences could not be detected by Northern analysis because of low copy number.

It seems likely that promoter II and I.3 are involved in basal transcription of the *CYP19* gene in adipose stromal cells. In cells treated with glucocorticoid and IL-11 or serum, total P450arom transcripts are elevated due to increased expression from exon I.4. However, there are also more exon I.3 (truncate)-specific sequences. So possibly there are other factors

which regulate the *CYP19* gene by utilizing promoter I.3. Most interestingly, from these results, we observed that in human adipose stromal cells and ovarian granulosa cells, the exon I.3-specific transcripts being amplified were the truncated version.[23] There was no amplification of full-length exon I.3-specific transcripts. Although the 5′ end primer (RT-2) was chosen from the sequence which was common in both exon I.3 and exon I.3 (truncate) transcripts, the size difference of 106 bp between both amplified PCR products is very clear. Earlier, by RACE cloning, both full-length and truncated exon I.3-specific transcripts were detected in cultured cells.[23] At this point we are unable to explain this difference.

We conclude that by using this very sensitive competitive RT-PCR method, we were able to amplify and quantify P450arom transcripts present in very low copy number. Most importantly, we were able to differentiate amplified products of only 5–6 bp difference in size. This methodology will be very useful to study the regulation of the *CYP19* gene in different tissues and in different disease states. Experiments are already in progress in our laboratory to determine the levels of the various exon-specific transcripts, present in the endometrium of patients with endometriosis and in adipose tissue of breast cancer patients. Furthermore, this procedure is not only useful for detecting alternatively spliced transcripts of the *CYP19* gene, but it can in principal be applied to detect multiple transcripts expressed from other genes which are regulated in this fashion.

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Aromatase Expression in Endometriosis*

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ABSTRACT

The conversion of C_{19} steroids to estrogens occurs in a number of tissues, such as the ovary and placenta, and is catalyzed by aromatase P450 (P450arom; the product of the CYP19 gene). P450arom expression has also been detected in a number of uterine tumors, such as leiomyomas and endometrial cancer. On the other hand, P450arom expression was undetectable in normal endometrial and myometrial tissues. The present study was conducted to determine the presence or absence of aromatase expression in peritoneal endometriotic implants and in the eutopic endometrium of women with endometriosis. Endometriotic implants in pelvic peritoneum (n = 17; *e.g.* posterior culdesac, bladder, and anterior culdesac) and eutopic endometrial curettings (n = 11) of 14 patients with histologically documented pelvic endometriosis were obtained at the time of laparoscopy or laparotomy. Pelvic peritoneal biopsies distal to endometriotic implants as well as normal endometrial tissues (n = 7) from disease-free women were used as negative controls. We used competitive RT-PCR technology employing an internal standard to amplify P450arom transcripts in total ribonucleic acid (RNA) isolated from these tissues. P450arom transcripts were detected in all endometriotic implants and in all eutopic endometrial tissues from patients with endometriosis. P450arom messenger RNA species were not detectable in endometrial tissues from disease-free women or in endometriosis-free peritoneal tissues. The highest levels of transcripts were detected in an endometriotic implant that involved the full thickness of the anterior abdominal wall. The P450arom transcript level within the core of this endometriotic mass was 4-fold higher than that in the surrounding adipose tissue. It has been shown recently that aromatase expression in various human tissues is regulated by the use of tissue-specific promoters via alternative splicing. To analyze promoter usage, we amplified by RT-PCR the most likely promoter-specific untranslated 5′-termini of P450arom transcripts in 2 endometriotic implants. It appears that these endometriotic implants use both the adipose-type promoter I.4 and gonadal-type promoter II for aromatase expression. The use of promoter I.4 for aromatase expression in adipose tissue has been recently observed to be regulated by members of the interleukin-6 (IL-6) cytokine family. Based on these findings, we examined by RT-PCR, IL-6 and IL-11 messenger RNA expression in 5 endometriotic tissues and 1 eutopic endometrial sample from a patient with endometriosis. We detected IL-6 and IL-11 transcripts in all endometriotic tissues and in the eutopic endometrial tissue sample studied. Our findings indicate that both eutopic endometrial tissues and endometriotic implants from patients with endometriosis are biochemically different from normal endometrial tissues of disease-free women. The presence of aromatase expression in eutopic endometrial tissues from patients with endometriosis may be related to the capability of implantation of these tissues on peritoneal surfaces. Furthermore, the possibility of estrogen production in these implants may serve to promote their growth. Increased IL-6 and IL-11 expression in these tissues suggests that P450arom expression in endometriosis may be regulated in part by these cytokines. (*J Clin Endocrinol Metab* **81:** 174–179, 1996)

ENDOMETRIOSIS is defined as the presence of both endometrial glands and stroma outside the uterine cavity that are histologically similar to eutopic endometrium. These endometriotic tissues exhibit a growth pattern that is invasive but nonneoplastic. Although the true prevalence of endometriosis remains unknown, it is estimated that approximately 10% of 50 million American women of reproductive age are affected with this disease (1). Endometriosis is responsible for a significant portion of gynecological surgery and represents one of the most common diagnoses made at the time of laparoscopy for chronic pelvic pain and infertility (2, 3). A variety of other potentially disabling symptoms, such as dyspareunia, dysmenorrhea, and dysfunctional uterine bleeding, are often associated with endometriosis.

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Considerable circumstantial evidence suggests that endometriosis is an estrogen-dependent disease (4). Endometriosis has not been reported in prepubertal or premenarchal women (5). However, it primarily affects women of reproductive age and occasionally is diagnosed in postmenopausal patients with relatively high estrogen levels. Moreover, patients with gonadal dysgenesis have developed endometriosis after the administration of exogenous hormonal therapy. There are also several case reports of histological endometriosis in men undergoing high dose estrogen therapy for prostatic carcinoma (6). In most patients with endometriosis, induction of hypoestrogenism using GnRH analogs generally results in temporary involution, but not complete regression, of the endometriotic implants (7).

Laboratory evidence obtained from *in vivo* and *in vitro* studies has demonstrated that endometriotic implants contain estrogen, progesterone, and androgen receptors (8). These clinical observations and laboratory evidence suggest that estrogens play a paramount role in the establishment and maintenance of endometriosis.

The formation of estrogens from C_{19} steroids is catalyzed by a specific form of P450, namely aromatase P450 (P450arom, the product of the CYP19 gene). Tissue-specific expression of P450arom has been found to be under the

control of several alternative promoters (Fig. 1) and in each tissue is regulated by binding of specific *trans*-activating factors to defined genomic response elements upstream of the promoter used in that particular tissue. Aromatase expression occurs in a number of human tissues and cell types, including the granulosa cells of the ovary (9), the syncytiotrophoblast of the placenta (10), and the Leydig cells of the testis as well as extraglandular tissue sites such as genital skin fibroblasts (11), brain (12), and adipose tissue (13, 14). On the other hand, normal endometrial and myometrial tissues do not contain P450arom transcripts (15). However, the neoplastic counterparts of these uterine tissues, namely endometrial cancer and leiomyomas, were demonstrated to express aromatase (16, 17). Thus, estrogens formed *in situ* may play a role in the regulation of the growth of these tumors.

To our knowledge, there are no previous reports in the literature regarding aromatase expression in endometriotic implants. In the present study, we investigated whether P450arom transcripts are present or absent in endometriotic implants and eutopic endometrial samples from women with endometriosis. Endometriosis-free pelvic peritoneum and normal endometrial tissues of disease-free women were used as negative controls. P450arom transcripts in endometriotic implants exist in sufficiently low abundance copy numbers that they are undetectable by Northern analysis, similar to the situation in adipose tissue. Therefore, a more sensitive method to detect P450arom messenger ribonucleic acid (mRNA) was employed using RT-PCR (18).

We detected P450arom transcripts in all endometriotic tissue implants and in all the eutopic endometrial samples of patients with endometriosis, whereas normal peritoneum from these patients and endometrium from disease-free patients did not contain P450arom transcripts. In addition, we analyzed alternative promoter usage for P450arom expression in endometriotic tissue samples.

In the present study we observed that P450arom transcripts present in endometriotic tissues are primarily those specific for promoters I.4 and II. It has been recently shown in human adipose stromal cells that the stimulatory effects of serum on aromatase expression can be mimicked by members of the interleukin-6 (IL-6), IL-11, oncostatin M, and leukemia inhibitory factor lymphokine family when expression is regulated by promoter I.4 (19). This stimulation is mediated by means of a Jak/STAT signaling pathway. Based on these findings and the previously reported postulated interaction between cytokines and endometriosis (38), we determined the expression of IL-6 and IL-11 mRNA in endometriotic implants and found that both were expressed in all such samples examined.

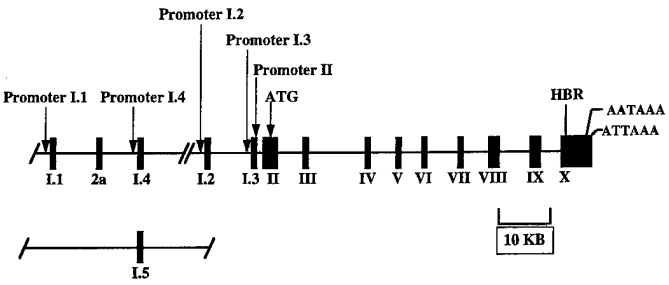

FIG. 1. Structure of the CYP19 (P450arom) gene. The coding region spans nine exons beginning with exon II. The gene contains a number of tissue-specific promoters that direct aromatase expression in human placenta (PI.1), adipose stromal cells (PI.4), and ovary (PII). Transcripts in different tissues have different 5′-termini, but the protein encoded by these transcripts is always the same, regardless of the tissue site of expression. Thus, there is only one human P450arom enzyme encoded by a single gene.

Materials and Methods

Tissue acquisition and processing

At the time of laparoscopy or laparotomy, the following samples were obtained from a total of 21 patients: 1) endometriotic peritoneal implants distant from the ovary (n = 17), 2) eutopic endometrial tissues from patients with endometriosis (by curettage; n = 11), 3) normal peritoneal biopsies from patients with endometriosis (n = 7), and 4) endometrial tissues from disease-free patients of reproductive age undergoing hysterectomy for uterine prolapse, leiomyoma, or cervical pathology (n = 7). All samples were histologically confirmed. Samples were frozen in liquid nitrogen and stored at −70 C. The ages of the patients ranged from 19–39 yr, and endometriosis stage (American Fertility Society classification) varied from II–IV. Written consent was obtained before surgical procedures, including a consent form and protocol approved by the Institutional Review Board for Human Research of the University of Texas Southwestern Medical Center.

Human RNA isolation and rat P450arom complementary RNA (cRNA) synthesis

Human total RNA was isolated from surgically removed tissues stored at −70 C by the guanidinium thiocyanate-cesium chloride method (20). Rat P450arom cRNA was synthesized from a rat complementary DNA (cDNA) clone, as previously described (15, 18).

Determination of P450arom transcripts levels using competitive RT-PCR

Briefly, PCR amplification of a region of the coding sequence of P450arom transcripts after reverse transcription was performed as previously described (18). This procedure involved primer extension by reverse transcription of 10 μg total RNA from surgically obtained tissues, followed by use of a 3′-oligonucleotide specific for coding exon IV to synthesize a single stranded cDNA. (We arbitrarily chose to use total RNA to normalize our results, because total RNA provides a rough index of the general metabolic rate.) This template was amplified by 25 cycles at 93 C for 1 min, 45 C for 1 min, and 72 C for 1 min using a specific 5′-oligonucleotide complementary to coding exon II in conjunction with the previously used 3′-primer. The generated 272-bp PCR products were then fractionated on a 1.8% agarose gel for Southern blot analysis or transferred directly to nylon membranes by slot blot. These membranes were hybridized to ^{32}P-labeled oligonucleotide probes. Autoradiographs were exposed to blotting membranes. Samples containing breast adipose stromal cells treated with dexamethasone in the presence or absence of serum were included in the experiments as positive controls.

Detection of promoter-specific P450arom transcripts in endometriotic tissues

Amplification of the unique 5′-termini of the promoter-specific P450arom transcripts from two endometriotic tissue samples was accomplished by using exon-specific oligonucleotides, as described previously (21, 22). Initial primer extension using 10 μg total RNA was performed using a 20-mer 3′-oligonucleotide complementary to coding exon III of the P450arom gene. Then, the newly synthesized cDNA templates were separately amplified using promoter-specific 5′-oligonucleotide primers, the sequences of which are shown in Table 1. Transcripts with 5′-termini specific for promoters II, I.4, I.3, and coding region were amplified, size-fractionated on 1.8% agarose gels, and trans-

TABLE 1. Sequences of oligonucleotides used as primers and probes for analysis of 5′-termini of P450arom transcripts

	Sense primer	[^{32}P]-Labeled probe
Exon I.4-specific	5′-GTAGAACGTGACCAACTGG-3′	5′-GGTTTGATGGGCTGACCAG-3′
Exon 1.3-specific	5′-GATAAGGTTCTATCAGACC-3′	5′-GCAGCATTTCTGACCTTGG-3′
Promoter II-specific	5′-GCAACAGGAGCTATAGAT-3′	5′-TGTGGAATCAAAGGGACAGA-3′
Coding exon II-specific	5′-TCTGAGGTCAAGGAACAC-3′	5′-CAGGCACGATGCTGGTGATG-3′

3′-Antisense oligonucleotide (complementary to the 3-end of coding exon II) used for initial primer extension of total RNA. 5′CTGGTATTGAGGATATGCCCTCATAAT-3′.

Sequences of oligonucleotides used in RT-PCR of IL-6 and IL-11

	Sense primer	Antisense primer	^{32}P-Labeled probe
IL-6	5′-GCC TTC GGT CCA GTT GCC TTC-3′	5′-CTG CAG GAA CTG GAT CAG GAC-3′	5′-AGG GTT CAG GTT GTT TTC TCG CAG-3′
IL-11	5′-CTG CAG GAA CTG GAT CAG GAC-3′	5′-GCA GCC TTG TCA GCA CAC CTG GGA GCT GTA-3′	5′-TCG AGG GGG GCC AGG TGG TGG CCC-3′

ferred to a blotting nylon membrane by capillary elution. Southern hybridization with the ^{32}P-labeled oligonucleotide probes was continued overnight. Autoradiographs were exposed to blotting membranes for 4–16 h. Samples containing adipose stromal cells treated with dexamethasone in the presence or absence of serum were used as controls. A no RNA sample was included in each experiment to preclude product carry-over contamination.

Detection of IL-6 and IL-11 mRNA expression

Total human RNA isolated previously from endometriotic tissues (n = 5), one eutopic endometrial tissue sample from a patient with endometriosis, one adipose tissue sample adjacent to an abdominal endometriosis mass, and a normal endometrial control were used. Primer extension was performed at 42 C for 60 min using 3 μg random primers (Promega, Madison, WI), a 1 mmol/L final concentration of each deoxy-NTP, 1 × first strand buffer (Life Technologies, Grand Island, NY), 10 mmol/L dithiothreitol, 400 U mouse mammary leukemia virus-RT (BRL, Gaithersburg, MD), and 40 U RNAsin (Promega) in a final volume of 20 μL. Each amplification master mix contained 10 pmol of the 5′-oligonucleotide and 10 pmol of the 3′-oligonucleotide specific for IL-6 and IL-11, a 500 μmol/L final concentration of each deoxy-NTP, 1 × PCR buffer (Perkin-Elmer/Cetus, Norwalk, CT), in a final volume of 20 μL consisting of 14.8 μL amplification master mix, 0.2 μL (1.3 U) AmpliTaq DNA polymerase (Perkin-Elmer/Cetus), and 5 μL initial random primer extension reaction mix. PCR amplification of 30 cycles was used to obtain IL-6 and IL-11 amplification products. The amplified products were treated with deoxyribonuclease-free ribonuclease (Boehringer, Indianapolis, IN) for 30 min at 37 C, size-fractionated by electrophoresis on a 1.8% agarose gel, and transferred to blotting membranes by capillary elution. Southern hybridization with oligonucleotide probes (end labeled with ^{32}P) specific for IL-6 and IL-11 was continued overnight at Tm−5C for each probe. Oligonucleotide sequences are shown in Table 1.

Results

P450arom transcripts in endometriotic and eutopic endometrial tissues

P450arom transcripts were detected in all endometriotic implants (n = 17) and in all the eutopic endometrial tissues from patients with endometriosis (n = 11), whereas normal pelvic peritoneum from endometriosis patients (n = 7) and endometrial tissue samples from disease-free patients (n = 7) did not contain any P450arom transcripts. These results are summarized in Table 1, and representative experiments are shown in Figs. 2 and 3.

The highest level of transcripts were detected in an 8 × 8 × 7-cm endometriotic implant that involved the full thickness of the anterior abdominal wall. The P450arom transcript level within the core of this endometriotic mass was 4-fold higher than that in the surrounding adipose tissue (Fig. 4).

Determination of promoter usage for P450arom gene expression in endometriosis

In two endometriotic tissue samples, transcripts containing exon I.4- and promoter II-specific sequences comprised the majority of P450arom transcripts, whereas many fewer exon I.3-specific transcripts were noted (Fig. 5). Therefore, it follows that promoter I.4 and promoter II are primarily responsible for aromatase expression in endometriotic tissues.

Determination of IL-6 and IL-11 expression in endometriosis

All endometriotic samples tested (n = 5) showed the presence of IL-6 and IL-11 transcripts (Fig. 6). A sample from adipose tissue surrounding an abdominal endometriosis mass, eutopic endometrial tissue from a women with endometriosis, and endometrial tissue from a disease-free control also showed the presence of both IL-6 and IL-11 transcripts. Although we did not use RT-PCR internal standards to accurately quantify IL-6 and IL-11 transcripts, it was evident that transcript levels of both cytokines are higher overall in endometriotic tissues than levels in eutopic endometrial samples or those from disease-free women; these results are, in turn, qualitative and not quantitative (Fig. 6).

TABLE 2. Summary of results (RT-PCR) from various human tissues

Tissue location	n	P450arom transcripts present	P450arom transcripts absent
Endometriotic implants	17	17	0
Eutopic endometrium/endometriosis	11	11	0
Endometrium disease-free	7	0	7
Disease-free peritoneum/from endometriosis patients	7	0	7

Total patients, n = 21; patients with histologically documented endometriosis, n = 14.

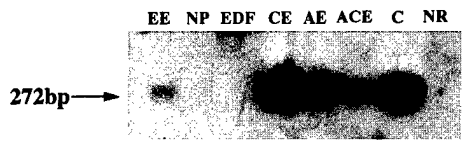

FIG. 2. A representative Southern blot autoradiograph of P450arom transcripts after RT-PCR (30 cycles) from endometriosis tissues. EE, Eutopic endometrium from a patient with endometriosis; NP, normal peritoneum from a patient with endometriosis; EDF, endometrium from a disease-free patient; CE, posterior culdesac endometriosis; AE, abdominal wall endometriosis mass; ACE, anterior culdesac endometriotic implant; C, positive control (adipose stromal cells); NR, "no RNA" control.

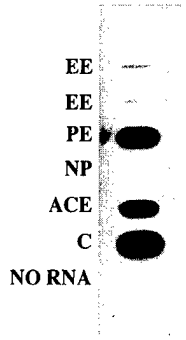

FIG. 3. Representative slot blot autoradiograph of RT-PCR products of two different eutopic endometrial tissues from patients with endometriosis (EE), one sample of a peritoneal endometriotic implant (PE), an anterior culdesac endometriotic implant (ACE), a normal peritoneal sample from a patient with endometriosis (NP), a sample from a positive control (adipose stromal cells; C), and a "no RNA' sample.

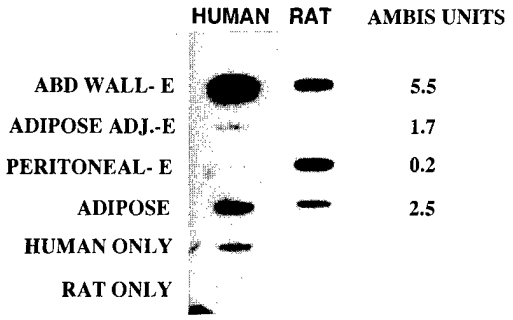

FIG. 4. P450arom transcripts quantified by competitive RT-PCR from abdominal wall endometriosis mass (ABD WALL-E), adipose adjacent to abdominal wall endometriosis mass (ADIPOSE ADJ.-E), pelvic endometriotic implant (PERITONEAL E), control adipose tissue, human RNA only (from adipose tissue), and rat RNA only. Using competitive RT-PCR, we compared P450arom mRNA levels in these tissues using 10 μg total RNA. Human RNA was coamplified with 1 pg rat P450arom cRNA for 20 cycles. PCR products from each sample were divided in half, and each portion was transferred to a separate blotting membrane through corresponding slots. The PCR products in the membrane on the *left* were probed with human-specific oligonucleotide, whereas the membrane on the *right* was hybridized with the rat-specific oligonucleotide probe. Radioactivity on the blotting membranes was quantified by an Ambis radioimaging system (Ambis, San Diego, CA). Ambis reading was calculated for each horizontal pair of slots by dividing the value for the human sample by the value for the coamplified rat sample.

Discussion

The molecular and cellular mechanisms underlying endometriosis are far from understood. Endometriosis appears to be confined to higher primates undergoing long periods of uninterrupted cyclic menses. Several hypotheses have been proposed for the development of endometriosis. These include retrograde menstruation of endometrial tissue into the pelvic peritoneum, lymphatic or hematogenous dissemination, and celomic metaplasia (23–26). Immune system alterations have also been implicated in the development of endometriosis (5, 27). Considering that retrograde menstruation appears to occur to some degree in almost 95% of women (28–30) and that only approximately 10% of these women will develop endometriosis, the presence of viable endometrial cells in the peritoneal cavity *per se* is unlikely to be a causative factor. Olive and Hammond (31) proposed that a combination of the amount of retrograde menstruation and the efficiency of the immune response that eliminates endometrial cells plays an important role in the development of endometriosis. Additionally, evidence has accumulated pointing out marked biochemical differences between the eutopic endometrium from patients with endometriosis and the endometrium from disease-free controls. These differences may play a role in the initiation of the disease process. Examples of such biochemical dissimilarities are the failure of β_3-integrin expression in eutopic endometrial tissues from patients with endometriosis during the luteal phase of the cycle (32), expression of C3 complement component in the eutopic endometrium of patients with endometriosis (33), and higher chemotactic activity toward neutrophils and macrophages in the endometrium of patients with endometriosis compared to that in normal controls (34). In addition, Takahashi *et al.* (35) compared estradiol levels in peripheral and menstrual blood of women with normal menstrual cycles, patients with adenomyosis, and patients with peritoneal endometriosis and found no significant differences in estradiol levels in peripheral blood samples. In contrast, the menstrual blood estradiol level was highest in women with adenomyosis, followed by the group of patients with endometriosis; the lowest levels were found in the group of patients with normal ovulatory cycles, suggesting the possibility of localized estrogen biosynthesis in patients with adenomyosis and endometriosis.

Aromatase is not expressed in normal endometrial tissues (15, 36). P450arom transcripts (17) and aromatase activity (37) have been documented to be present in endometrial cancer samples. We demonstrated that P450arom transcripts are present in the eutopic endometrium of patients with endometriosis, whereas normal endometrial controls do not contain P450arom transcripts. Therefore, this study offers further evidence supporting the differences between normal endometrial tissues and the endometrium of patients with endometriosis. Aromatase expression and, hence, possible localized estrogen production in the endometrium of these patients may contribute to the capacity of these cells to implant on peritoneal surfaces. It is not clear whether aromatase expression in endometrial tissues of patients with endometriosis is an inherent property of these cells or the result of the disease process. Endometriosis is known to elicit an inflammatory response in the peritoneal cavity mainly mediated by peritoneal macrophages and mononuclear phago-

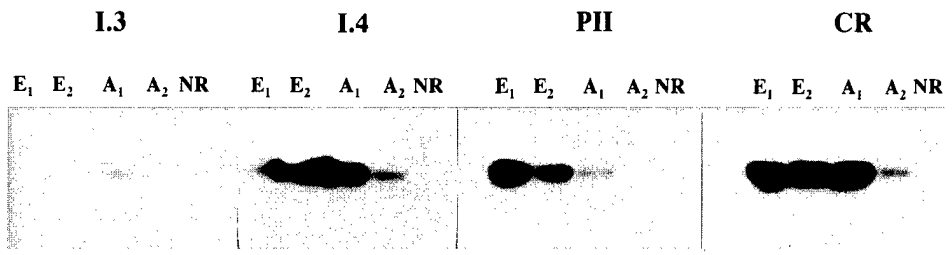

FIG. 5. RT-PCR analysis of untranslated 5′-termini of P450arom by Southern blot hybridization of samples from various tissues. Specific oligonucleotides were used as primers and probes to amplify exon I.3-specific, exon I.4-specific (adipose-type promoter-specific sequence), and promoter II-specific (gonadal-type) sequences. The last set of samples, CR, represents amplification of a sequence in the coding region common to all of these tissues, which was used as a positive control. E_1, An endometriotic tissue sample from the culdesac; E_2, a sample from an abdominal wall endometriosis tissue; A_1 and A_2 (controls), adipose stromal cells treated in the presence of serum and dexamethasone and in the absence of serum, respectively. These give rise to transcripts with the expected untranslated 5′-termini. Endometriosis sample E_1 appears to contain promoter II-specific transcripts to a greater extent than exon I.4-specific transcripts, whereas sample E_2 contains exon I.4-specific transcripts to a greater extent than those containing promoter II. Note that sample E_2 shows abundant amplification products of exon I.4-specific transcripts that smear into sample E_1, which contains a much less prominent band.

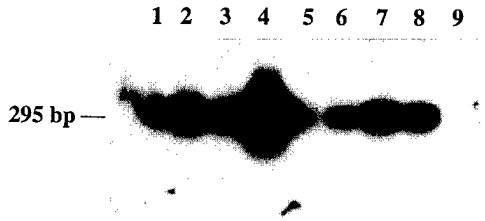

FIG. 6. A representative Southern blot autoradiograph of IL-11 transcripts from various tissues after RT-PCR. 1, Abdominal wall endometriosis; 2, adipose tissue adjacent to abdominal endometriosis implant; 3, pelvic endometriotic implant; 4, posterior culdesac endometriotic nodule; 5, anterior culdesac endometriotic implant; 6, disease-free endometrium; 7, eutopic endometrium/endometriosis patient; 8, IL-11 control sample (U-937 cells); 9 "no RNA" sample.

cytes, which are known to be closely related to the growth and maintenance of endometriosis (38, 39). Macrophage-derived growth substances and members of the IL-6 cytokine family have been found to be present in the peritoneal fluid of patients with endometriosis (40). Cytokines such as IL-6, IL-11, leukemia inhibitory factor, and oncostatin M have been shown to be potent stimulators of aromatase expression in adipose stromal cells in culture (19). The results of the present study suggest that the expression of aromatase in endometriotic implants may be regulated by a complex interaction of macrophage-derived growth substances in an autocrine, paracrine, or intracrine fashion.

Androstenedione of adrenal origin is the major substrate for aromatase in peripheral tissues, such as adipose tissue. Estrone, the product of the enzymatic reaction catalyzed by aromatase from androstenedione, is weakly estrogenic and requires conversion to estradiol by the enzyme 17β-hydroxysteroid dehydrogenase (17βHSD; estradiol dehydrogenase) to attain full estrogenic potency. The presence or absence of this enzyme in endometriosis is not yet known. The role of local estrogen production in the regulation of growth and maintenance of estrogen-dependent tumors such as endometrial cancer, uterine leiomyoma, and endometriosis remains unclear.

In the endometriotic samples examined, it appears that the gonadal-type promoter II and the adipose-type promoter I.4 are primarily used for aromatase expression. P450arom expression is known to be greatly stimulated by members of the IL-6 cytokine family in human adipose fibroblasts in the presence of glucocorticoids (19). These cytokines act via a Jak/STAT pathway and promoter I.4 to stimulate P450arom transcription in these cells (19). Given the presence of IL-6 and IL-11 transcripts in endometriotic tissues coupled with the postulated interaction between macrophage-derived growth factors and cytokines for the growth and maintenance of endometriosis, one can envision that this regulatory mechanism may play an important role in aromatase expression in endometriosis. Based on our findings in promoter usage for aromatase expression in tissues of Mullerian origin (17, 41) and gonads (21), it is unlikely that the placental-type promoter I.1 is used in endometriosis. Therefore, we did not attempt to amplify exon I.1-specific sequences or other minor splice variants (I.2 or 2a) that were detected in placenta. The sizes of promoter II- and promoter I.4-specific bands compared with simultaneous amplification of the coding region suggest that these two promoters are primarily responsible for aromatase expression in endometriosis.

The presence of P450arom transcripts in endometriotic tissues and the eutopic endometrium of patients with endometriosis contrasts with the absence of transcripts in the peritoneum of these patients and in normal endometrial samples from disease-free patients. It is possible that aromatase expression in the eutopic endometrium of patients with endometriosis is an inherent property of these cells that could influence the capability of implantation outside the uterus, or it could be the result of the disease process itself, resulting from the interaction of growth factors and cytokines inducing aromatase expression in eutopic endometrial tissues. Thus, the expression of P450arom mRNA in endometriotic implants suggests the possibility of localized estrogen production, that, acting in an autocrine or paracrine fashion, may promote the growth of the implants, perhaps in concert with macrophage-derived substances and cytokines.

Further studies directed toward the regulatory mechanisms of aromatase expression in endometriosis, employing cells from endometriosis samples in culture, are necessary to answer these questions and provide further insight into the pathogenesis of this enigmatic disease.

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CLINICAL REVIEW 78

Aromatase Deficiency in Women and Men: Would You Have Predicted the Phenotypes?*

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THE PHYSIOLOGICAL roles of estrogens in women include the development of secondary sexual characteristics, follicular maturation, regulation of gonadotropin secretion for ovulation, preparation of tissues for progesterone response, maintenance of bone mass, regulation of lipoprotein synthesis, prevention of urogenital atrophy, and possibly regulation of insulin responsiveness (1). However, the important physiological roles of estrogens in men were largely unanticipated until recently, when estrogen was found to be necessary for fusion of epiphyses and prevention of bone loss (2, 3). Estrogen deficiency manifested by sexual infantilism in a 46,XX individual can be caused by an inherited enzymatic defect. Any of the ovarian steroidogenic enzymes can be affected, but the common feature is absent negative feedback inhibition of estrogen on pituitary gonadotropin secretion. The clinical manifestations are the consequences of both deficient synthesis of estrogen and excessive secretion of precursor steroids that are synthesized before the defective step. Aromatase catalyzes the final reaction of this pathway in the ovary and converts C_{19} steroids to estrogens (4). It is also expressed in the human placenta, testis, adipose tissue, and brain (4). MacDonald *et al.* (5) demonstrated that in men, estrogens are formed primarily by aromatization of plasma precursors in extraglandular sites. It is currently believed that the adipose tissue is the primary site for estrogen production in men (6). There have been several recent reports in the world literature describing aromatase deficiency, following the first description in 1991 of a Japanese newborn girl with an aromatase P450 (P450arom) gene defect (7, 8). Until then, aromatase deficiency had been considered incompatible with life, and this dogma may have undermined the efforts of investigators to entertain this diagnosis in suspected cases. It should be noted that Mango *et al.* (9) described a pregnancy in which aromatase activity in the placenta was low; however, the newborn girl did not have genital ambiguity, and the P450arom gene in this individual has not been sequenced. To date, one Japanese female infant (7), one American adolescent girl (10), and two American adult siblings, a woman and a man (3), have been found to have P450arom gene defects. Convincingly, estrogen biosynthesis in all of these patients was virtually absent, giving rise to a number of anticipated as well as unexpected symptoms. As a result, it is known now that aromatase deficiency is an autosomal recessive condition manifest in 46,XX fetuses by female pseudohermaphroditism and, in the case of adult men, extreme tallness due to unfused epiphyses. In two of three cases, transient maternal virilization during pregnancy was noted.

The human placenta develops from the trophectoderm of the blastocyst and is genetically fetal tissue. Among mammalian placentas, the human placenta is uniquely capable of aromatizing massive amounts of C_{19} steroids into estrogens most efficiently. In pregnant women at or near term, there is a daily production of 70 μmol (20 mg) estradiol and 300–450 μmol (80–120 mg) estriol (11). At 38 weeks gestation, it is very common to detect maternal serum estradiol levels above the 100,000 pmol/L (27,000 pg/mL) and estriol levels above the 55,000 pmol/L ranges. The physiological role of this massive placental estrogen production during pregnancy is not understood. However, when the aromatization capacity of the placenta is exceeded by the overproduction of C_{19} steroids of maternal origin, *e.g.* luteomas, early during pregnancy (12), virilization of the female fetus and the mother has been noted. By contrast, serum estradiol (1650 pmol/L or 450 pg/mL) or estriol (134 pmol/L or 37 pg/mL) levels were extremely low in the 38-week pregnant mother of the infant with aromatase deficiency (7). Placental tissue with aromatase deficiency fails to convert C_{19} precursors into estrogens. As a result, the androgen and estrogen precursor, dehydroepiandrosterone sulfate (DHEAS), derived primarily from the fetal adrenal, is converted in the placenta to androstenedione and testosterone. (The extent of conversion of placental androstenedione to testosterone at maternal peripheral sites is not clear.) Thus, both the female fetus and mother become virilized. Severe genital ambiguity noted in the female fetuses (3, 7, 10) implies exposure of external

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genitalia to testosterone and dihydrotestosterone much earlier than the 12th week of gestation (13).

Genotype vs. phenotype

Estrogen biosynthesis is catalyzed by an enzyme located in the endoplasmic reticulum of estrogen-producing cells. This enzyme is a member of the cytochrome P450 superfamily, namely aromatase P450 (P450arom), the product of the *CYP19* gene (4). P450arom has the capacity to metabolize the three precursors, androstenedione, testosterone, and 16α-hydroxydehydroepiandrosterone sulfate (after conversion to 16α-hydroxyandrostenedione), into estrone, estradiol, and estriol, respectively (14). In humans, the *CYP19* gene and its product P450arom are expressed in ovary, testis, placenta, adipose tissue, and brain. Estrogen levels in the circulation are primarily maintained by aromatase activity in the ovarian granulosa cells of ovulatory women and adipose tissue of men (5) and postmenopausal women (15). The *CYP19* (P450arom) gene is 75 kilobases long, and its tissue-specific expression is regulated by the use of tissue-specific promoters involving alternative splicing (16, 17). Despite the size and complexity of the P450arom gene, only four definitively characterized cases of aromatase deficiency have been reported to date, as indicated above, although deficiencies of most other steroidogenic forms of P450s (17α-hydroxylase, 11β-hydroxylase, and 21β-hydroxylase) have been well characterized. Examination of genomic DNA from the Japanese patient (7, 8) revealed that a consensus splice acceptor site between the coding exon 6 and intron 6 was mutated, resulting in the use of a cryptic acceptor site further downstream in intron 6. This homozygous mutation added an insert of 87 bp to P450arom messenger ribonucleic acid, resulting in translation of an abnormal protein with 29 extra amino acids (8). The Japanese infant was born with severely virilized external genitalia. At this time, it is not known whether she will have pubertal failure at the expected age. It is possible that in tissues other than the placenta, *e.g.* the ovary, the original splice site would be recognized despite the point mutation in intron 6 (8). This might lead to normal estrogen production in the ovary.

The second case was an 18-yr-old girl with primary amenorrhea and female pseudohermaphroditism (10). She was found to be a compound heterozygote for two different missense mutations in the heme-binding region of the P450arom gene (4), one of which comprises the fifth coordinating ligand of the heme iron and is conserved in all P450 family members. Finally, studies of a 27-yr-old woman and her 24-yr-old brother, both affected by aromatase deficiency, have recently been reported (3). Both were found to have the same homozygous missense mutation in a highly conserved region of the P450arom gene believed to guard the substrate access channel. In all of these cases, *in vitro* transient expression of the mutant complementary DNAs gave rise to only trace amounts of aromatase activity. Various point mutations in the *CYP19* (P450arom) gene in females have given rise to certain common phenotypic features, *e.g.* congenital genital ambiguity, pubertal failure, hypergonadotropic hypogonadism, and multicystic ovaries. The male phenotype for aromatase deficiency was similar in many respects to the previously described case of estrogen receptor deficiency (2), namely extremely tall stature due to incomplete epiphyseal closure, continued growth into adulthood, and osteoporosis.

Clinical presentation and diagnosis (Tables 1 and 2)

Maternal virilization during pregnancy may or may not be a consistent presentation, because, to date, two of three mothers of aromatase-deficient fetuses were reported to develop virilization during the second half of pregnancy. The mother of the two siblings with aromatase deficiency (3) consistently developed virilization from the 18th to 20th weeks of gestation during both pregnancies, whereas the mother of the Japanese patient (7) became progressively virilized from the 30th week on. Symptoms consist of severe acne, lowered voice, hirsutism, and clitoromegaly. Androgen precursors of fetoplacental and maternal origin that do not become aromatized in placenta are converted into testosterone in placenta and possibly peripheral maternal tissues to cause maternal virilization. These symptoms gradually disappeared after delivery. During pregnancy, extremely low maternal serum estradiol and estriol levels within the range of 0.1–2% of normal values will differentiate aromatase deficiency from virilizing maternal ovarian tumors or maternal ingestion of androgens. Low levels of estriol in maternal plasma or urine may also be detected in placental sulfatase deficiency and in the case of an anencephalic fetus (11). However, in these conditions, maternal estriol or estradiol levels are not as low as those in aromatase deficiency; testosterone is not elevated; and maternal virilization is absent. For differential diagnosis, one should rarely need to perform the DHEAS loading test (7, 11).

Labioscrotal fusion and a greatly enlarged phallus with a single meatus at its base were noted in all female patients at birth. The karyotype was 46,XX. Urogenitogram and sonographic examination of pelvis revealed the presence of a

TABLE 1. Clinical findings in aromatase deficiency

	Female	Male
Fetal life	Virilization of the mother during second half of pregnancy	Virilization of the mother during second half of pregnancy
Genitalia at birth	Clitoromegaly and posterior labioscrotal fusion	Normal male
Childhood	Unremarkable	Unremarkable
Puberty	Absent growth spurt, absent breast development, primary amenorrhea, further enlargement of clitoris, normal development of pubic and axillary hair	Normal pubertal development
Adult	Severe estrogen deficiency, virilization, multicystic ovaries, tallness?	Extremely tall (>3 SD) with continued linear growth into adulthood, osteoporosis, macroorchidism infertility?

TABLE 2. Laboratory findings in aromatase deficiency

Mother (pregnant woman)	Extremely low maternal serum estradiol or estriol, markedly elevated maternal serum testosterone
Infancy (girls)	Elevated FSH and undetectable serum estradiol
Puberty (girls)	Sonographic examination: multicystic ovaries, retarded bone age, markedly elevated FSH and LH, mildly elevated levels of testosterone and androstenedione
Adult (man)	Undetectable estrone or estradiol in serum despite markedly elevated FSH and LH, severely retarded bone age with unfused epiphyses, densitometric and biochemical evidence of osteoporosis, elevated basal insulin level, decreased HDL/LDL cholesterol ratio, abnormal semen analysis?

urogenital sinus, vagina, and uterus. Lack of progressive virilization and normal basal or ACTH-stimulated 17α-hydroxyprogesterone, 11-deoxycortisol, and Δ^5/Δ^4-steroid ratios will rule out congenital virilizing adrenal hyperplasia. True hermaphroditism may be ruled out by the lack of an elevated testosterone response to hCG administration.

During the first 2 yr of life in the American adolescent girl (10), both basal and GnRH-stimulated FSH levels (75 and 200 mIU/mL, respectively) were far greater than those in normal subjects, whereas estradiol and estrone were either too near to the detection limit of RIA or undetectable. Basal LH was normal (5 mIU/mL) at this infancy stage. From late infancy until puberty, serum FSH and LH levels were in the normal (either undetectable or barely detectable) range. Serum DHEAS, 17α-hydroxyprogesterone, androstenedione, and testosterone were also found to be in the normal range during childhood. Both aromatase-deficient American girls (3, 10) grew and developed normally during infancy and childhood. However, at puberty, these girls did not experience breast development or the normal growth spurt. They did develop acne, pubic and axillary hair, and further clitoral enlargement. Both ovaries were found to contain multiple large (4- to 8-cm) cysts. Basal FSH (16–75 mIU/mL), LH (17–18 mIU/mL), and testosterone (3.3–5.2 nmol/L) levels were elevated. Mild elevations were also noted in levels of androstenedione and 17α-hydroxyprogesterone, measured serially (3, 10). Bone age was 8 months to 4 yr behind chronological age at puberty. Both American patients, the 18-yr-old girl and the 27-yr-old woman, were started on oral estrogen treatment indefinitely at the onset of puberty. Breast development and growth spurt ensued after estrogen replacement in both individuals. The final height of the 18-yr-old girl was 166.4 cm and normal. The extreme tallness [final height, 177.6 cm (+3.5 SD)] of the 27-yr-old woman may be related to aromatase deficiency. However, a bone age that was only 8 months behind the chronological age at puberty and the fact that she has been receiving high dose estrogen replacement since 13 yr of age suggest that her final height might have been determined by factors other than estrogen deficiency.

The only male thus far diagnosed with aromatase deficiency went through an unremarkable puberty. Although his height of 170 cm was regarded as normal at the age of 15 yr, his linear growth continued into adulthood. When first seen, he was 24 yr old and 204 cm (+3.7 SD) tall. Bone age was 14 yr, and iliac apophyses were not ossified. The recently reported estrogen-resistant man (2) had eunuchoid body proportions; however, this information has not been provided for the aromatase-deficient man (3). In the aromatase-deficient man, clear densitometric and biochemical evidence of osteoporosis was noted. Fasting insulin and total and low density lipoprotein cholesterol levels were increased, whereas the high density lipoprotein cholesterol level was low. Despite evidence of insulin resistance, glucose levels were normal. Orally administered conjugated estrogens lowered insulin levels. The important clinical features of aromatase deficiency are categorized in Table 1. In Table 2, some of the diagnostic laboratory tests are listed.

Spotted hyena: a natural animal model for human aromatase deficiency?

The publication by Yalcinkaya *et al.* (18) regarding steroid metabolism in the placenta of the spotted hyena has increased our understanding of the mechanism by which virilization of the female spotted hyenas occurs *in utero*, which is a normal physiological process in these animals. The female spotted hyena is born with fused labia to form a pseudoscrotum, and the clitoris is enlarged to form a male-like phallus through which the urogenital sinus traverses. Analogous to DHEAS of human fetal adrenal origin, extremely large quantities of an androgen precursor, namely androstenedione, is produced by ovarian stroma during late term pregnancy in the hyena. However, as in other nonprimates whose placental aromatase expression has been studied, aromatase activity in spotted hyena placenta is very low compared with that in human placenta (18). Yalcinkaya *et al.* also showed that, as in the human placenta, the conversion of androstenedione to testosterone was efficiently carried out in spotted hyena placentas (18). Testosterone production by the spotted hyena placenta must start very early during embryonic life to explain the severe virilization of female external genitalia. Although there are apparent similarities between female pseudohermaphroditism due to human aromatase deficiency and female spotted hyenas, there are profound differences between molecular mechanisms of virilization and phenotypic features. First, the major precursor for testosterone in hyena placenta is androstenedione, which is secreted in large quantities by maternal ovaries. In humans, DHEAS, a circulating precursor (19) secreted by the fetal adrenal gland, would primarily contribute to placental testosterone production in the absence of sufficient aromatase activity. Secondly, the adult female spotted hyena can ovulate and reproduce, which is suggestive of adequate ovarian aromatase expression in response to FSH. In contrast, aromatase-deficient women are incapable of ovulating, because their ovaries do not produce estrogens due to mutations in the coding region of the P450arom gene giving rise to inactive

proteins (8, 20). Finally, androgen excess during pregnancy in hyenas is not clinically detectable in already virilized mothers. It has also been suggested that *in utero* androgen exposure of the female hyena causes depletion of the primordial follicles replaced by stroma (18). In the aromatase-deficient adolescent girl (10), an ovarian biopsy at 17 months of age revealed many closely packed primordial follicles, which was considered normal. The aromatase-deficient adult woman (3) underwent ovarian biopsy at 13 yr of age, and excessive atresia of follicles was reported. Rates of follicular atresia in aromatase-deficient women during various stages of development need to be further clarified. Therefore, the *in utero* virilization of female spotted hyenas and aromatase-deficient human females may appear related; however, the (patho)physiological significance and the underlying molecular mechanisms are greatly different in these two species. To date, a transgenic mouse with a disrupted *CYP19* (P450arom) gene has not been produced.

What did we learn from studying patients with aromatase deficiency?

First, aromatase deficiency is not a universally lethal defect as once believed. This pertains, as two homozygous siblings were conceived (3). Estrogens may not be necessary for successful implantation or embryonic or fetal development. On the other hand, aromatase expression in human placenta appears to be necessary for the clearance of DHEAS from fetal and maternal adrenals by conversion into estrogens. Otherwise, placental sulfatase, 3β-hydroxysteroid dehydrogenase Δ^{4-5}-isomerase and 17β-hydroxysteroid dehydrogenase have the capacity to convert this precursor into testosterone. This results in enough testosterone production to create severe virilization of female external genitalia in early embryonic life. The same level of androgen exposure of the aromatase-deficient male fetus does not cause any genital abnormalities at birth, which is consistent with the findings in male fetuses affected by congenital adrenal hyperplasia.

Sonographic imaging of the two aromatase-deficient girls at puberty (3, 10) revealed the presence of multiple large ovarian cysts. This is probably due to very high levels of gonadotropins in the absence of estrogen and possibly inhibin. The ovarian biopsy result from one of the aromatase-deficient girls at puberty was reported to be similar to the polycystic ovary syndrome (3). Comparable histological features were found in the ovaries of estrogen receptor (ER)-disrupted transgenic mice (21). Ovaries of ER-disrupted adult mice showed multiple hemorrhagic cysts with few, if any, granulosa cells. Aromatase expression appears to be necessary for normal follicular development; however, aromatase deficiency or estrogen resistance do not prevent uncontrolled follicular growth, which seems to be mediated primarily by gonadotropins. In aromatase-deficient women, more histological information regarding theca and granulosa cells comprising the follicle walls and immunohistochemical assessment of their gonadotropin receptor content may provide new insights into the *in vivo* role of estrogens in follicular development. This may also increase our understanding of the pathophysiology of polycystic ovary syndrome. For example, although not yet described, gene defects that cause milder degrees of aromatase deficiency or estrogen resistance may be common and associated with symptoms of polycystic ovary syndrome, *e.g.* anovulation, multicystic ovaries, and insulin resistance. Because of abnormal follicular growth in aromatase-deficient women, decreased numbers of granulosa cells result in insufficient inhibin production, which may explain the extremely high FSH levels. During a laparotomy at 13 yr of age, one aromatase-deficient girl was noted to have infantile appearing uterus and tubes (3), although patency was not examined. This finding is consistent with the hypoplastic uterine horns found in ER-disrupted adult female mice (21), emphasizing the importance of estrogen action on the final development of müllerian ducts.

In certain avian species, estrogen biosynthesis in the brain has been implicated in sex-related behavior such as mating responses, and frequently, a marked sexually dimorphic difference has been demonstrated (22). The possible impact of lack of estrogen representation in the brain on psychosexual development of both women and men with aromatase deficiency was considered in two reports (3, 10). To date, no evidence is available to suggest gender identity or libido problems in these patients.

In the adult man who fails to synthesize estrogens (3), some findings were extremely striking. Circulating levels of LH as well as FSH were extremely high despite elevated (3) testosterone levels, suggesting an important role for estrogens, whether circulating (23) or locally produced in the brain (24), in suppressing LH secretion. Epiphyseal fusion and maintenance of bone mass are two recently recognized consequences of estrogen action in men (2, 3). Estrogens have been shown to exert their suppressive effects on osteoclast development and action through regulation of cytokine expression in the bone (25, 26). Insulin resistance in the aromatase-deficient man improved after estrogen administration (3). This is consistent with the similar decrease in basal insulin levels in postmenopausal women treated with transdermal estradiol (1). On the other hand, the estrogen-resistant man (2) showed biochemical evidence of both insulin resistance and glucose intolerance, which did not respond to very high doses of estrogen. Although the mechanism of insulin resistance in the aromatase-deficient man (3) is not known, it is not likely to be caused by elevated testosterone levels, because the estrogen-resistant man (2) had a much severer degree of insulin resistance despite normal serum testosterone concentrations. Any information regarding insulin responsiveness in ER-disrupted mice (21) has not been reported. The macroorchidism noted in the aromatase-deficient man may be a result of elevated gonadotropin levels (3). It should also be noted that the sizes of the estrogen-resistant man's testes were within the upper limit of the normal range (2). Although a semen analysis of the aromatase-deficient man had not been performed, the normal sperm count but low motility noted in the estrogen-resistant man (2) suggest that infertility may be another symptom associated with deficient estrogen action in the testis. Most of the ER-disrupted adult mice were infertile due to low sperm counts (21). Sperm motility was not evaluated in these animals.

Another crucial role of estrogens in men has recently been demonstrated to be the regulation of lipoprotein synthesis

(27). Aromatase inhibitor treatment of normal men was observed to decrease serum high density lipoprotein cholesterol levels (27). The abnormally high low density lipoprotein/high density lipoprotein cholesterol ratio in the aromatase-deficient man (3) emphasizes this critical role of estrogen action in men, which is the prevention of cardiovascular disease. We have yet to see whether this abnormal lipid profile in the aromatase-deficient man can be reversed with estrogen replacement.

Finally, one can envision mutations in the *CYP19* gene causing milder degrees of aromatase deficiency analogous to other steroidogenic P450 defects, *e.g.* late-onset 21β-hydroxylase deficiency. These may give rise to a whole host of symptoms with varying degrees of severity, such as polycystic ovary syndrome and incomplete pubertal development in women, and infertility, insulin resistance, extreme tallness, and a tendency to develop cardiovascular disease in both men and women. As another twist, as tissue-specific expression of the *CYP19* (P450arom) gene is regulated in part by tissue-specific promoters (4), mutations in any of these promoter regions or splice junctions may result in the loss of estrogen formation in one organ only, *e.g.* placenta, ovary, brain, or adipose tissue. Analysis of such cases would provide insight into the relative roles of estrogens produced in each of these sites.

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Distribution of Aromatase P450 Transcripts and Adipose Fibroblasts in the Human Breast*

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ABSTRACT

The fibroblast component of adipose tissue is the primary extragladular site of aromatase P450 (P450arom) expression, which is responsible for the conversion of C_{19} steroids to estrogens. Previously, we have shown positive correlations between the level of P450arom transcripts and the ratio of fibroblasts to mature adipocytes in adipose tissue samples proximal to breast tumors. The present study was conducted to determine the distribution of P450arom messenger ribonucleic acid (RNA) levels and fibroblast to adipocyte ratios in disease-free breasts of premenopausal women. Adipose tissue samples were collected from 3 regions (outer, upper, and inner) of both breasts of 13 women undergoing reduction mammoplasty. The histological composition of adipose tissue (n = 12) was determined by morphometry using a computerized image analysis program. We used a competitive RT-PCR method employing rat P450arom complementary RNA as an internal standard to quantify adipose P450arom transcripts in tissue total RNA samples (n = 11). Overall, 67% of the highest fibroblast to adipocyte ratios and 64% of the highest P450arom transcript levels were detected in an outer breast region, whereas in only 1 patient were the highest values detected in an inner region. Parametric ANOVA showed significant differences between the fibroblast content of the regions [p(F) = 0.037]. This distribution pattern directly correlates with the most common or the least common sites of carcinoma in the breast, the outer and inner regions, respectively. Moreover, a direct relationship was demonstrated between adipose fibroblasts and P450arom transcripts within the breast, in that regions with the highest fibroblast to adipocyte ratios contained the highest P450arom transcript levels (by ANOVA of contrast variables, $P = 0.0009$). These results suggest that, similar to our previous findings in the breast bearing a tumor, adipose tissue aromatase expression in the disease-free breast is determined by the local ratio of fibroblasts to adipocytes. (*J Clin Endocrinol Metab* **81:** 1273–1277, 1996)

THE CONVERSION of C_{19} steroids to estrogens by aromatase P450 (P450arom) takes place in a number of human cells, *e.g.* the ovarian granulosa cell (1), the placental syncytiotrophoblast (2), and the adipose fibroblast (3, 4). Aromatase expression does not occur in mature adipocytes, but, rather, in the fibroblasts that are dispersed among the adipocytes (3, 4). Adipose fibroblasts are believed to be the preadipocytes that give rise to mature adipocytes in human adipose tissue. Aromatase expression in adipose tissue has long been implicated in the pathophysiology of breast cancer growth (5–8). Potent aromatase inhibitors have recently been widely used in the treatment of postmenopausal breast cancer (9–11). Peripheral estrogen production in whole body adipose tissue may be enough to effectively increase circulating levels of estrogens (5). Estrogen produced by adipose tissue within the breast also may act locally to promote the growth of breast tumors; O'Neill and co-workers (7) demonstrated that the breast quadrant displaying the highest level of aromatase activity was consistently involved with tumor. Furthermore, we found the highest levels of P450arom transcripts in fat from the quadrants bearing tumors in 69% of the cases (8). In the same study, the quadrants containing the highest fibroblast to adipocyte ratios had the highest P450arom levels.

The present study was conducted to ascertain the distributions of adipose fibroblast content and P450arom transcript levels in tumor-free breasts of women undergoing reduction mammoplasty. We previously demonstrated that both aromatase activity (3) and P450arom transcript levels (4) primarily reside within the fibroblast fraction of the adipose tissue, and we observed a positive correlation between fibroblast to adipocyte proportions and P450arom transcript levels in breast adipose tissue from mastectomy specimens bearing tumors (8). However, the presence of a tumor in breast adipose tissue in this instance may have influenced the distribution of P450arom expression in the breast. Therefore, the present study used reduction mammoplasty samples to investigate the distribution patterns of fibroblasts and P450arom transcript levels in the disease-free breasts of premenopausal women. Additionally, we investigated the possible effects of side (left *vs.* right) or position (outer, upper, or inner) on these distribution patterns.

Materials and Methods

Tissue acquisition and processing

Breast fat was obtained at the time of surgery from both breasts of 13 women undergoing reduction mammoplasty for symptomatic macromastia (6 samples/patient). Written consent was obtained be-

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fore surgery, including a consent form and protocol approved by the Institutional Review Board for Human Research of the University of Texas Southwestern Medical Center. Whole breast adipose tissue samples were frozen in liquid nitrogen and stored at −70 C.

Adipose tissue samples were obtained from the midportions of the outer and inner breast halves. A third sample was also obtained from the middle of the upper breast fat, which practically is the midpoint between the outer and inner samples above the nipple. This mode of sampling was dictated by the technique of the reduction mammoplasty operation.

Human ribonucleic acid (RNA) isolation and rat P450arom complementary RNA (cRNA) synthesis

Human total RNA was isolated from frozen tissue of 11 women by the guanidinium thiocyanate-cesium chloride method (12). Rat P450arom cRNA was synthesized as previously described (4, 8).

Quantitative PCR after RT-PCR and hybridization

RT-PCR using synthesized oligonucleotides as primers and radiolabeled probes was performed as previously described (4, 8). Briefly, an antisense oligonucleotide complementary to coding exon IV was used for primer extension. The created single stranded P450arom complementary DNA template was then amplified by PCR using the same antisense oligonucleotide and a sense oligonucleotide identical to a sequence in coding exon II. As an internal standard, 1 pg of a homologous rat P450arom cRNA (4, 8) was reverse transcribed and coamplified in each human breast total RNA sample to control and correct for the differences in amplification efficiency between these samples. The antisense and sense primers were designed as such to recognize identical sequences in both human and rat complementary DNAs. Amplified products were divided in half and transferred to separate membranes. Each membrane was hybridized with a species-specific labeled oligonucleotide probe designed to recognize the heterologous midportion of the amplified human or rat product. An AMBIS image acquisition and analysis system (AMBIS, Inc., San Diego, CA) was used to quantify radioactivity on each membrane. As each sample initially contained an equal amount of rat cRNA, the ratio of human to rat amplification product obtained for the P450arom messenger RNA (mRNA) level in each adipose tissue sample was reported as an arbitrary unit.

Histology

Representative sections were obtained from each breast region (n = 12) using the same frozen adipose tissue fragments, parts of which were previously used for RNA isolation. Sections (3 μm thick) of paraffin-embedded tissue were stained with hematoxylin-eosin. Adipocytes, fibroblasts, acellular stroma, ductal epithelium, and endothelium-lined space components were quantified by a computer-assisted image processing program (Image 1.51, NIH, Bethesda, MD) and expressed as percentages of the total image in any particular field.

Statistical analysis

Parametric repeated measures ANOVA followed by Newman-Keuls multiple comparisons test were used to compare fibroblast proportions and P450arom transcript levels between regions of individual breasts. Multivariate ANOVA for repeated measures and ANOVA of contrast variables were used to test the correlation between fibroblast proportions and P450arom levels. Simple linear regression analysis was used in establishing the standard curve.

Results

Standard curve (Fig. 1)

Initially, we established a range of starting amounts of human breast adipose total RNA (2–16 μg) that yielded linear increases in amplification product in the presence of a constant amount of coamplified rat P450arom cRNA (Fig. 1). The arbitrary units for P450arom mRNA levels were plotted against the initial breast adipose tissue total RNA quantities to create a standard simple regression line (r = 0.987; $P < 0.0001$). The samples were amplified in duplicate, and the mean value for each duplicate was reported as the P450arom transcript level for each sample. Individual values of the standard curve experiment remained within ±15–25% of the mean. This experiment was repeated twice with similar results. Using individual values instead of means of duplicates did not change the precision of this assay. Subsequent experiments employed 10 μg breast adipose RNA and 20 cycles of amplification, because under these conditions the intensity of the radioactivity on each slot fell within the linear range of increase in the human amplification product.

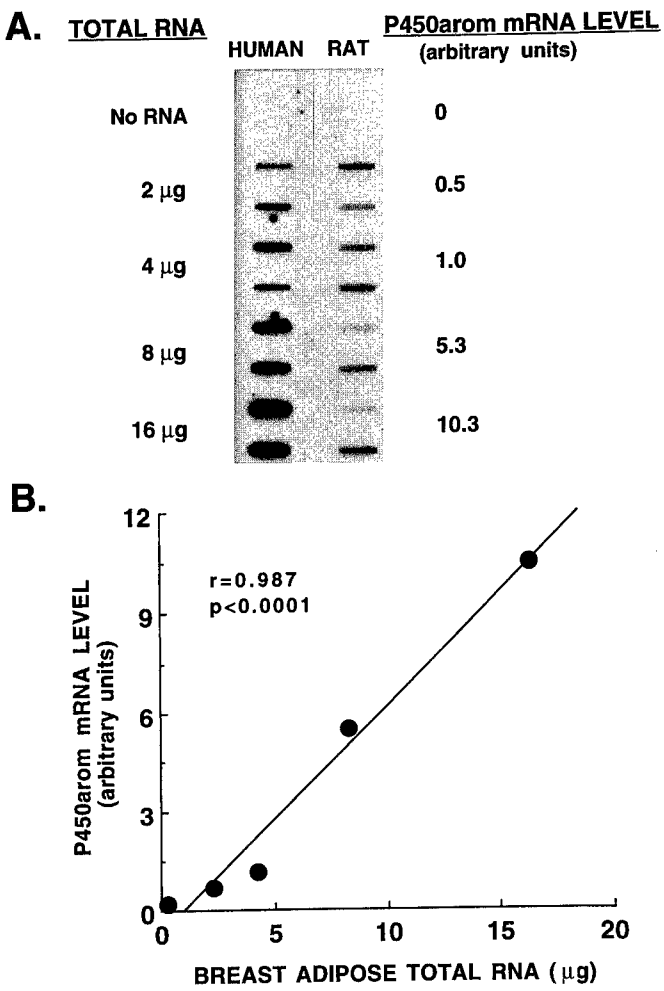

FIG. 1. Standard curve, revealing simple linear relationship between increasing amounts of total breast adipose tissue RNA and corresponding arbitrary units for P450arom transcript levels. A, Coamplification of increasing amounts of breast adipose tissue total RNA and a constant quantity of rat P450arom cRNA internal standard (1 pg). Samples were amplified in duplicate. B, A linear increase in breast P450arom levels (ratios of human to rat amplification products) was observed with increasing amounts of breast adipose tissue RNA. Statistical analysis was performed using simple linear regression with a coefficient of determination of 0.987 at a level of significance of $\alpha = 0.05$ and $P < 0.0001$.

P450arom transcript levels in breast regions (Fig. 2 and Table 1)

Figure 2 illustrates a representative experiment for 1 of the 11 patients. Eleven such separate experiments were performed, and P450arom transcript levels are listed in Table 1. P450arom transcript levels from 6 breast regions (3 on each side) were compared for each woman. P450arom transcripts were detected in all samples by autoradiography and the AMBIS Radioanalytic Imaging System. Parametric repeated measures ANOVA revealed a linear trend, with the highest levels found in the outer region followed by the upper region, and the lowest levels found in the inner region; however, this relationship did not reach statistical significance [p(F) = 0.110]. In 64% of the women, the highest levels were localized to an outer breast region, whereas in only 1 patient was the highest level found in an inner region. This distribution followed the same order as the fibroblast to adipocyte ratio distribution among the breast regions (see below).

Distribution of fibroblasts in breast regions (Fig. 3 and Table 2)

Sections of breast regions from 12 women revealed marked differences in the ratio of fibroblasts to adipocytes (1%-15%;

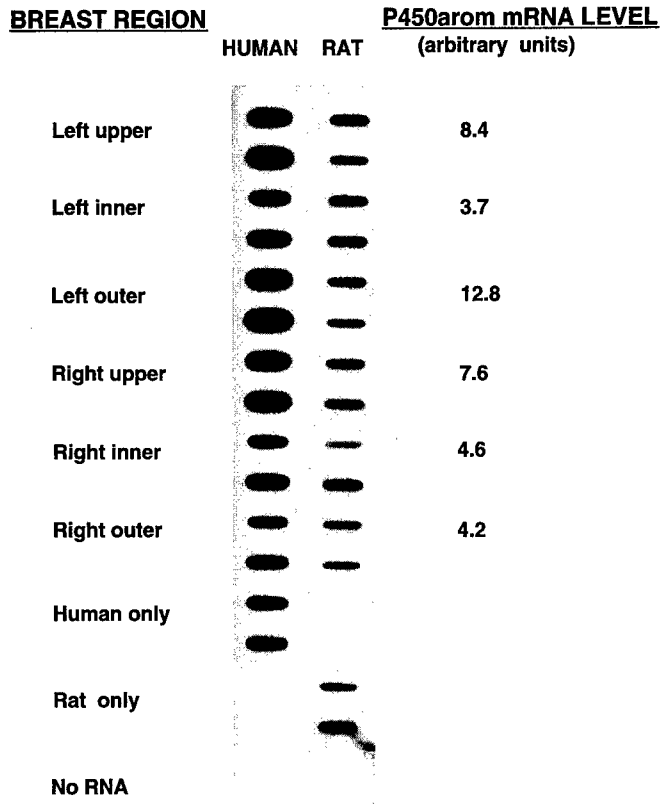

FIG. 2. A representative slot blot autoradiograph from one patient is shown. We compared P450arom transcript levels in breast fat using 10 μg total RNA from each quadrant. Each sample was assayed in duplicate (variation in duplicate samples ranged from 2–8%), and radioactivity on the blotting membranes was quantified by an AMBIS radioimaging system. A corrected AMBIS reading was calculated and recorded as an arbitrary unit for the P450arom transcript level for each quadrant, as described in Fig. 1.

TABLE 1. Adipose tissue P450arom transcript levels in breast regions

Patient No.	Left			Right		
	Inner	Upper	Outer	Inner	Upper	Outer
1	0.21	0.28	0.12	0.21	0.10	0.07
2	1.31	1.80	2.23	1.23	2.47	1.74
3	0.13	0.13	0.18	0.11	0.14	0.22
4	2.76	2.80	2.29	3.85	1.98	2.23
5	1.01	0.80	0.33	1.58	1.24	2.54
6	0.07	0.03	0.08	0.05	0.01	0.07
7	3.67	8.40	12.80	4.60	7.60	4.20
8	0.96	1.60	1.01	1.07	0.77	1.90
9	1.30	4.60	4.70	2.30	3.30	4.84
10	1.25	3.78	2.03	3.46	3.40	3.08
11	1.20	1.89	1.03	1.87	1.31	1.95

Fig. 3). Mature adipocytes comprised the remainder of the sections. Glandular epithelium, vascular endothelial cells, and acellular stroma were noted in some samples in negligible proportions. Fibroblast to adipocyte ratios are listed in Table 2. Sixty-seven percent of the highest ratios were found in an outer breast region, whereas only 1 patient had the highest fibroblast to adipocyte ratio in an inner region. Parametric repeated measures ANOVA revealed significant differences between regions of individual breasts [p(F) = 0.037]. Newman-Keuls multiple comparisons test showed significant differences between the outer and inner or outer and upper regions ($P < 0.5$), but no significant difference was found between upper and inner regions.

P450arom transcript levels and fibroblast content were determined in the identical breast adipose tissue samples of 10 women. There was a statistically significant direct relationship between the distributions of fibroblasts and P450arom mRNA levels in the breast. In other words, proportions of fibroblasts in the 6 breast regions of a woman increased in the same order as the P450arom transcript levels. Multivariate ANOVA comparing regional fibroblast to adipocyte ratios with regional P450arom mRNA levels revealed a direct relationship ($P = 0.023$). Additionally, ANOVA of contrast variables showed that regional fibroblast to adipocyte ratios in the breast increased, with significant differences between each other when they were arranged to match regions with increasing order of P450arom mRNA levels ($P = 0.0009$). No influence of side (left *vs.* right) was noted on the distribution of P450arom transcripts or fibroblasts.

Discussion

Adipose tissue has a similar uniform histological appearance in various parts of the human body; however, evidence from several laboratories, including ours, suggests that the endocrinological behavior of this tissue may be different in the breast, abdomen, buttock, and thigh of a woman (13–15). Differences in adipose tissue aromatase expression at various body sites of women have been well characterized. Aromatase expression in adipose tissue samples of women was highest in the buttock region, followed by that in the thigh, and the lowest in the abdomen (13). Thus far, breast adipose tissue aromatase expression has not been directly compared to that at other body sites. We found that different promoters are responsible for aromatase expression in adipose tissue in

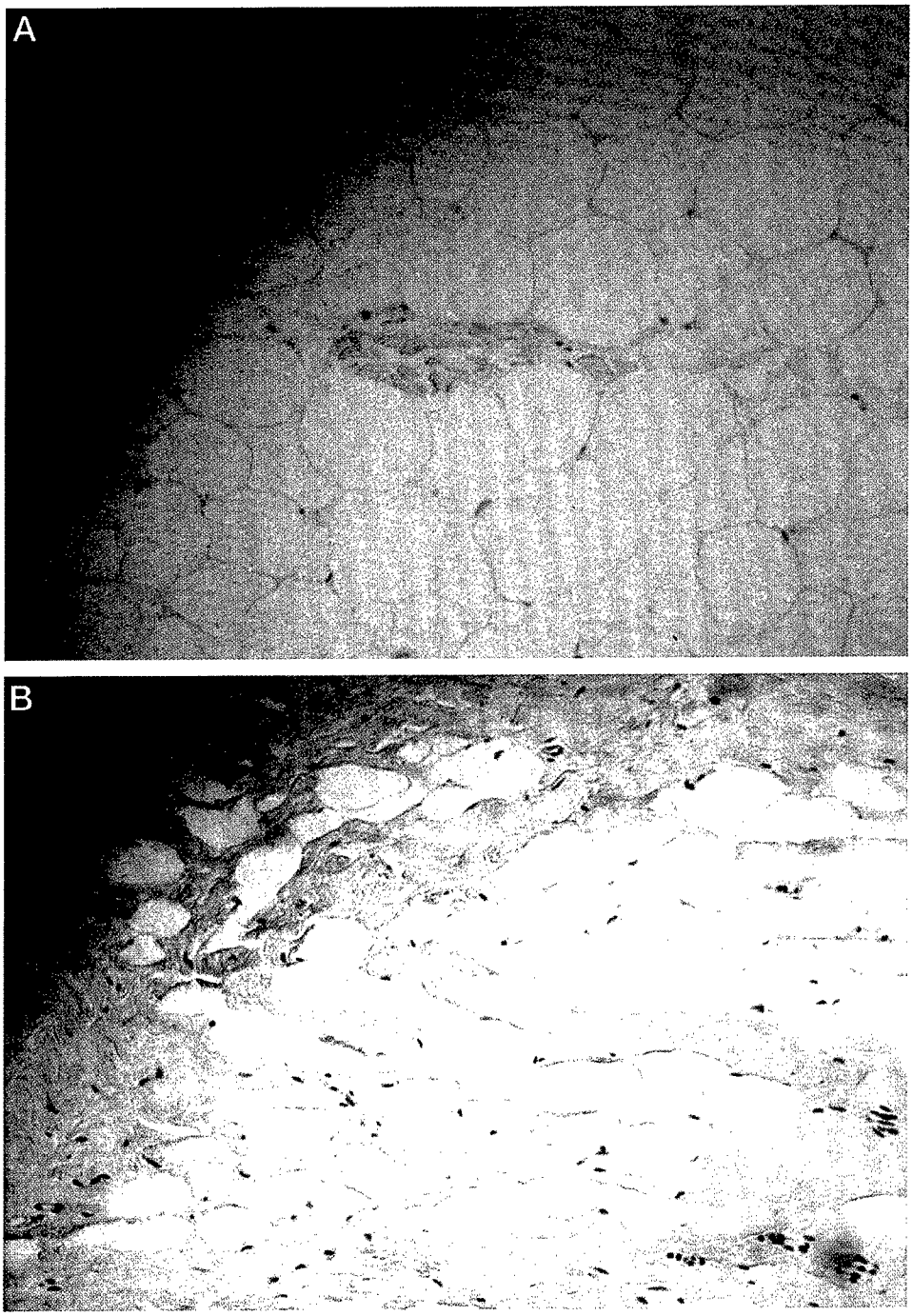

FIG. 3. A, A representative section of the breast adipose tissue with low fibroblast content. Whereas mature adipocytes (large cells with cytoplasms distended by lipid material and peripheral nuclei) constitute the majority of this section, a few scattered fibroblasts dispersed between mature adipocytes are seen. A very low P450arom mRNA level (normalized to total RNA content) was detected in this sample. B, A breast fat specimen showing higher numbers of fibroblasts. Fibroblasts are oval or spindle-shaped and do not contain conspicuous lipid in their cytoplasms. A markedly higher level of P450arom mRNA was found in this sample. Hematoxylin and eosin stain; magnification, ×200. Note that handling and magnification for both sections are the same, whereas the sizes of the adipocytes vary. Tissue components were quantified by computerized morphometry. A positive correlation was found between the tissue fibroblast content and the P450arom transcript level in breast adipose tissue.

breast and lower thighs (16). This suggests that different signal transduction pathways are responsible for aromatase expression at these two sites. Finally, in contrast to variable expression in breast regions, the marked differences in aromatase expression in the abdomen, buttock, and thigh are not related to the fibroblast to adipocyte ratios at these body sites (our unpublished observations). This suggests that in contrast to breast regions, intrinsic differences in the specific aromatase expression among the fibroblasts from these other body sites, rather than fibroblast numbers, determine local estrogen biosynthesis.

The studied group of women undergoing reduction mammoplasty suffered symptomatic macromastia. The etiology of this condition is not known, and it is possible that breast aromatase expression in these women may be significantly different from that in women with smaller breasts. Therefore, our results should be viewed with a degree of caution when applied to the general population. Nevertheless, the subjects in this study represent a premenopausal group of women with cancer-free breasts, in contrast to our previous study group that was comprised of postmenopausal women with breast cancer (8). Theoretically, general anesthesia and its associated stress may give rise to elevated cortisol levels, which may effect aromatase expression in adipose tissue.

TABLE 2. Fibroblast to adipocyte ratios in breast regions

Patient No.	Left			Right		
	Inner	Upper	Outer	Inner	Upper	Outer
1	0.03	0.15	0.12	0.09	0.05	0.05
2	0.02	0.06	0.10	0.04	0.05	0.06
3	0.12	0.11	0.13	0.04	0.07	0.11
4	0.12	0.02	0.05	0.12	0.04	0.06
5	0.06	0.04	0.09	0.10	0.10	0.05
6	0.05	0.03	0.11	0.04	0.03	0.06
7	0.05	0.15	0.05	0.06	0.09	0.15
8	0.10	0.06	0.08	0.09	0.05	0.12
9	0.03	0.04	0.01	0.02	0.02	0.07
10	0.03	0.05	0.06	0.10	0.04	0.10
11	0.05	0.04	0.06	0.07	0.10	0.12
12	0.04	0.03	0.10	0.07	0.05	0.11

However, pharmacological doses of glucocorticoids are capable of causing a detectable increase in aromatase activity of adipose fibroblasts after 3 h of treatment (17), whereas the samples for this study were collected within 1 h after initiation of general anesthesia. Therefore, an effect of general anesthesia on our results through this mechanism is unlikely. Finally, in this study, P450arom transcripts determined in breast adipose tissue are assumed to be translated into P450arom protein with aromatase activity, which gives rise to local estrogen biosynthesis. At least two published studies from this laboratory support this assumption. Significant levels of aromatase activity have been demonstrated in adipose tissue (3), and aromatase activity of adipose fibroblasts has been shown to be primarily regulated by changes in P450arom transcript levels (18).

One of every nine American women will develop breast cancer. More than two thirds of breast carcinomas develop in women over the age of 50 yr. The present study provides data of an associative nature and does not necessarily demonstrate a cause and effect relationship between local estrogen biosynthesis and the occurrence of breast cancer. However, it suggests that local factors in the breast may influence carcinogenesis. It has been hypothesized that 5–15 yr are required for a single malignant cell to develop into a clinically detectable breast cancer tissue mass. One may then assume that conditions that predispose a postmenopausal woman to develop breast cancer may preexist in her younger years. High local estrogen concentrations in the breast may predispose cancers initiated in these areas to develop more aggressively than those in others. Breast cancer occurs in the outer quadrants in 71% of the cases in a large series (19). The present study reveals that 64–67% of the highest P450arom transcript levels and adipose fibroblast content were also found in an outer breast region. On the other hand, we have previously shown in mastectomy specimens that the highest transcript levels were detected in regions bearing tumors regardless of the tumor location (8). Tumors secrete products (20), *e.g.* members of the interleukin-6 cytokine subfamily, that stimulate aromatase expression in the surrounding adipose tissue (21). Moreover, estradiol stimulates cytokine secretion by T-47D breast cancer cells (our unpublished observations). These data collectively suggest that tumors preferentially develop in breast regions with the highest aromatase expression. Once developed, tumor growth is further promoted by high local estrogen concentrations that are maintained by the positive feedback relationship between adipose tissue aromatase expression and tumor-derived cytokines.

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Rapid Paper

Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells

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Abstract

The expression of transcripts of cytokines of the interleukin-6 (IL-6) family has been examined in human breast tumors, breast cancer cell lines, and adipose stromal cells, by means of reverse transcription polymerase chain reaction amplification. Of the six breast tumor samples examined, all expressed transcripts encoding IL-6 and Leukemia Inhibitory Factor (LIF). Four of the samples also expressed transcripts for oncostatin M (OSM) and IL-11, and three expressed the IL-6 receptor. Adipose stromal cells expressed IL-6, IL-11 and LIF, but not the IL-6 receptor, consistent with previous conclusions that IL-6 activity in these cells required addition of IL-6 soluble receptor. In the case of T47D cells, expression of IL-11 protein was confirmed by immunotitration. Moreover, in these cells, expression of IL-11 transcripts was induced 3-fold by addition of estradiol to the culture medium. These results add credence to our previous proposal that breast cancer development is regulated in part by local autocrine and paracrine mechanisms via epithelial/mesenchymal interactions, in which estrogen produced by stromal cells surrounding the tumor acts to stimulate the production of growth factors and cytokines by the tumor cells. Some of these may act to stimulate further the growth and development of the tumor, while these or other factors may act on the surrounding mesenchymal cells in a paracrine fashion to stimulate aromatase expression in the presence of glucocorticoids. Thus, a positive feedback loop is established which leads to the development and growth of the tumor.

Keywords: Aromatase; Cytokines; Breast cancer; Adipose

1. Introduction

Estrogen biosynthesis is catalyzed by aromatase P450 ($P450_{arom}$, the product of the CYP19 gene). Aromatase activity is found in a variety of tissues including adipose, ovary, placenta, testis and brain [1–6]. In postmenopausal women adipose tissue is the primary site of estrogen biosynthesis [7–10]. Studies from a number of laboratories have examined aromatase activity or else aromatase expression, as determined by RT-PCR, in breast adipose tissue from cancer patients [11–16]. The results have indicated that aromatase expression is higher in adipose tissue proximal to a tumor, as compared to that distal to a tumor. Although such a correlation was not observed in one study [17], these observations suggest that there is crosstalk between the tumor and the surrounding tissue in terms of the ability of the latter to synthesize estrogens, and that factors produced by tumors stimulate the surrounding stromal cells to produce estrogens. These in turn stimulate the tumor cells to produce more growth factors, and thus stimulate the growth of the tumor in a paracrine fashion [18,19].

In order to study the regulation of aromatase expression in adipose tissue, we have utilized a cell model, namely adipose stromal cells in primary culture [20]. Tissue-specific regulation of the $P450_{arom}$ gene is due, in part, to the use of alternative transcriptional start sites as a consequence of tissue-specific promoters [21–25]. In adipose tissue three promoters are used, promoter II, promoter I.3 and promoter I.4 [26,27]. Furthermore, different hormonal conditions will also determine which

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of the three promoters is used in adipose stromal cells in monolayer culture [26]. Glucocorticoids, in the presence of serum, induce the expression of aromatase via promoter I.4. Promoter I.4 is a distal TATA-less promoter which contains an upstream glucocorticoid response element (GRE), an interferon-γ activation site (GAS) and an Sp1 site [28]. Reporter gene analysis of this promoter demonstrated that the GRE and Sp1 binding site were required for transcription in the presence of serum and glucocorticoids [28]. Earlier work had demonstrated the requirement for serum in the induction of aromatase activity in the presence of glucocorticoids, however, no good candidate for the 'serum factor' could be found [29]. Recently, conditioned medium from breast cancer cell lines, as well as other cell types, has been found to mimic the action of serum in the stimulation of aromatase by glucocorticoids in adipose stromal cells in culture [30,31], as well as tumor fibroblasts [12]. Similarly, members of the gp130 receptor family of cytokines, namely interleukin-11 (IL-11), leukemia inhibitory factor (LIF), and oncostatin M (OSM), have been shown to replace serum under these circumstances [32]. This suggests that one or more members of this cytokine finally could be present in the conditioned media from these cells. In contrast to the other members of this cytokine family, IL-6 could not induce aromatase activity on its own. However, IL-6 was as active as IL-II and OSM when the IL-6 soluble receptor was added to the culture medium [32]. Previously, Reed and colleagues had shown that IL-6 was present in breast cystic fluid and could stimulate aromatase expression of breast tumor fibroblasts in the presence of dexamethasone [12].

Further analysis of the promoter I.4 region revealed that the GAS element, to which members of the STAT family of transcription factors are known to bind, was essential for cytokine-induced expression of aromatase. Recent findings from this laboratory have shown that stimulation of aromatase by members of this cytokine family and glucocorticoids is mediated by a member of the Jak family of tyrosine kinases (JAK1) as well as a STAT transcription factor (STAT3) which binds to the GAS element [32].

In the work presented here, the expression of these cytokines by adipose stromal cells, a breast cancer cell line and breast tumor samples was evaluated. The results indicate that most of the tumor samples and cell lines tested express one or more of these cytokines. These findings lend further support to the hypothesis that cytokines produced by breast tumors, as well as by adipose stromal cells, may up-regulate the expression of aromatase in surrounding tissue, and the resulting increase in local estrogen production could influence, in turn, the growth of the tumor.

2. Material and methods

2.1. Culture and treatment of adipose stromal cells

Subcutaneous adipose tissue was obtained from women at the time of reduction abdominoplasty or reduction mammoplasty. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were prepared as described [20] and maintained in primary culture in Waymouth's enriched medium containing 10% Nu serum (10% v/v) (Collaborative Research Inc.) and allowed to grow to confluence (5–6 days) before treatment. At this time serum was removed for 24 h, and the cells were treated with 250 nM dexamethasone for 48 h before cytokines or conditioned medium from T47D cells was added. Aromatase activity was determined by the incorporation of tritium into [H^3]water from (1β-H^3]androstenedione as described previously [20].

2.2. Culture and treatment of T47D cells

T47D cells were cultured in RPMI (without phenol red), 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were grown to confluence before treatment. At this time serum was removed for 24 h (with two changes of media during this period to ensure complete removal of serum), and the cells were treated with estradiol for 24 h or left untreated. The conditioned medium was collected and centrifuged to remove cellular debris.

2.3. Culture of control cell lines

As controls, cell lines were cultured which were reported to express the individual cytokines: U937, a human histiocytic lymphoma cell line, which expresses IL-11 and IL-6; HCT-8/HRT-18, a human ileocecal adenocarcinoma, which expresses LIP; and CESS, a human lymphoblastoma, which expresses IL-6 soluble receptor. All cell lines were obtained from ATCC and cultured according to their instructions.

2.4. RNA preparation from cells in culture

RNA was prepared using RNasolB (Biotecx Inc, Houston, TX), according to the manufacturer's instructions.

2.5. RNA preparation from tumor samples

RNA was prepared by the guanidinium thiocyanate/cesium chloride method as described [33]. RNA concentration was determined by spectrophotometric absorption at 260 nm.

2.6. RT-PCR

RNA was denatured at 65°C for 2 min and annealed to random hexamers (Gibco-BRL) by allowing the samples to cool slowly to 37°C. To synthesize the complementary DNA (cDNA), the annealed RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) at 37°C for 1 h in the presence of 200 μM of each dNTP, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DDT and 5 units of RNasin (Gibco-BRL). cDNA was amplified using the polymerase chain reaction in the presence of 500 μM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin (w/v), 10 pmol of the specific oligonucleotide (see Table 1) and 4 units of *Taq* polymerase (Perkin-Elmer) in a final volume of 20 μl.

PCR conditions were: denaturing at 94°C for 30 s, annealing at 50°C for IL-6, LIF, OSM and IL-6 receptor and 55°C for IL-11 for 30 s, and extension at 72°C for 1 min, for 25 cycles. The PCR was performed in a Gene Amp PCR system 9600 (Perkin-Elmer). GAPDH sequence was also amplified from each sample as a separate check on amplification efficiency (data not shown). The amplification products were run on a 1.5% agarose gel and transferred to Hybond N^+ (Amersham) by capillary elution in 0.4 M NaOH solution. Southern hybridization was carried out with specific oligonucleotides, internal to the sense and antisense oligonucleotides used in the PCR amplification, which were end-labeled with [^{32}P]phosphate. Hybridization was carried out overnight at room temperature followed by a series of stringent washes at 5°C below the T_m of the oligonucleotide. Radioactivity on the membrane was determined by autoradiography.

Table 1
Oligonucleotides used for RT-PCR amplification of specific cytokines

Interleukin 11 (Sequence described in [37]).	
Sense:	5′-CACATGAACTGTGTTTG CCGCCTGGT-3′
Antisense:	5′-GCAGCCTTGTCAGCAC ACCTGGGAGCTGTAGA-3′
Internal:	5′-TCGAGGGGGGCCAGGT GGTGGCCC-3′
Interleukin 6 (Sequence described in [38]).	
Sense:	5′-GCCTTCGGTCCAGTTGC CTTC-3′
Antisense:	5′-CTGCAGGAACTGGATC AGGAC-3′
Internal:	5′-AAGGTTCAGGTTGTTTT CTGCCAG-3′
Interleukin 6 soluble receptor (Sequence described in [39]).	
Sense:	5′-ATGCTGGCCGTCGGCT GCGCG-3′
Antisense:	5′-GAAAGAGCTGTCTCCC TCCGG-3′
Internal:	5′-CCGGTAGCATGAATAG TTTCCAGA-3′
Oncostatin M (Sequence described in [40]).	
Sense:	5′-ACGCTGCTCAGTCTGG TCCTT-3′
Antisense:	5′-CACCCCCTTCCTCAGGG CCTG-3′
Internal:	5′-GATGTTCAGCCCAGAC CTCTCCAA-3′
Leukemia Inhibitory Factor (Sequence described in [41])	
Sense:	5′-AAACATGGGGCGGGGA GCCCC-3′
Antisense:	5′-GCACTGGGGTTGAGGA TCTTC-3′
Internal:	5′-AAAGAGGGCATTGGCA CTGCCATT-3′

3. Results

3.1. Reverse transcriptase polymerase chain reaction analysis of breast tumor samples, breast cancer cell lines and adipose stromal cells

The RT-PCR analysis is shown in Fig. 1 and a summary of the results is given in Table 2. The RT-PCR method enables a range of tumor samples and cell lines to be assessed for the expression of transcripts for a particular cytokine. All the tumor samples expressed one or more of the cytokines examined, namely IL-6, IL-11, LIF, OSM, as well as the IL-6 receptor, with wide variation in the pattern and levels observed between patients. Adipose stromal cells expressed all the transcripts except IL-6 receptor and OSM. T47D cells expressed only IL-11 and IL-6 receptor. Estradiol (10^- 7 M) induced the expression by T47D cells of IL-11 transcripts, but not of IL-6 receptor.

3.2. The effect of conditioned media from estradiol-treated T47D cells on aromatase activity in adipose stromal cells in culture

As can be seen from Fig. 2, conditioned media from T47D cells stimulated $P450_{arom}$ activity of adipose stromal cells maintained in the presence of dexamethasone. The stimulatory factor(s) present in the conditioned media was completely titrated by addition of an anti-IL-11 antibody in a concentration dependent fashion. However, the antibody had no effect on basal activity in the absence of conditioned medium.

4. Discussion

From the results of the present study, it is apparent that of the six breast tumor samples examined, all were capable of expressing transcripts for LIF and IL-6. Of the six, four were also capable of expressing transcripts for oncostatin M and IL-11. Moreover, three of the

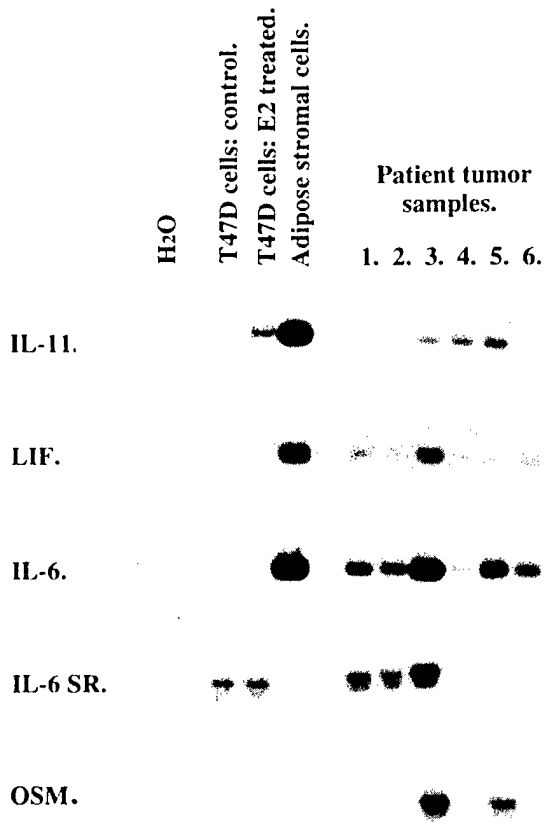

Fig. 1. Reverse transcriptase polymerase chain reaction analysis of breast tumor samples, T47D cells and adipose stromal cells. RNA was prepared using the guanidinium thiocyanate-cesium chloride method. The RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase. Specific oligonucleotides for the polymerase chain reaction were designed to amplify portions of the respective cDNAs, which spanned exons within each gene. After PCR the samples were run on a 1.5% agarose gel, then transferred to nitrocellulose. In order to identify specific PCR products, the nitrocellulose was probed with oligonucleotides complementary to specific sequence for the gene and internal to those used for amplification.

tumor samples expressed the receptor for IL-6. Thus it would appear that breast tumors are capable of synthesizing factors which stimulate aromatase and enhance estrogen production by surrounding adipose stromal cells, although obviously caution must be exercised in extrapolating from an expressed transcript to a secreted protein. Interestingly, a breast cancer cell line, namely T47D, was capable of expressing only transcripts for IL-11 and the IL-6 receptor. However, the expression of IL-11 transcripts was induced by treatment of the cells with estradiol, although expression of the IL-6 receptor was not. Previously we observed that conditioned medium from T47D cells was capable of stimulating aromatase activity by adipose stromal cells [30]. This stimulatory activity was increased by estradiol in a concentration-dependent fashion [30], and moreover could be blocked by an antibody to IL-11 (Fig. 2). Thus it is likely that the factor present in T47D cell-conditioned media which stimulates aromatase activity of adipose stromal cells is IL-11, and moreover its expression and secretion by the T47D cells is stimulated by estrogens. So in this case, at least, expression of the transcript results in secretion of a bioactive protein.

If extrapolated to the in vivo situation, these results add credence to our previous proposal that breast cancer development is regulated in part by local autocrine and paracrine mechanisms via epithelial/stromal interactions, in which estrogen produced by stromal cells surrounding the tumor acts to stimulate the production of growth factors and cytokines by the tumor cells. Some of these may act to stimulate further the growth and development of the tumor, while these or other factors may act on the local adipose stromal cells in a paracrine fashion to stimulate aromatase expression in the presence of glucocorticoids. Thus, a positive feedback loop is established which leads to the development and growth of the tumor.

An important unanswered question relates to the nature of the cell type(s) within the tumor samples which are expressing these cytokines. In a recent study, Purohit et al [19] stress the importance of infiltrating macrophages in the production of cytokines such as IL-6 at breast tumor sites. In a study of a patient with a chronic inflammatory response to silicon injection for the purposes of breast augmentation, aromatase activity in breast tissue was very high and correlated with IL-6 production [19].

From the results of the present study, it is apparent that adipose stromal cells themselves have the capacity to express a number of these cytokines, although they do not express the receptor for IL-6, consistent with previous observations that IL-6 has no effect to stimulate aromatase expression by these cells, except when the soluble receptor is added [32]. However, the fact that the cells can also express IL-11 and LIF, raises the possibility that aromatase expression by adipose stromal cells themselves is regulated in part by paracrine and autocrine mechanisms involving cytokine secretion in the absence of any breast tumor or other heterologous cell type. It is conceivable therefore that a major factor regulating aromatase expression in adipose is local production by adipose cells of such stimulatory factors. Since IL-6 levels in plasma are reported to be increased with aging in both humans [34] and rodents [35], it is conceivable that the previous observation of increased adipose tissue aromatase expression with aging [36] may also be the consequence of production of aromatase-stimulating cytokines, either in the adipose tissue itself or in other body sites.

Table 2
Summary of expression of cytokines in breast tumor samples, T47D breast cancer cells and adipose stromal cells, as determined by RT-PCR

Cell line		Expressed product				
		IL-11	LIF	IL-6	IL-6R	OSM
T47D	Control	+	–	–	+	–
	E2 treated	++	–	–	+	–
Adipose stromal cells		++++	+++	++++	–	–
Patient tumor samples						
	1.	–	+	++	++	–
	2.	–	+	++	+	–
	3.	+	++	+++	+++	+++
	4.	+	+	+	–	+
	5.	++	+	++	–	++
	6.	+	+	++	–	+

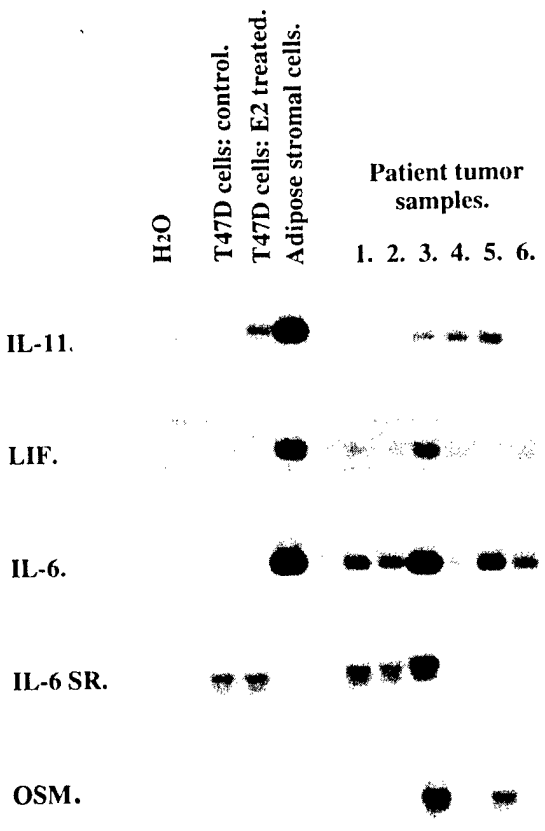

Fig. 2. The effect of conditioned media from estradiol-treated T47D cells on aromatase activity in adipose stromal cells in culture. T47D cells were grown to confluence, then maintained for two successive 12-h periods in serum-free medium, after which time serum-free medium was left on the cells for 24 h, then removed and applied to the adipose stromal cells in primary culture, in the presence or absence of anti-IL-11 antibody in various concentrations. Aromatase activity was then determined following a 2-h incubation with [1β-^{3}H]androstenedione. CM: conditioned medium from T47D cells applied to adipose stromal cells. DEX: dexamethasone. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 h, and represent the mean ± S.E.M. of triplicate replicate dishes.

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Cellular Characterization of Adipose Tissue from Various Body Sites of Women*

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ABSTRACT

Adipose tissue is the primary site of estrogen biosynthesis in postmenopausal women. The two main histologic components of adipose tissue are mature adipocytes and fibroblasts. Aromatase P450 expressed in the fibroblast component of adipose tissue is responsible for catalyzing conversion of C_{19} steroids to estrogens. We previously have demonstrated that, in women, aromatase expression in adipose tissue of various body sites increases with age and that aromatase expression in the hip is markedly higher than in the abdomen. To determine whether this age- and regional-dependent variation in aromatase expression is caused by an alteration in the ratio of fibroblasts to mature adipocytes, we collected sc adipose tissue samples from 19 women (age range: 21–93 yr) at the time of autopsy. Using a computerized image analysis system, we determined by morphometry the proportions of adipocytes, fibroblasts, and vascular endothelial cells within histologic sections of adipose tissue from midabdomen, both breasts, and both hips. The percentage of each cell component at each body site was expressed as the mean of triplicate replicates. Statistical analysis of our results did not indicate any correlation between advancing age and fibroblast to adipocyte ratios in the breast, abdomen, or hip. Fibroblast to adipocyte ratios were found to be significantly higher in the breast and abdomen compared with the hip ($P < 0.05$). No statistical differences were found between the breast and abdomen. These findings suggest that the increase in aromatase expression with advancing age and the higher aromatase expression in the hip compared with the abdomen in women may be caused by alterations in specific signal transduction mechanisms rather than a simple increase in local adipose fibroblast numbers. (*J Clin Endocrinol Metab* **81:** 2443–2447, 1996)

ESTROGENS have diverse actions at different body sites of a woman. Estradiol is produced in the ovarian granulosa cells of premenopausal women, whereas estriol and estradiol are secreted by the placenta (1). In both women and men, estrone is produced in the adipose tissue (2), and a substantial part of this estrone is further converted into estradiol in the periphery (3, 4). Aromatase P450 ($P450_{arom}$) is responsible for conversion of C_{19} steroids to estrogens in all of these tissues (5). Adipose tissue is the major source of estrogen synthesis in men and postmenopausal women (3, 6, 7). This occurs primarily by aromatization of circulating androstenedione from the adrenal cortex. In both women and men, MacDonald and co-workers (8) have observed a progressive increase in the efficiency of the extraglandular conversion of circulating androstenedione to estrone with advancing age and obesity. Subsequently, a direct correlation between increasing age and levels of specific adipose aromatase activity in abdominal adipose fibroblast cultures of women has been demonstrated (9). Finally, we have shown that $P450_{arom}$ transcript levels in adipose tissue biopsies from the abdomens, hips, and thighs of women increase with advancing age regardless of body fat mass (10). We also have demonstrated clear regional differences in $P450_{arom}$ transcript levels, in that the levels were the highest in the hip, followed by the thigh, and lowest in the abdomen (10).

Adipose tissue is composed of mature adipocytes, fibroblasts, and vascular endothelial cells. In addition, glandular tissue dispersed within the adipose tissue is found in the breast. Estrogen production in adipose tissue occurs primarily in the nonadipocyte fraction of the adipose tissue (2, 11). In this nonadipocyte fraction, fibroblasts (12, 13) and vascular endothelial cells (our unpublished observations) were immunohistochemically demonstrated to express aromatase. Fibroblasts comprise 87% of the nonadipocyte fraction (11), whereas only 7% of this fraction is accounted for by vascular endothelial cells (11). Fibroblasts are presumed to be preadipocytes that are the precursors for mature adipocytes. We have previously shown that aromatase activity in adipose fibroblasts in culture is regulated primarily by changes in the level of messenger RNA-encoding $P450_{arom}$ (14). Two recent studies from our laboratory have provided evidence that adipose tissue aromatase expression in the breast bearing a tumor (15) and in the disease-free breast (16) is, in part, determined by local fibroblast populations. Regional fibroblast to adipocyte ratios in the breast were found to be directly proportional to adipose tissue $P450_{arom}$ transcript levels. These findings collectively prompted the current investigation, in which we investigated whether the increase in estrogen synthesis in the periphery, as a function of age

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and body site, is also related to increased numbers of fibroblasts in the adipose tissue. To achieve this, we used computerized morphometry to determine the distribution of cellular components of the adipose tissue samples as a function of age and body site.

Materials and Methods

Acquisition of human tissues

Adipose tissue samples were collected at the time of autopsy from 19 women between the ages of 21 and 93 yr from a variety of ethnic backgrounds. This study has been approved by the Human Institutional Review Board of the University of Texas Southwestern Medical Center. Subjects are characterized in Table 1 as to age, height, weight, and BMI (body mass index = weight/height^2). BMI was included as an indirect measure for body fat content. Three adipose tissue samples, each measuring 1 × 1 × 1 cm, were taken at 2 cm from each other in each of five body sites: right and left breasts (4 cm from the skin surface around the projection of the nipple), right and left hips (4 cm from the skin surface in the middle of each hip), and abdomen (4 cm from the skin surface in the midline). Therefore, at least 15 adipose tissue samples were obtained from each subject. Samples in triplicate were also taken from the lower tip of the omentum in 8 women.

Histologic morphometry

Specimens were immediately fixed in 10% neutral buffered formalin. Samples remained in formalin for 24 h before making hematoxylin- and eosin-stained slides. Each slide was examined at 150× magnification. A computerized image analyzer (NIH Image V1.54) was used to achieve random sampling as described by Gunderson and Jensen (17, 18). Fields to be examined were chosen randomly. A grid was overlaid on each field examined and cells landing on intersections of the grid were counted. Mature adipocytes were defined as differentiated cells distended with lipid material. They contain peripherally displaced inconspicuous nuclei (Fig. 1). Fibroblasts, on the other hand, are spindly cells with eosinophilic cytoplasms and dark and elongated nuclei (19). Fibroblasts in adipose tissue are either clustered in connective tissue or dispersed between adipocytes (Fig. 1). A cell was counted as a fibroblast only if the eosinophilic cytoplasm was clearly identified. Endothelial cells are flattened fibroblast-like cells (Fig. 1C) that line the capillaries or small lymphatic vessels (20). In breast adipose tissue samples, epithelial cells lining occasional glandular structures were noted and counted as glandular cells. The quantity of each of the above cell types was expressed as a percentage of the total area. The three samples from each body site were averaged. The results were expressed as the mean ± SEM. The SEM of triplicate samples ranged from ±0.1 to ±3.9% with 54% of SEM less than ±1.0%. The ratio of fibroblasts to adipocytes was calculated and plotted against age, weight, and BMI. Percentage of fibroblasts and percentage of fibroblasts plus endothelial cells were also plotted against age, weight, and BMI.

Statistical analysis

Simple linear correlation analysis and two-way parametric ANOVA followed by Newman-Keuls multiple comparisons procedure were used as statistical methods.

Results

Table 2 describes the means ± SEM and the ranges of each cell type in 19 subjects. Each cell type is expressed as a percentage of total cells counted for three body sites. The percentages of fibroblasts in the abdominal, left, and right breast samples are greater than those found in the left or right hip samples. As shown in Table 3, no statistically significant correlations were found between the cellular composition of the adipose tissue (in the breast, hip, or abdomen) and age or body fat content of women (n = 19). For the significance level of α:0.05, correlation coefficients for none of the body sites exceeded the minimum absolute R value, which is 0.456 for a sample size of 19 (21). These results did not change when patients at the extreme ends of the BMI spectrum (2nd, 4th, 7th, 11th 16th, 17th, and 19th patients, Table 1) were excluded. The fibroblast to adipocyte ratio in the omentum seemed to decrease in women (n = 8) with advancing age; however, this negative trend did not reach the level of statistical significance (minimum significant R value = 0.707).

Two-way parametric ANOVA indicated a statistically significant difference between the percentages of fibroblasts within the adipose tissue or fibroblast to adipocyte ratios in the breast, abdomen, and hip [p(F) = 0.029]. Fibroblast to adipocyte ratios in both the abdomen and breast are significantly higher than those found in the hip (Fig. 2). Newman-Keuls multiple comparisons analysis showed statistically significant differences between the breast and hip ($P < 0.05$), as well as between the abdomen and hip ($P < 0.05$) but not

TABLE 1. Characterization of women by age, height, weight, BMI, and race

Age	Height (in.)	Weight (lbs.)	BMI	Race	Cause of Death	Medical History
21	63.0	187	33	L	H	alcohol/cocaine abuse
23	64.0	330	55	A	U	seizure disorder
26	65.0	149	25	C	U	UR
27	64.0	110	19	C	H	UR
32	65.5	152	25	C	H	UR
36	67.0	202	31	A	CA	UR
38	60.0	220	42.5	A	MI	CHD
38	63.5	130	22.5	A	CA	UR
45	68.0	140	21	A	OD	cocaine/heroin abuse
45	65.5	187	30	C	OD	alcohol/cocaine abuse
50	63.5	207	36	C	L	liver cirrhosis
54	65.0	168	27.5	C	MI	CHD
58	61.0	130	24.5	A	CA	UR
61	66.0	212	34	C	MI	CHD
71	59.5	112	22.5	L	MI	insulin dependent diabetes
74	61.0	210	39	C	CA	peripheral vascular disease
74	59.5	182	37	C	CA	hypertension
76	63.5	142	24.5	C	MI	CHD
93	62.0	106	19	C	CA	UR

BMI, body mass index; weight/height^2. Race: A, African-American; C, Caucasian; L, Latin-American. H, homicide; U, unknown; CA, car accident; MI, myocardial infarction; OD, cocaine overdose; L, liver failure; UR, unremarkable; CHD, coronary heart disease.

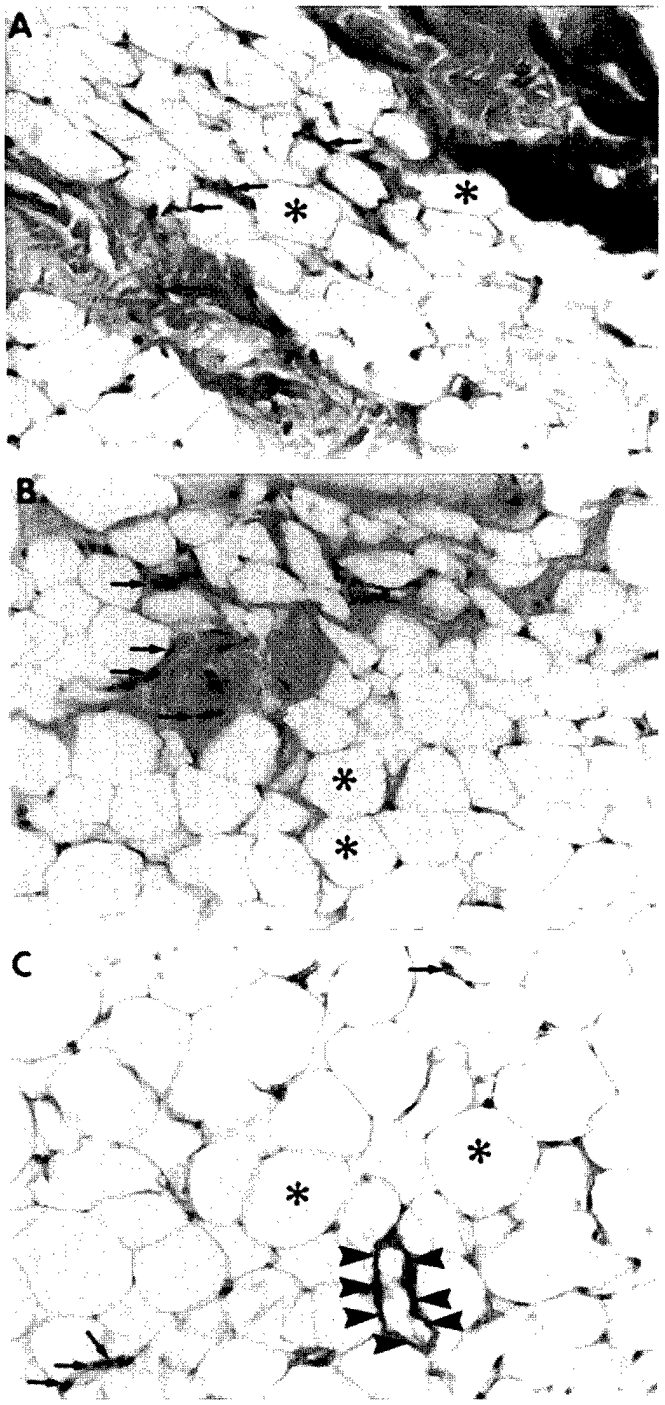

FIG. 1. Hematoxylin and eosin-stained slides of adipose tissue samples from A) breast; B) abdomen; and C) hip. Breast and abdominal samples show relative abundance of fibroblasts (*black arrows*) seen in clusters within dense connective tissue or dispersed among mature adipocytes (*asterisks*). On the other hand, hip sample (C) contains less fibroblasts. Rare endothelium-lined vascular spaces were noted in adipose tissue samples. *Arrowheads* outline a vessel lined with endothelial cells (C).

between the breast and abdomen. This is represented graphically in Fig. 2.

Figure 1, A and B, is hematoxylin and eosin (H&E)-stained slides from breast and abdominal samples, respectively. Figure 1C is an H&E-stained slide from a hip sample. This figure demonstrates the greater fibroblast to adipocyte ratio seen in breast and abdomen samples compared with hip sample.

TABLE 2. Mean ± SEM and range (expressed as a percentage of total cells) for each cell component found in body regions of 19 women

		Abdomen	Hips	Breasts
FB	range:	1.7–10%	1.7–9.3%	1.7–11.7%
	mean ± SEM:	5.91 ± 0.41	4.92 ± 0.41	6.07 ± 0.52
Ad	range:	88.9–98%	89–98.3%	88.7–98.6%
	mean ± SEM:	92.78 ± 0.51	94.02 ± 0.5	92.54 ± 0.6
FB/Ad	range:	0.017–0.112	0.017–0.104	0.024–0.136
	mean ± SEM:	$6.42 \times 10^{-2} \pm .47 \times 10^{-2}$	$5.27 \times 10^{-2} \pm .47 \times 10^{-2}$	$6.63 \times 10^{-2} \pm .61 \times 10^{-2}$
EC	range:	0.3–3.3%	0.0–4.3%	0.0–4.3%
	mean ± SEM:	1.32 ± 0.2	1.06 ± 0.2	1.30 ± 0.2

FB, fibroblasts; Ad, adipocytes; FB/Ad, ratio of fibroblasts to adipocytes; EC, vascular endothelial cells.

TABLE 3. Correlation coefficient for age, weight, and BMI plotted against percentage of FB, percentage of FB + EC, and ratio of FB to adipocytes from six different body sites

	R values					
	A	LH	RH	LB	RB	O
FB *vs.* age	+.184	−.272	+.141	+.110	−.321	−.636
FB + EC *vs.* age	+.182	−.303	+.055	+.045	−.033	−.643
FB/Ad *vs.* age	+.200	−.265	+.141	+.063	−.348	−.589
FB *vs.* wt.	−.263	−.077	0	−.358	−.255	+.411
FB + EC *vs.* wt.	−.283	−.095	0	−.261	−.288	+.456
FB/Ad *vs.* wt.	−.333	−.110	0	−.356	−.228	+.392
FB *vs.* BMI	−.330	−.118	0	−.366	−.288	+.279
FB + EC *vs.* BMI	−.359	−.110	+.032	−.311	−.336	+.361
FB/Ad *vs.* BMI	−.390	−.158	0	−.374	−.265	+.243

R, correlation coefficient. A, LH, RH, LB, RB, O refer to body site from which specimens were taken and cell numbers counted. A, abdomen; LH, left hip; RH, right hip; LB, left breast; RB, right breast; O, omentum; FB, Fibroblasts; EC, vascular endothelial cells; FB/Ad, ratio of fibroblasts to adipocytes; wt., weight BMI, (body mass index) weight/height^2. All cell counts were expressed as percentages.

Discussion

Specific aromatase expression per unit adipose tissue increases with advancing age irrespective of total body fat mass (9, 10). It has also been demonstrated that aromatase expression resides in the fibroblast component of adipose tissue (2, 11). The next natural question is to ask whether this age-dependent increase in aromatase expression is caused by a change in fibroblast to adipocyte ratio in women. In the present study, we did not find any significant correlation between advancing age and fibroblast to adipocyte proportions in breast, abdomen, and hips, suggesting that alterations in adipose fibroblast numbers is not a mechanism responsible for increased estrogen production with advancing age. The population we studied encompassed a wide age range (21–93 yr) and a sample size (n = 19) large enough, at least, to indicate a trend in reference to our previous study, which included 10 women (10), demonstrating a statistically significant relationship between age and $P450_{arom}$ transcript levels. It should be pointed out here that certain steroid hormones, *e.g.* estradiol and glucocorticoids, can affect body

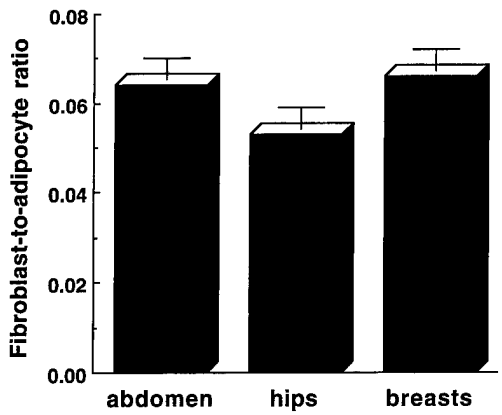

FIG. 2. Graph representing fibroblast to adipocyte ratios in three body sites.

fat distribution. However, the *in vivo* effects of steroids on the cellular composition of body fat are not known. Nonetheless, none of the subjects in the present study was known to have glucocorticoid excess. Exclusion of subjects with extreme obesity to rule out estrogen excess also did not change our results.

Another unexpected result was the fibroblast content of body sites. We had anticipated that fibroblast numbers might follow $P450_{arom}$ transcript levels (10) in that the highest fibroblast to adipocyte ratios would be found in the hip. However, the present study indicated that both breast and abdominal tissue samples showed greater fibroblast to adipocyte ratios than did hip samples. This suggests that variation in adipose tissue aromatase expression between body sites is not mediated by changes in local fibroblast to adipocyte ratios. Mechanisms other than fibroblast content of adipose tissue must be responsible for region- and age-dependent changes in aromatase expression. Areas with the highest fibroblast to adipocyte ratios in tumor-bearing breasts (15) and in disease-free breasts (16) have been shown to contain the highest $P450_{arom}$ transcript levels. Thus, variations in aromatase expression within a region (such as breast, abdomen, and hips) may reflect changes in the ratios of these cell types within that region, whereas variations from one region to another, *e.g.* differences between hips and abdomen, may be related to changes in the specific expression of aromatase in fibroblasts from these sites.

Two mechanisms that may contribute to age-dependent increases in specific aromatase expression, as well as regional variation in expression, may be: 1) increased responsiveness of fibroblasts to hormones or growth substances that stimulate $P450_{arom}$ expression or increased concentration of such factors; and/or 2) decreased responsiveness to inhibitory mechanisms. Thus far, members of the interleukin (IL)-6 cytokine family were found to be the most potent stimulators of aromatase expression in adipose fibroblasts in culture (22). This induction requires the presence of glucocorticoids in the medium (22). Local cortisol levels in the adipose tissue regulated by 11β-hydroxysteroid dehydrogenase recently have been shown to regulate aromatase activity of adipose fibroblasts (23). Changes in local production of cytokines and cortisol levels are candidate mechanisms for age- and region-dependent increases in estrogen production in adipose tissue. For example, circulating IL-6 levels are elevated in elderly mice and humans, compared with young controls (24, 25). Cyclic adenosine monophosphate analogues also stimulate aromatase expression in adipose fibroblasts; however, this induction seems to be inhibited by serum or growth factors (14). Therefore, any combination of the above mechanisms may be responsible for age- or body site-dependent variation in aromatase expression.

In vitro studies have shown that a number of substances influence differentiation of preadipocytes (fibroblasts) to mature adipocytes. Stimulatory substances include the adipogenic transcription factors peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα) (26). On the other hand, IL-11 (27), tumor necrosis factor-α, and C/EBP-homologous protein (28) are capable of inhibiting differentiation of a murine preadipocyte cell line. Also of interest is that IL-11 (22) and tumor necrosis factor-α (our unpublished observations) stimulate aromatase expression in human adipose fibroblasts. Effects of estrogen on expression of these substances are not known yet. Finally, their physiologic roles in determination of cellular composition of human adipose tissue are presently not known.

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Use of Alternative Promoters to Express the Aromatase Cytochrome P450 (CYP19) Gene in Breast Adipose Tissues of Cancer-Free and Breast Cancer Patients*

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ABSTRACT

Estrogen biosynthesis in adipose tissue has assumed great significance in terms of a number of estrogen-related diseases. Recent evidence suggests that estrogen synthesized locally in the breast is of singular significance in the development of breast cancer in elderly women. The biosynthesis of estrogen from C_{19} steroids is catalyzed by a specific form of cytochrome P450, namely aromatase cytochrome P450 (P450arom; the product of the CYP19 gene). The human CYP19 gene comprises nine coding exons, II-X, and its transcripts are expressed in the ovary, placenta, testes, adipose tissue, and brain. Tissue-specific expression of the CYP19 gene is determined, at least in part, by the use of tissue-specific promoters, which give rise to transcripts with unique 5′-noncoding termini. Transcripts in adipose tissue contain 5′-termini derived from specific untranslated exons, corresponding to expression derived from the proximal promoter II and its splice variant I.3, as well as a distal promoter, I.4. The object of the present study was to determine the distribution of these various exon-specific transcripts in breast adipose tissues from cancer-free women undergoing reduction mammoplasty and from patients with breast cancer, because this would provide important clues as to the nature of the factors regulating aromatase expression in these sites. To achieve this, we employed competitive RT-PCR, utilizing an internal standard for each exon-specific transcript of the CYP19 gene, as well as for the coding region, to evaluate total CYP19 gene transcripts. In cancer patients (n = 18), total CYP19 gene transcript levels were significantly higher in adipose tissue proximal to a tumor in comparison with adipose tissue distal to a tumor, in agreement with previous findings. Moreover, total transcript levels were higher in breast adipose tissue of cancer patients in comparison with those of cancer-free individuals (n = 9), even when the adipose tissue from the cancer patient was taken from a quadrant with no detectable tumor. We observed that exon I.4-specific transcripts were predominant in breast adipose obtained from cancer-free women. In this tissue, promoter-II-specific and exon I.3-specific transcripts were present in low copy number. On the other hand, in breast cancer patients, CYP19 gene transcripts from breast adipose tissue had primarily promoter-II-specific and exon I.3-specific sequence, whereas comparatively few transcripts had exon I.4-specific sequence at the 5′-terminus. We conclude that CYP19 gene transcription in breast adipose tissue of cancer-free individuals uses preferably promoter I.4, implicating a role of glucocorticoids and members of the IL-6 cytokine family in the regulation of this expression. On the other hand, the increased expression in breast adipose tissue bearing a carcinoma results from expression from promoters II and I.3, which are regulated by unknown factors acting via increased cAMP formation, which are presumably secreted by the tumor or associated cells. (*J Clin Endocrinol Metab* **81:** 3843–3849, 1996)

ADIPOSE tissue is the major site of estrogen biosynthesis in postmenopausal women (1, 2). In adipose tissue, estrogen biosynthesis increases with obesity but also with advancing age (3–8). Increased estrogen production in elderly obese women is believed to play a role in the pathogenesis of endometrial cancer (4). Furthermore, estrogen produced by adipose tissue within the breast may act locally to promote the growth of breast carcinomas (9). Estrogen biosynthesis is catalyzed by an enzyme known as aromatase cytochrome P450 (P450arom; the product of the CYP19 gene) (10–12). Aromatase is a member of the P450 superfamily of genes, which currently contains over 300 members in some 36 gene families (13). In the human, aromatase expression occurs in a number of human tissues and cell types, including syncytiotrophoblasts of the placenta (14), ovarian granulosa cells and corpus luteum (15, 16), testicular Leydig cells (17–19), various sites in the brain (20, 21), and in adipose tissue (2, 22–25). In women, aromatase activity and transcripts have been detected both in breast adipose and in breast tumor tissues. Within adipose tissue, aromatase expression occurs primarily, if not exclusively, in the fibroblasts (22, 26). A few years ago, we and others cloned and characterized the CYP19 gene, which encodes P450arom (11, 12). The coding region spans nine exons, beginning with exon II. Tissue-specific expression of the CYP19 gene is determined, at least in part, by the use of tissue-specific promoters, which give rise to transcripts with unique 5′-noncoding termini (27). Placental transcripts contain, at their 5′-end, untranslated exon I.1, which is located at least 40 kb upstream from the start of translation in exon II. This is because placental expression is

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driven by a powerful distal placental promoter, I.1, upstream of exon I.1. On the other hand, transcripts in the ovary contain sequence at their 5′-end that is immediately upstream of the start of translation. This is because expression of the gene in the ovary uses a proximal promoter, promoter II. By contrast, transcripts in adipose tissue contain yet another distal untranslated exon, I.4, located in the gene 20 kb downstream from exon I.1. A number of other untranslated exons have been characterized by us and others, including one that apparently is specific for brain (27–30).

A number of studies have attempted to relate tumor site to either aromatase activity (9, 31–34) or else aromatase expression (35) in adipose tissue of breast quadrants. In most cases (9, 31, 33, 35), a direct relationship has been found between the presence of a tumor and aromatase expression in the tumor-bearing quadrant. Moreover, we have demonstrated recently that, in women undergoing reduction mammoplasty, the highest adipose aromatase transcript levels were detected in lateral regions of the breast, the most common site where cancer develops (36). These results suggest that breast tumors may preferentially develop in areas with higher aromatase expression; and once developed, the tumors may secrete substances that influence aromatase expression in adjacent stroma. Recently, a number of cytokines have been found to stimulate aromatase expression in adipose fibroblasts (37) or else tumor-derived fibroblasts in culture (32). These include members of the class I cytokine family, namely interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), and leukemia-inhibitory factor (LIF). Several of these factors have been shown to be elaborated either by cells derived from adipose tissue (38) or else by tumor-derived fibroblasts (39) or breast tumor tissue (31, 38). The latter may include, in addition to transformed mammary epithelial cells, infiltrating macrophages and monocytes (31). Thus, it is possible to envision the establishment of a positive-feedback loop whereby fibroblasts surrounding a developing tumor produce estrogens that stimulate the tumor to produce a variety of cytokines and other factors. Some of these act to stimulate the further growth and development of the tumor in a paracrine and autocrine fashion. Additionally, these or other factors act to stimulate proliferation of the surrounding fibroblasts and expression of aromatase in these cells. Thus, a positive feedback loop is established via mesenchymal-epithelial interactions, which leads to the continuing growth and development of the tumor.

Sequencing of RACE-generated complementary DNA (cDNA) clones from adipose tissue reveals that, in addition to promoter I.4-specific transcripts, transcripts derived from promoter I.3 also are present (27). Interestingly, when adipose fibroblasts are placed in culture, the proportions of promoter I.4, II, and I.3-specific transcripts depend on the stimulatory factors present in the culture medium (27). Thus, when aromatase expression is stimulated by members of the class I cytokine family, such as IL-6 or IL-11, in the presence of glucocorticoids, the transcripts that are present are those derived from promoter I.4 (37). On the other hand, when expression is stimulated by dibutyryl cAMP in the presence or absence of phorbol esters, the transcripts that are present are those specific for promoters II and I.3 (27). Recently we have defined the second messenger pathway whereby class I cytokines stimulate aromatase expression via promoter I.4 (37). This stimulation is mediated by a Jak 1 kinase and a STAT 3 transcription factor that binds to a GAS (interferon-γ-activating sequence) element within promoter I.4 of the P450arom gene. On the other hand, factors that might regulate aromatase expression in adipose tissue via promoter II and I.3, presumably involving stimulation of adenylate cyclase, have yet to be defined.

As indicated above, expression of aromatase transcripts in breast adipose tissue seems to be higher in sites proximal to a tumor than at sites distal to a tumor (35). An important question then arises as to the nature of the 5′-termini of the aromatase transcripts that are expressed at higher levels at sites proximal to a tumor, as this might give clues to the nature of the factors produced by the tumor that are stimulating aromatase expression. To achieve this goal, it was necessary to develop a competitive RT-PCR method to amplify each of the exon-specific transcripts in the presence of the other transcripts, employing an internal standard specific for each exon-specific transcript. Validation of this methodology has been published elsewhere (40). In the present study, we report the quantitative detection of exon-specific aromatase transcripts in breast adipose and tumor samples derived from patients with breast cancer and from cancer-free individuals employing the above mentioned competitive RT-PCR method. This study has provided new insights into the regulation of aromatase expression in breast adipose at sites proximal and distal to a developing tumor.

Materials and Methods

Tissue acquisition and processing

Breast adipose tissue was obtained at the time of surgery from 18 women undergoing mastectomy for histologically confirmed breast cancer and from breast specimens belonging to 9 women undergoing reduction mammoplasty for symptomatic macromastia. Age, race, and tumor type of breast cancer patients are given in Table 1. Nine breast cancer patients had tumor in the upper lateral (outer) quadrant, 4 in the lower lateral (outer) quadrant, 2 in the upper medial (inner) quadrant. Three patients had a central tumor. This distribution of tumor location is consistent with published data of 5 different studies performed on a large number of patients in which it was found that tumors are located in the upper outer quadrant of 40–50% of breast cancer patients (41). Thus, our patient population was representative of the patient population in general. The ages of the women undergoing reduction mammoplasty ranged from 23–40 yr. Written consent was obtained before surgery, including a consent form and protocol approved by the Institutional Review Board for Human Research of The University of Texas Southwestern Medical Center. Whole breast adipose tissue and carcinoma samples were frozen in liquid nitrogen and stored at −70 C. Adipose tissue was sampled from each of the 4 quadrants in mastectomy specimens in the following fashion (Fig. 1): Sampling was performed 8–10 cm from the projection of the nipple at the posterior aspect of each specimen. In the quadrant bearing the tumor (Q1), fat was obtained 4–6 cm from the outer border of the neoplasm. Adipose tissue was dissected free of glandular and vascular tissue components of the breast. During reduction mammoplasty, because of the surgical technique, samples were obtained from 3 divided breast regions (Fig. 1): the superior region (S), the medial region (M), and the lateral region (L). Samples were taken from the center of each region.

RNA isolation and RT-PCR

Human total RNA was isolated from the frozen tissues by the guanidinium thiocyanate-cesium chloride method (42). RT-PCR was performed according to a recently standardized competitive RT-PCR

TABLE 1. Clinical and pathological parameters of 18 breast cancer patients

Patient	Age	Race	Tumor	Grade	Size (cm)	Axillary lymph node involvement
1	64	Black	IL	III	3.5	+
2	41	Black	IL	I	2.5	
3	47	Black	IL	III	6.0	+
4	65	Black	ID		3.2	
5	51	Black	Medullary	I	1.8	
6	51	White	ID	II	5	+
7	58	Hispanic	ID	I	1.3	
8	65	Black	ID	II	5	+
9	72	Black	ID	II	1.5	
10	55	Asian	ID	I	5	
11	53	Black	ID	III	4	+
12	39	Black	ID	III	2.3	+
13	54	Black	ID	III	3.5	+
14	59	Black	ID	III	1.8	+
15	36	Hispanic	ID	I	1	
16	84	Black	IL	III	2.8	+
17	47	Hispanic	ID	III	4	+
18	50	Black	ID	II	3	+

IL, Infiltrating lobular; ID, Infiltrating ductal.

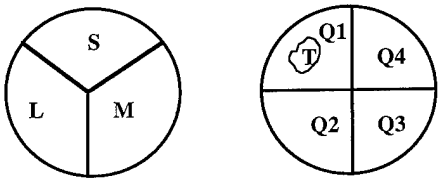

FIG. 1. Location of breast adipose tissues obtained from cancer-free subjects (A) and breast cancer patients (B). L, lateral; S, superior; M, medial; T, tumor; Q1, tumor bearing quadrant; Q2, Q3, Q4, nontumor-bearing quadrants.

method that we developed for this purpose (40). RNA was treated initially with DNase I to remove any contaminating DNA. Total RNA was then reverse-transcribed, using random hexamers. Complementary DNA was used in subsequent PCR amplifications for 25 cycles. Specific 5′-end primers were used to amplify the various 5′-termini. The 3′-end primer was the same in all reactions (40). A trace amount of [^{32}P] dCTP was added to each PCR mix. The reaction products were analyzed on 4% nondenaturing polyacrylamide gels. Gels were either autoradiographed with x-ray film or scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantitatively analyzed using ImageQuant software. For quantitative analysis, specific internal standards generated by inserting a 27-bp random AT-rich region (the looped-oligo method) (40) were employed for exon-specific transcripts containing each 5′-terminus, as well as for the coding region. The latter represents all of the P450arom transcripts present in the sample, regardless of the 5′-termini. The expected sizes of amplification products are given in Table 2. Quantification of each transcript in the cancer-free breast tissue was performed using two or three different copy numbers of each internal standard, together with the fixed quantity of target RNA. The copy number of internal standard that yielded an intensity value equivalent to that of the target was considered to be equivalent to the copy number of target transcripts corrected for size difference; for details, see Ref. 40. In mastectomy specimens, specific transcript levels were expressed as arbitrary units normalized to GAPDH. To check the integrity and comparative quantity of RNA used in amplification of CYP19 gene transcripts from all samples, transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, were amplified by the RT-PCR method described earlier (40).

Statistical analysis

We used three statistical methods in the following order to analyze the data: Initially, two-way parametric repeated measures ANOVA was applied to determine overall significance. A Newman-Keuls multiple-comparisons test was then applied to evaluate levels of significance among specific transcripts (I.3, PII, I.4) within each group of women (cancer-free *vs.* women with breast cancer). To evaluate the interaction between group and specific transcript distribution, a two-way parametric repeated-measures ANOVA model was applied. Finally, within-subject paired *t* test with Bonferroni adjustment was used to evaluate interaction of specific transcripts in quadrants (quadrant bearing a tumor *vs.* average of nontumor-bearing quadrants).

TABLE 2. Expected sizes of PCR-amplified products

Alternative Exon I (5′-Termini)	Target	Internal standard
Exon I.4-Exon III	294 bp	321 bp
Exon I.3-Exon III	289 bp	422 bp
Exon II-Exon III (promoter II-specific)	305 bp	332 bp
Exon II-Exon III (coding region)	194 bp	221 bp

Results

Alternative transcripts of the CYP19 gene in cancer-free subjects

Firstly, we examined the distribution of P450arom (CYP19 gene) transcripts with unique 5′-ends in breast tissue of a group of cancer-free women (n = 9) who underwent a reduction mammoplasty procedure. The adipose tissue was sampled from roughly equal regions of both breasts, namely from superior (S), lateral (L), and medial (M) regions (Fig. 1). To quantify the transcripts, known copy numbers of each specific internal standard transcript were added with the target RNA (40) in the RT-PCR reaction. Fig. 2 depicts a representative experiment employing one sample and shows the amplification of the coding region (total transcripts) and transcripts specific for three 5′-termini: promoter II-specific, exon I.3-, and exon I.4-specific, each with their respective internal standards. By adding the copy numbers of all three exon-specific transcripts, good agreement was obtained with the copy number of the coding region (Table 3), indicating that these three species accounted essentially for all of the P450arom transcripts present in the RNA. We also performed experiments without internal standards, although values were still normalized to GAPDH. Then we compared both

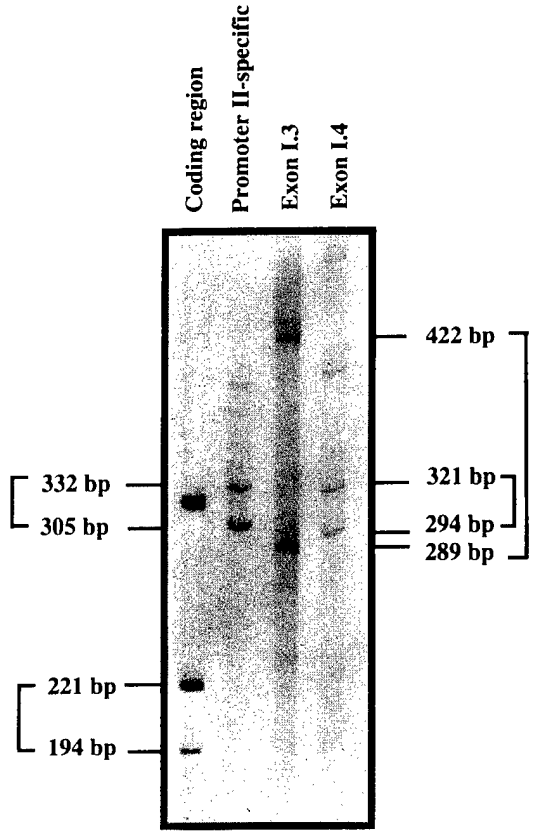

FIG. 2. Amplification of coding region (total CYP19 gene transcripts) and exon-specific CYP19 gene transcripts from RNA isolated from adipose tissue of cancer-free subjects, together with the corresponding internal standard. cDNA from 1 μg RNA was used for each reaction with respective internal standards. The experiment shown is representative of experiments performed using RNA from adipose tissue of cancer-free subjects. Each target and its corresponding internal standard are linked for illustrative purposes by brackets. The size difference between target amplified products and internal standard was 27 bp (Table 2) except for exon I.3. For exon I.3, internal standards were synthesized from full length exon I.3 sequence, whereas amplified product from target RNA was truncated exon I.3. For details, see references (27, 40).

sets of data (employing internal standard and without internal standard). We found a statistically indistinguishable ratio of each exon-specific transcript to total transcripts whether internal standards were employed or not. Thus, from the data obtained from cancer-free women, we were able to demonstrate that in our hands (although the use of the internal standards was necessary for absolute quantification purposes), for the purpose of comparing levels of transcripts to one another, it was not necessary to employ these standards. Exon-specific transcript levels measured in the absence of internal standards were expressed as percent of coding region transcripts (total transcripts), and this is shown in the *left-hand panel* (CF, *i.e.* cancer-free) of Fig. 3. A two-way parametric repeated-measures ANOVA revealed significant differences among the levels of these three types of P450arom transcripts [$P(F)<0.0001$]. A Newman-Keuls multiple-comparisons test showed that, overall, I.4-specific transcript levels (mean = 3.46) were significantly higher ($P < 0.0001$) than both 1.3- (mean = 2.11) and PII-specific (mean = 1.47) transcript levels. No statistically significant difference was detected between I.3- and PII-specific transcript levels (*first set of columns*, Fig. 3). For illustrative purposes (Fig. 3), exon I.3-, PII-, and I.4-specific transcripts are expressed as percentages of the total transcript levels instead of as arbitrary units. (The statistical significance levels were enhanced over parametric analysis after this conversion.) A trend was observed in both breasts, indicating higher total transcript levels in superior and lateral regions, compared with the medial region. This observation is in agreement with a previous study (36).

Total CYP19 gene transcript levels in patients with breast cancer

The fact that it was not necessary to employ internal standards for the purpose of comparing levels of transcripts to one another was a critical point in analyzing the samples obtained from cancer patients, because insufficient tissue was available from these individuals to allow the use of the internal standards in the methodology (although the GAPDH normalization was still employed). Thus, we were unable to determine the absolute copy number of CYP19 gene transcripts. Instead, we determined the comparative values of transcript levels in the samples obtained from breast cancer patients. From the 18 breast cancer patients, breast adipose tissue was sampled from four roughly-equal regions (quadrants Q1, Q2, Q3, Q4, Fig. 1). The tumor-bearing quadrant was designated as Q1, whereas Q2-Q4 were tumor-free. Tumor RNA [neoplastic tissue (T)] was also processed. Thus, five samples were obtained from each patient. In nonneoplastic adipose tissue proximal to the tumor (tumor bearing quadrant, Q1), total (coding region) CYP19 transcript levels were significantly higher ($P = 0.018$) in comparison with levels found in tissue distal to the tumor (average of three nontumor bearing quadrants, Q2-Q4) (Fig. 4). The differences between the tumor (T) *vs.* Q1, and tumor (T) *vs.* Q2-Q4, were not significant (Fig. 4). A within-subject paired *t* test was used for this statistical analysis. Total P450arom transcript levels in quadrants Q2-Q4 were found to be significantly higher ($P = 0.03$; after Bonferroni adjustment) from those in adipose tissue from cancer-free subjects (CF) (Fig. 4).

Alternative transcripts of the CYP19 gene in breast cancer patients

The distribution of exon-specific transcript levels in patients with cancer was quite different from that observed in cancer-free individuals (Fig. 3). Transcripts containing exon I.3-specific and promoter-II-specific sequences were the most abundant (Fig. 3), whereas exon I.4-specific transcripts were present but in lower copy number (Fig. 3). Overall, a two-way parametric repeated-measures ANOVA revealed significant differences [$P(F) = 0.0004$] among these three groups of transcripts. Subsequently, Newman-Keuls multiple-comparisons analysis showed significant differences between I.3- (mean = 11.41) and I.4- (mean = 3.85) specific transcripts ($P = 0.002$), as well as between PII- (mean = 7.97) and I.4-specific transcripts ($P = 0.008$) but not between I.3- and PII-specific transcripts. The within-subject paired *t* test (with Bonferroni adjustment) also was applied to evaluate differences among transcripts containing I.3 and I.4, I.3 and PII, and I.4 and PII (expressed as percentages of total transcript levels, Fig. 3) in each quadrant [Q1, average of Q2-Q4 and

TABLE 3. Number of transcripts present in 1 μg of RNA isolated from breast adipose tissue of cancer-free subjects

Variable[a]	Coding region	Promoter II-specific	Exon I.3-specific	Exon I.4-specific
RS	4.15 ± 1.91 (9)	0.93 ± 0.55 (8)	1.08 ± 0.55 (8)	2.11 ± 0.89 (8)
RM	3.15 ± 1.67 (8)	0.89 ± 0.44 (8)	0.92 ± 0.59 (8)	1.45 ± 0.72 (8)
RL	3.32 ± 1.78 (9)	0.57 ± 0.38 (9)	1.01 ± 0.89 (9)	1.92 ± 1.07 (9)
LS	3.41 ± 1.83 (9)	0.71 ± 0.48 (9)	0.96 ± 0.55 (9)	1.78 ± 0.97 (9)
LM	3.35 ± 1.29 (9)	0.74 ± 0.46 (8)	1.03 ± 0.34 (9)	1.44 ± 0.38 (9)
LL	3.66 ± 1.29 (9)	0.66 ± 0.24 (9)	1.34 ± 0.72 (9)	1.70 ± 0.56 (9)

Data are presented as mean ± SEM (n) × 10^5, where n = no. of subjects.
[a] R, right; L, left; S, superior; M, medial; L, lateral.

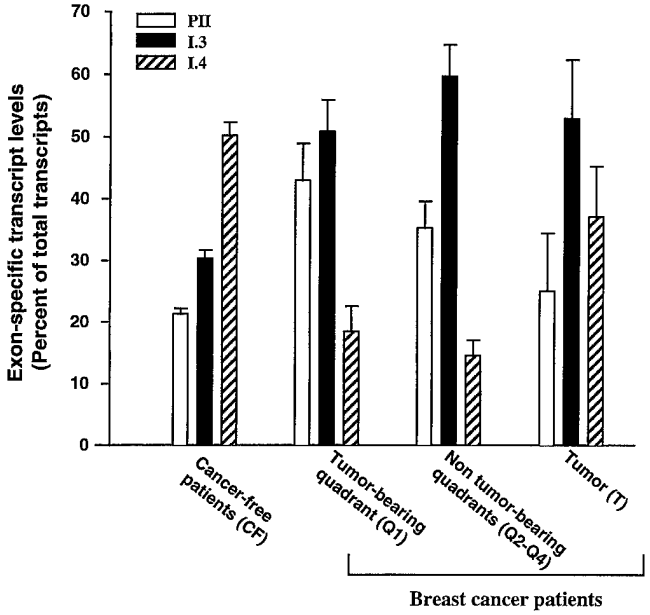

FIG. 3. Amplification of exon-specific CYP19 gene transcripts in cDNA from 1 μg RNA isolated from different breast quadrants of cancer patients and cancer-free individuals. Data are presented as mean ± SEM of percent of total transcripts (*i.e.* coding region) and are normalized to GAPDH. PII, promoter II-specific transcripts; I.3, exon I.3-specific transcripts; I.4, exon I.4-specific transcripts. Q1 and Q2-Q4 are the different quadrants of cancer patients. T, Tumor (n = 9); Q1, tumor-bearing quadrant (n = 15); Q2-Q4, average of nontumor-bearing quadrants Q2, Q3, and Q4 (n = 15). CF, Average of 3 regions from both breasts of 9 cancer-free patients. Percentage was calculated considering coding region as 100.

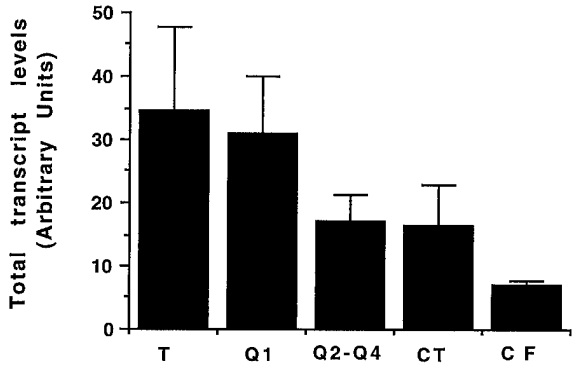

FIG. 4. Amplification of coding region (total transcripts) of CYP19 gene transcripts in cDNA from 1 μg RNA isolated from different breast quadrants of cancer patients and cancer-free individuals. Data are presented as mean ± SEM and are normalized to GAPDH. CT, average of four quadrants of 3 cancer patients with a centrally-located tumor. Other abbreviations are as in the legend to Fig. 3.

tumor (T)]. It was found that I.3-specific transcripts were significantly higher than those containing I.4 in Q1 (P = 0.001) and in Q2-Q4 (P = 0.004) and not significantly higher in tumor (T) (Fig. 5). I.3-specific transcripts were also significantly higher than those containing PII in Q2-Q4 (P = 0.004). Transcripts containing I.4 were significantly lower than PII-specific transcripts in Q1 (P = 0.024) and in Q2-Q4 (P = 0.054) (Fig. 5). There were no significant differences among tumor (T) and Q1, tumor (T) and Q2-Q4, or Q1 and Q2-Q4 for any of the three exon-specific transcripts.

A two-way parametric repeated-measures ANOVA model was applied to evaluate the effect of group (cancer-free women *vs.* women with breast cancer) on the distribution of exon-specific transcripts (I.3, PII, and I.4) in the breast. A significant interaction was found between the group of women and the transcript type (P = 0.0009). This statistical significance was amplified when arbitrary units for transcript levels were converted to express each specific transcript as a percent of the total transcript levels.

Three cancer patients had tumors located in the center portion of the breast (CT; Fig. 4). No significant difference was observed in the levels of the P450arom transcripts among the quadrants. Therefore, the mean of values from the four quadrants was calculated for further comparisons. Total transcript levels were similar to those of the Q2-Q4 quadrants of the other breast cancer patients but were significantly higher (Fig. 4) in comparison with cancer-free subjects. Again, transcripts with promoter II-specific and exon I.3-specific 5′-termini were present at higher levels in comparison with those with exon I.4-specific 5′-termini (data not shown).

The data were further analyzed to observe if there was a difference in the expression of CYP19 gene transcripts in terms of age. Five of the breast cancer patients (Table 1) were under 50 yr of age. There were no significant differences in either total transcript levels or with respect to any of the 5′-termini studied between the over-50 and under-50 groups. Likewise, there seemed to be no significant difference in CYP19 gene transcript levels between the younger and older women undergoing reduction mammoplasty, but because of the small number of samples, the possibility of age-related effects should not be ruled out. Additionally, because most of our patients were black, it was not possible to determine a role of ethnic origin on CYP19 gene transcripts levels.

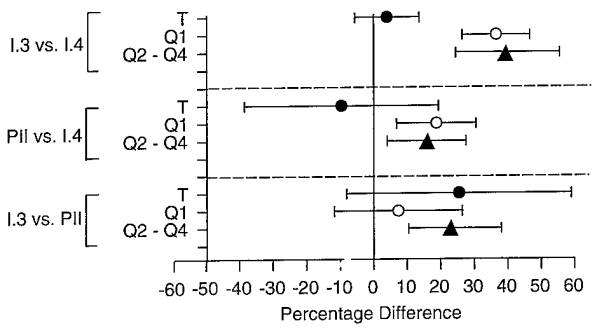

FIG. 5. Mean ± 95% confidence interval of percentage difference in expression level of pairs of transcripts by site. Under the normality assumption, the difference is considered significant at the level of alpha = 0.05 if the 95% confidence interval does not cross the zero line. Abbreviations are as described in the legend to Fig. 3.

Discussion

Consistent with the notion that breast tumors elaborate factors that stimulate local expression of aromatase, is our observation that transcript expression in the quadrant that contains the tumor is greater than that in the remaining three quadrants. This is consistent with the findings of a number of studies that show that either aromatase activity (9, 31, 33) or expression (35) is highest in adipose tissue proximal to a tumor, as compared with tissues distal to a tumor. Only one published study (34) has, so far, failed to show such a distribution. However, the present study shows, for the first time, that breast adipose tissue from cancer-free individuals has a lower level of aromatase expression than adipose tissue from cancer patients, whether it be from the quadrant that contained the tumor or from the remaining three quadrants derived from the same breast. Thus, it seems that, within the tumor-containing breast, there is a gradient of aromatase-stimulating activity emanating from the tumor site. Another difference between the cancer patients and the cancer-free individuals relates to age, namely, that the latter are a younger group than the former. Earlier studies (3, 5) have shown an increase of aromatase activity and expression with age in other body sites, *e.g.* buttock, thigh, and abdomen. However, no such link has been established in breast adipose tissue. In the present study, there was no detectable difference in aromatase expression among women of different ages in either group. So we conclude that age is unlikely to be a major factor influencing the results reported here, although considering the small number of samples, the possibility of an effect of age on breast aromatase expression can not be ruled out.

To gain insight into the factors that stimulate aromatase expression in the breast tissue of individuals with breast cancer, in the present study we have examined the distribution of the various 5′-termini-specific transcripts in samples of breast adipose tissue removed from cancer-free individuals and individuals diagnosed as having breast cancer. In breast adipose tissue samples from cancer-free individuals, we found that promoter I.4-specific transcripts were the most numerous, comprising at least 50% of the total P450arom transcripts, whereas promoter I.3- and promoter II-specific transcripts together made up the remaining 50% (Fig. 3). This is consistent with aromatase expression in adipose tissue of cancer-free individuals being regulated mainly by class I cytokines. IL-6, IL-11, and LIF all have been shown to be elaborated by adipose stromal cells in culture (38). Thus, in normal human adipose tissue, it seems that aromatase expression is under local control by such factors via paracrine and autocrine mechanisms. The issue, as to whether breast adipose tissue obtained as a consequence of reduction mammoplasty should be considered normal, is unclear at this time because this procedure is most commonly undertaken as a result of symptomatic macromastia. Nevertheless, in the absence of any evidence to the contrary, for the purposes of this study, such tissue is considered normal in the sense that it is derived from individuals who are cancer-free.

Of considerable surprise to us, therefore, was the observation that the increase in total P450arom expression that occurs in breast adipose tissue of patients with cancer was accompanied by only a small increase in promoter I.4-driven expression, but rather, was largely the result of an increase in expression from promoter II and promoter I.3 (Fig. 3). This was true not only in the quadrant that contained the tumor but in the other breast quadrants as well (Fig. 3). This conclusion is illustrated diagrammatically in Fig. 6. A similar result was briefly mentioned in reference (43) as a small study employing RT-PCR of five patients, in which three showed such a reversal. However, in that report, the methodology was not validated by the use of internal standards to control for variations in amplification.

Schor *et al.* (44) has reported that breast adipose tissue from patients with cancer has intralobular fibroblasts with a fetal fibroblast phenotype, whereas tissues obtained from disease-free patients do not have this type of fibroblast (44). These fetal-like fibroblasts also were present in histologically normal adipose tissue adjacent to the carcinoma. Therefore, it seems that the presence of a breast tumor imposes several changes in the phenotype of the surrounding mesenchymal cells, of which aromatase expression is only one.

These results imply that, in the presence of a tumor, factors that stimulating aromatase expression via promoter I.4 are not primarily responsible for the increase in expression in surrounding adipose fibroblasts, but rather some other influence, stimulating expression via promoter II and I.3, is dominant. In adipose stromal cells in culture, expression from promoters II and I.3 is stimulated by dibutyryl cAMP (27). Moreover this action of cAMP analogs is dramatically potentiated by phorbol esters (27). Thus, it would appear that in the presence of a tumor, P450arom expression is driven

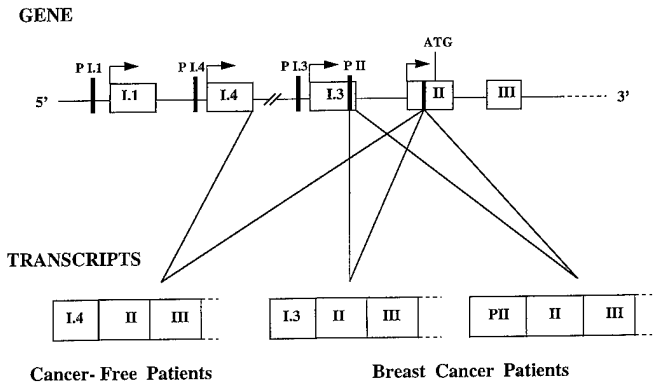

FIG. 6. Predominant exon-specific transcripts of the CYP19 gene in breast adipose tissue of cancer-free individuals and breast cancer patients, and their origin by alternative splicing.

primarily by a factor or factors that stimulate adenylyl cyclase, perhaps in conjunction with protein kinase C activation. This factor or factors are presumably produced either by the tumor cells themselves or recruited into the tumor site as a consequence of an inflammatory response. Clearly, the challenge for the future will be to determine the nature of this factor or factors. This is important not only to further our understanding of the relationship between breast cancer and estrogen biosynthesis in adipose, but additionally, delineation of this signaling pathway may lead to the development of inhibitors of this pathway that would, therefore, inhibit aromatase expression specifically in adipose tissue of individuals with breast cancer. This may, in turn, lead to the development of novel therapies for the endocrine management of breast cancer that specifically eliminate estrogen produced locally at the tumor site.

Acknowledgments

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CLINICAL CASE SEMINAR

Aromatase Expression in a Feminizing Adrenocortical Tumor

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The clinical, endocrinological and molecular biological aspects of an estrogen-secreting adrenal carcinoma in an adult male are described.

Case report

A 29-yr-old man was referred for evaluation of progressive bilateral gynecomastia, diminished libido, and impotence. There was no history of exposure to exogenous estrogen, androgen antagonists, or alcohol. Physical examination revealed symmetric gynecomastia (5 × 5 cm), a feminized body contour, and diminished testicular size (9 and 8 mL, respectively). Blood pressure was 130/85 mm Hg. Liver and thyroid functions were normal. The sperm count showed azoospermia. A computerized axial tomographic scan of the abdomen revealed a left adrenal mass (9 × 9 × 10 cm). The diagnosis was made based upon high plasma estrone (E_1) and estradiol (E_2) levels. After detailed endocrine preoperative testing and catheterization of adrenal veins, the patient underwent left adrenalectomy. An adrenal cortical carcinoma was removed without complications. The weight of the encapsulated adrenal mass was 210 g. Severe nuclear pleomorphism was present, with frequent mitoses and multiple foci of vascular invasion. After resection of the tumor, plasma E_1 and E_2 levels fell dramatically. Three months later, gynecomastia had regressed, and the patient had normal sexual intercourse. Four years after surgery, whereas E_1 and E_2 remained in the normal range, a chest computed tomography scan detected a lung nodule. Resection and histological analysis of this nodule confirmed the existence of a distant metastasis, and mitotane therapy was started.

Endocrine testing

Plasma hormone levels in a peripheral vein before and after surgery are shown in Table 1. In the preoperative period, plasma E_1 and E_2 levels were 10–20 times higher than those in normal subjects. Plasma testosterone (T) levels were low, and plasma androstenedione (A) was in the normal range, but dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) were low. Plasma 11-deoxycortisol (S) was increased. Basal cortisol (F) at 0800 h was in the normal range, but the diurnal variation was abolished (data not shown). Basal plasma ACTH levels were low and did not increase after a CRH test (100 μg, iv). PRA was in the normal range.

Plasma steroid levels in the adrenal veins are shown in Table 2. On the tumor side, plasma E_1 levels were very high (8-fold greater than in the peripheral vein). The E_1/E_2 ratio was 13.9. Plasma E_2 levels were elevated, but in contrast to plasma E_1, on the same order of magnitude as in peripheral blood. The plasma A level was normal, but DHEA and DHEAS levels were low. Plasma F and S levels greater in the adrenal vein than in the peripheral vein demonstrated that the adrenal tumor was the source of F and suggested a partial 11β-hydroxylase deficiency in the tumor. However, plasma deoxycorticosterone levels were not increased. The plasma aldosterone level was extremely low, indicating destruction of the glomerulosa zone by the tumor. In the right adrenal vein, the elevated aldosterone levels confirmed the correct position of the catheter in this vein.

In the preoperative period, LH and FSH were almost undetectable (0.5 IU/L and 0.4 IU, respectively) and did not increase after GnRH injection (100 μg, iv; Fig. 1). Pulsatile LH secretion (sampling every 10 min for 5 h) was suppressed. Pulsatile GnRH administration (20 μg/pulse, sc) failed to induce a normal pattern of endogenous LH secretion and did not restore the gonadotropin response to the GnRH test. In contrast, 9 days after surgery, a pulsatile LH profile was restored, with a frequency of one pulse per h, and normal responses of both gonadotropins to the GnRH test were observed (Fig. 1).

Aromatase expression in the tumor

Aromatase microsomal activities in three normal human adrenals, the adrenal tumor, and human placenta are presented in Fig. 2. Aromatase activity was not detected in normal adrenals. In contrast, a very high level of aromatase activity was observed in the microsomes of the tumor tissue.

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TABLE 1. Plasma hormone levels in a peripheral vein before and 5 days after tumor resection

	Before surgery	After surgery	Normal range
E_1 (pmol/L)	2,790	40	100–280
E_2 (pmol/L)	1,890	70	50–130
T (nmol/L)	2.4	3.5	6.0–10.0
A (nmol/L)	4.9	1.9	2.8–4.9
DHEA (nmol/L)	2.0	1.5	3.5–38
DHEAS (nmol/L)	1,292	952	3,800–14,000
F (nmol/L)	548	312	250–650
S (nmol/L)	70	1.2	0–3.0
DOC (nmol/L)	0.17	0.21	0.12–0.5
Aldosterone (nmol/L)	0.15	0.12	0.09–0.3
17-OHP (nmol/L)	5.8	0.6	3.0–8.0
ACTH (pmol/L)	1.1	10.2	4.5–10

Data represent the mean values of three pooled samples taken at 30-min intervals.

TABLE 2. Plasma steroid levels in the adrenal veins

	Left adrenal vein (tumor)	Right adrenal vein	Normal range[a]
E_1 (pmol/L)	20,900	2,700	200–400
E_2 (pmol/L)	1,500	1,560	0–150
T (nmol/L)	7.8	4.3	3.0–12.0
A (nmol/L)	184	3.3	52–109
DHEA (nmol/L)	15.2	4.0	2,198–2884
DHEAS (nmol/L)	2,508	2,343	12,420–25,313
F (nmol/L)	2,430	648	2,300–4,500
S (nmol/L)	176	54	
DOC (nmol/L)	27.9	ND	12–87
Aldosterone (nmol/L)	0.15	13.9	7.3–15.4
17-OHP (nmol/L)	15.0	4.0	14–29

Data represent the mean values of three pooled samples taken at 30-min intervals. ND, Not done.

[a] Hormone levels in six men with a nonhormone-secreting adrenocortical tumor (personal data).

Aromatase P450 (P450arom) transcripts in the tumor were detected using both Northern blot analysis and reverse transcription-PCR, whereas they could not be demonstrated in a normal adult adrenal using either method (Figs. 3 and 4). In addition, in the adrenal tumor, the untranslated first exons in the 5′-terminals of P450arom transcripts were studied (Fig. 4). Only promoter II-specific transcripts were present, whereas exon I.3- and I.4-specific sequences were not detected. Therefore, promoter II, the gonadal-type promoter, was responsible for aromatase expression in this tumor.

Discussion

The present study reports the uncommon discovery of a feminizing adrenocortical carcinoma in a patient with gynecomastia. This malignant tumor is rare, and fewer than 100 cases have been reported (1, 2).

Adrenal androgens are substrates for estrogen production by peripheral tissues. However, it has not been demonstrated that estrogens could be synthesized by the normal adrenal. In contrast, the main characteristic of this adrenal tumor was its ability to produce estrogens. In this patient, circulating E_1 and E_2 levels were 10–20 times higher than those in normal subjects. Adrenal venous sampling confirmed the left adrenal mass to be the source of E_1 and its precursor A. This result suggested an increase in substrate (A) availability and the presence of aromatase activity within the tumor. Thus, the possibility of an extraadrenal aromatization of A as a source of estrogen excess was excluded. The enzymatic study performed in the tumor tissue confirmed this hypothesis. A very high level of aromatase activity was demonstrated in the tumor, whereas aromatase activity was not detected in normal adrenals. Although tumor aromatase activity was 34 times less than that in placenta, this was still an extremely high level. For example, it was 200 times higher than aromatase activity in adipose tissue (3). Taken together, *in vivo*

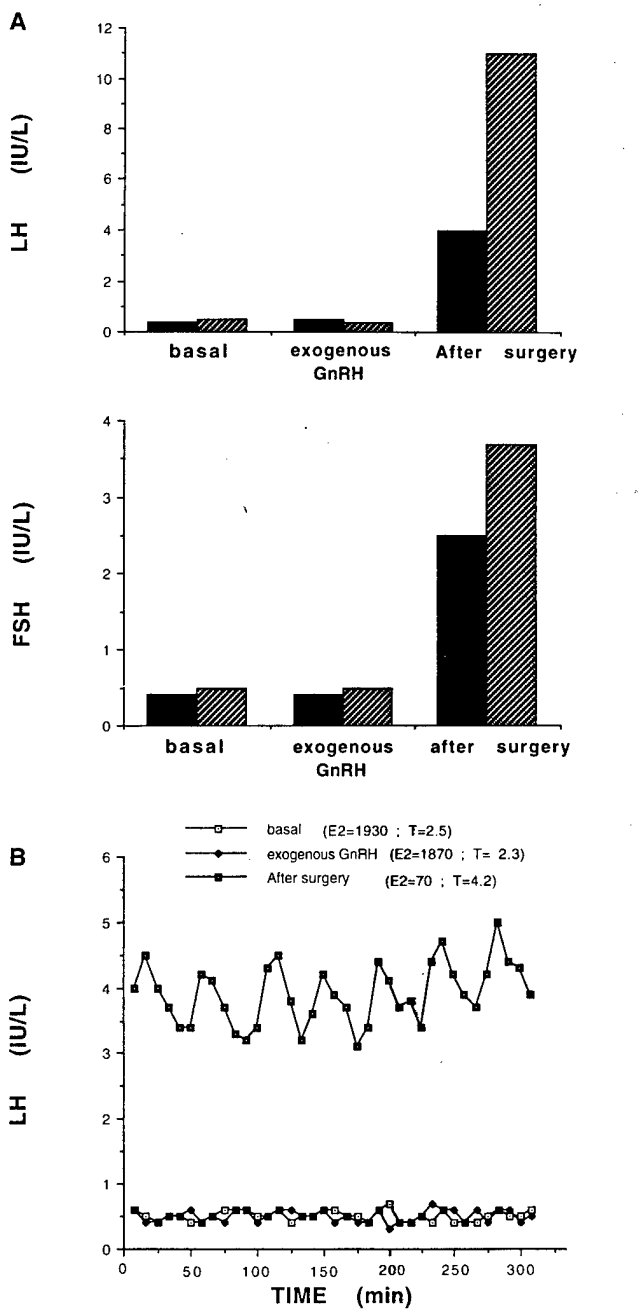

FIG. 1. Gonadotropin secretion before (basal) and 10 days after pulsatile exogenous GnRH administration during the preoperative period and 9 days after removal of the estrogen-secreting adrenal tumor. A, Basal and GnRH (100 μg, iv)-induced peak levels of LH and FSH. B, LH pulsatile profiles. Plasma E_2 (picomoles per L) and T levels (nanomoles per L).

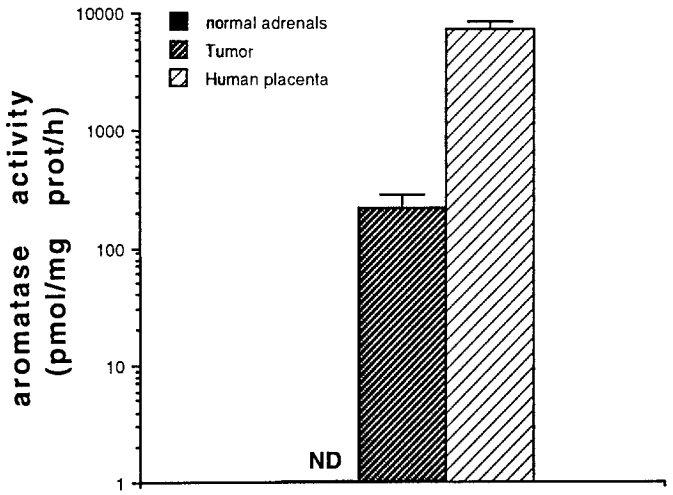

FIG. 2. Microsomal aromatase activity measured by the tritiated water technique in three normal human adrenals, in the adrenal tumor and in human placenta (used as positive control). Values are expressed as the mean ± SE. Note the log scale. ND, Not detectable.

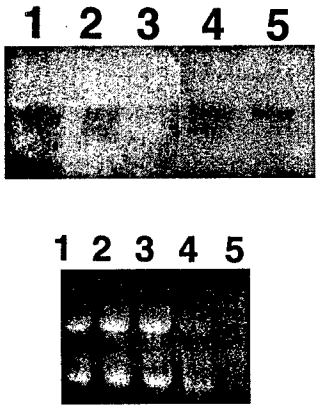

FIG. 3. *Upper panel*, Northern blot analysis revealing the presence of P450arom transcripts in an adrenal tumor ribonucleic acid (RNA) sample (lane 2). Two bands of the expected sizes (3.4 and 2.9 kilobases) were noted. No P450arom transcripts were detected in disease-free adrenal (lane 3). Readily hybridizable P450arom transcripts were detected in control tissue samples: fetal liver (lane 1), placenta (lane 4), and cAMP-treated adipose fibroblasts (lane 5). *Lower panel*, Ethidium bromide-stained 28S (*upper*) and 18S (*lower*) bands are demonstrated in the same RNA samples before transfer to a nylon membrane. Total RNA was used in the first three lanes (20 μg) and the fourth lane (10 μg). Polyadenylated RNA (0.5 μg) was used in lane 5. The first three samples were exposed to an autoradiograph for 72 h, whereas the exposure period was 2 h for the last two samples due to the abundance of P450arom transcripts in placenta and cAMP-treated adipose fibroblasts.

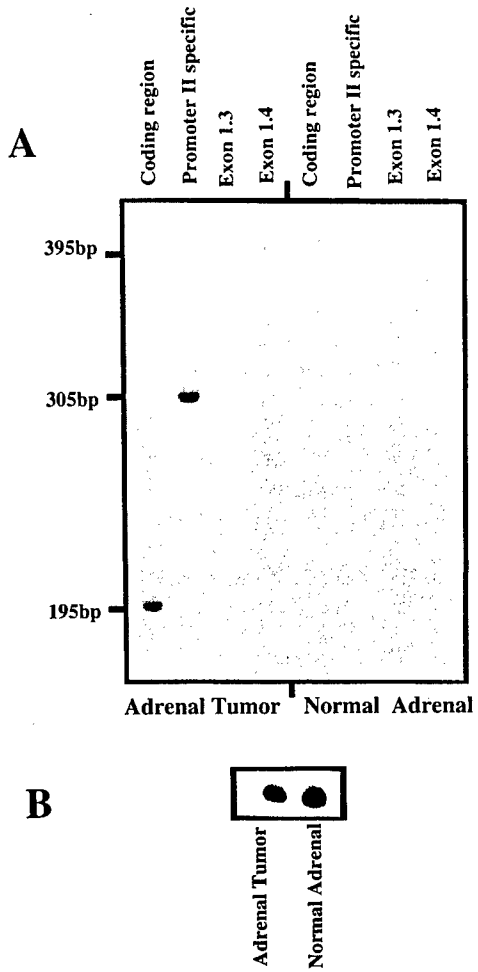

FIG. 4. A, After reverse transcription, P450arom complementary DNA samples were amplified using primers specific for unique untranslated 5′-ends of P450arom transcripts. Only promoter II-specific sequence could be amplified in adrenal tumor (*left panel*, second lane), whereas promoter I.3- and I.4-specific transcripts were not detected (lanes 3 and 4). As a control for the total P450arom transcript level, a coding region that is common to all P450arom transcripts, was amplified (first lane), and bands for promoter II-specific sequence and the common coding region were of similar intensity, indicating that only promoter II was responsible for aromatase expression in the adrenal tumor. No P450arom transcripts could be amplified in disease-free adrenal sample (*right panel*), confirming the results of Northern blot analysis. B, GADPH transcripts were amplified in total RNA samples to confirm the use of comparable amounts of intact RNA.

and *in vitro* data showed that the adrenal tumor had the capacity to synthesize E_1 very efficiently. Whereas the level of E_1 in the left adrenal vein was 8 times higher than peripheral levels, E_2 levels were not significantly different from peripheral values. Thus, it appears that almost all E_2 production originated from peripheral conversion of E_1, but not from adrenal secretion. The markedly higher E_1/E_2 ratio in the left adrenal vein than in peripheral blood similarly suggested the conversion of the weak estrogen E_1, secreted by the tumor, into the active estrogen E_2 by an extraadrenal 17β-hydroxysteroid dehydrogenase (4).

P450arom catalyzes the conversion of C_{19} steroids into estrogens in a number of human tissues, such as the ovary, testis, placenta, adipose tissue, and brain (5). Aromatase expression has also been detected in several tumors (5, 6). In this report, we present evidence for massive aromatization of C_{19} steroids by an adrenocortical tumor. Aromatase expression in human tissues is regulated in part by alternative use of tissue-specific promoters. The distal promoter I.1 is exclusively used in placenta, whereas promoters I.3 and I.4 are responsible for aromatase expression in adipose tissue (5). The classically located proximal promoter II directs P450arom gene transcription in the gonads (6). The present study demonstrates that promoter II is responsible for aro-

matase expression in an adrenocortical tumor, as previously shown in various tumors (5). Whereas this is not surprising in gonadal tumors, it is intriguing to consistently observe the use of promoter II in the other tumors, because disease-free tissues of origin, namely normal endometrium, myometrium, adult liver, and adult adrenal, do not express aromatase (7).

It is well known that T may inhibit gonadotropin secretion after aromatization to E_2 (8). The importance of estrogens in the regulation of the gonadotrope axis in men has been demonstrated in subjects treated with aromatase inhibitors (9). More recently, it has been reported in a man with a mutation in the estrogen receptor gene and in a man with aromatase deficiency by a mutation in the P450arom gene that gonadotropins were increased despite normal or high T levels (10, 11). In the present report, before surgery, the increased serum concentrations of E_1 and E_2 were associated with marked decreases in plasma LH and FSH, with severe androgen deficiency and azoospermia. The absence of a LH response to pulsatile GnRH administration contrasted with the rapid recovery of LH and FSH secretion after suppression of estrogen excess. These data strongly suggest that estrogen acted directly on the pituitary to decrease gonadotropin secretion.

In summary, the present study demonstrated for the first time an abnormal expression of the CYP19 (P450arom) gene responsible for the very high aromatase activity in a feminizing adrenocortical carcinoma. Only promoter II, the gonadal type, was responsible for aromatase expression in this tumor. In addition, the high plasma levels of estrogens were associated with a marked suppression of gonadotropin secretion at the pituitary level.

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Aromatase and 17β-Hydroxysteroid Dehydrogenase Type 1 in Human Breast Carcinoma*

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ABSTRACT

The *in situ* formation of estradiol plays an important role in the development and biological behavior of human breast cancer Aromatase and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD type 1) are two principal enzymes involved in *in situ* estradiol production. We evaluated the expression of aromatase and 17β-HSD type 1 by immunohistochemistry in 41 cases of invasive breast carcinoma (19 lobular and 22 ductal). We then examined the correlation among the expression of these enzymes, estrogen (ER) and progesterone (PR) receptor status, Ki67 labeling index of carcinoma cells, age, and the clinical stage of the patients. Marked aromatase immunoreactivity was observed in stromal cells around carcinomatous glands in 32 of 41 cases (78%), and 17β-HSD type 1 immunoreactivity was detected in carcinoma cells in 23 of 41 cases (56%). There was a significant correlation observed between expression of 17β-HSD type 1 and aromatase in invasive lobular carcinoma ($P = 0.0119$), but not in invasive ductal carcinoma. There was an inverse correlation between aromatase and ER status in invasive ductal carcinoma ($P = 0.0213$), but not in invasive lobular carcinoma. No other correlations were observed among 17β-HSD type 1, aromatase, PR, ER, clinical stage, age, and Ki67 labeling indexes. Aromatase and 17β-HSD are not always expressed simultaneously in human breast carcinoma, but their simultaneous expression is more frequent in invasive lobular carcinoma than invasive ductal carcinoma. Consequently, different mechanisms may be involved in the regulation of expression of these two enzymes in human breast carcinoma. (*J Clin Endocrinol Metab* **81:** 4042–4046, 1996)

NUMEROUS *in vitro* studies have demonstrated that the normal female breast and breast carcinomas can synthesize estradiol (1, 2). This estradiol production initially involves the production of estrone from C19 steroids of the adrenal and/or ovarian origin by the cytochrome P450 aromatase complex (1, 2). Estrone is the main product of the aromatization of C19 steroid and can be effectively converted to estradiol by 17β-hydroxysteroid dehydrogenases (17β-HSD), especially 17β-HSD type 1. Increased aromatase expression was recently reported in human breast carcinoma (3, 4) compared with its expression in normal breast tissue, and overexpression of aromatase is believed to play an important role in the development of human breast cancer. Similarly 17β-HSD type 1 expression has been reported in the human breast and in breast carcinoma (5, 6). The level of 17β-HSD expression is highly variable, and in contrast to aromatase, its concentration in breast cancer is not necessarily higher than that found in nonneoplastic breast tissue (5, 7). The correlation of the expression of these two enzymes, *i.e.* aromatase and 17β-HSD type 1, in the individual cases of human breast cancers may be important in understanding the *in situ* production of estradiol but has not yet been examined. Therefore, in this study, we immunolocalized aromatase and 17β-HSD type 1 in 41 cases of human breast carcinoma. In addition, we also examined the correlation of these findings with estrogen (ER) and progesterone (PR) receptor status and with Ki67 labeling index, which reflects proliferative activity, to evaluate the biological significance of aromatase and 17β-HSD type 1 expression in human breast cancer.

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Materials and Methods

Cases

The carcinomas examined in this study were retrieved from the surgical pathology files. The carcinoma specimens included 22 cases of invasive ductal carcinoma and 19 cases of invasive lobular carcinoma. Clinicopathological findings of the cases are summarized in Table 1.

Antibodies

The characteristics and dilutions of the primary antibodies employed were summarized in Table 2. Antiaromatase antibody was a rabbit immunoglobulin G (IgG) prepared against enzyme purified from human placenta (8). The antibody inhibited more than 80% of

TABLE 1. Summary of results

Case no.	Age	Stage	Arom[a]	17β-HSD[a]	ER[b]	PR[b]	Ki67[b]
Invasive lobular Carcinoma[c]							
1	60	2	+	−	92	55	62
2	55	2	++	±	87	71	52
3	60	3	+++	+	91	0	36
4	60	1		+	83	75	23
5	42	2	+		86	96	31
6	62	2	+++	++	81	51	6
7	48	1	+		96	88	24
8	54	2	++	+	95	98	21
9	50	4		±	0	0	36
10	45	2	++	++	91	83	68
11	81	2	+++	++	93	5	43
12	57	1	+		6	4	8
13	41	2			89	24	52
14	80	1	+++	++	76	0	5
15	73	3	++	++	86	78	43
16	62	2	+	+	93	82	28
17	39	3	+		86	83	22
18	59	2	+		83	57	15
19	47	1	±	+++	28	41	13
mean[d]					75.9 ± 29.6	52.2 ± 36.0	30.9 ± 18.7
Invasive ductal carcinoma							
20	36	4	++	±	0	0	18
21	54	N/A	+	±	86	0	21
22	48	2			85	92	22
23	83	N/A	+	+++	72	66	30
24	48	1	++	++	93	84	61
25	51	2	+	++	93	76	38
26	65	1	+	−	72	3	18
27	51	2	++	+	65	87	38
28	61	1	+++	+	83	23	11
29	51	2			99	68	64
30	58	2			98	99	72
31	56	2	+	+	0	12	61
32	42	2	++	±	82	31	24
33	48	1	+		63	58	28
34	46	2	±		0	0	33
35	58	2			94	91	59
36	46	2		±	93	89	18
37	68	2		++	98	93	20
38	66	2	++		86	83	43
39	37	2	+		0	0	52
40	59	2	++		0	8	31
41	60	1	+		0	0	41
mean[d]					61.9 ± 40.0	48.3 ± 40.0	36.5 ± 17.9
total					68.4 ± 35.9	50.1 ± 37.6	33.9 ± 18.2

[a] 17βHSD (17β-hydroxysteroid dehydrogenase type 1), arom (aromatase): (−) = no immunoreactivity; (±) = 0 to 5%; (+) = 5 to 25%; (++) = 25 to 50%; (+++) = more than 50% positive for the enzyme.
[b] The number represents the labeling index.
[c] Cases 1, 5, 6, 18 and 19 contained a foci of invasive ductal carcinoma.
[d] The mean represents the mean ± standard deviation.

Table 2. Characteristics of primary antibodies

Antibody	Animal	Antigen	Dilution	Source
Aromatase	polyclonal (rabbit)	placental protein	1:800	references 3, 8
17β-HSD type 1	polyclonal (rabbit)	placental protein	1:350	references 5, 11
ER	monoclonal (mouse)	recombinant protein (87kd)	1:1	Immunotech SA (Luminy, France)
PR	monoclonal (mouse)	synthetic peptide	1:50	Novocastra (Newcastle, UK)
Ki67 (MIB 1)	monoclonal (mouse)	recombinant protein	1:100	Immunotech SA (Luminy, France)

the aromatization activity *in vitro*, and a single band was obtained via immunoblotting (8). The authors have demonstrated similar patterns of expression between immunoreactivity employing this antiaromatase antibody and messenger RNA localization by *in situ* hybridization in human endometrial carcinoma (9) and normal cycling human ovary (10). Anti-17β-HSD antibody was a rabbit polyclonal antibody against enzyme purified from human placenta (11). The detailed characterization is described previously (11). Antibodies against ER and PR and Ki67 antibody were commercially obtained mouse monoclonal antibodies (Table 2).

Immunohistochemistry

The immunohistochemical procedure employed was described previously by the authors (12, 13). Autoclave treatment for 5 min at 121°C in 0.01 mol/L citrate buffer, pH 6.0 was used after deparaffinization for antigen retrieval for ER, PR, and Ki67. Immunoreactivity of these nuclear antigens could not be demonstrated without employing antigen retrieval methods. Patterns of immunolocalization of aromatase and 17β-HSD type 1 were essentially the same with or without autoclave treatment (data not shown). Therefore, autoclave treatment was employed only for immunostain of ER, PR, and Ki67. The immunohistochemical staining procedure used in this study was the biotin-streptavidin amplified method using the Histofine immunostaining system (Nichirei, Tokyo, Japan). Control sections were incubated with normal rabbit serum and 0.01 mol/L phosphate-buffered saline or normal mouse IgG for monoclonal antibodies instead of primary antibodies. No immunoreactivity was observed in these sections. In addition, aromatase immunoreactivity was completely obliterated by absorbing the antibody with excessive amount of purified antigen.

Scoring of immunoreactivity

Aromatase and 17β-HSD type 1. For evaluation of aromatase and 17β-HSD type 1 immunoreactivity, we determined the labeling index, *i.e.* the percentage of cells with relatively strong immunoreactivity in each carcinoma, according to the report by Watanabe *et al.* (9) with some modification. After completely reviewing the immunostained sections of each carcinoma, two of the authors (H.S. and R.S.) independently divided the carcinomas into the following six groups: (−) = no immunoreactivity; (±) = 0 to 5%; (+) = 5 to 25%; (++) = 25 to 50%; (+++) = more than 50% of cells positive for aromatase or 17β-HSD type 1. Cases with disconcordant results between observers were simultaneously reevaluated by the same two authors using double-headed light microscopy.

Ki67 and steroid receptors. Scoring of Ki67, ER, and PR in carcinoma cells was performed on high power fields (×400) using a standard light microscope. In each case, 200–500 tumor cells were counted independently by these same two authors, and the percentage of immunoreactivity, *i.e.* the labeling index, was determined. When interobserver differences were less than 5% of the greater value, the mean of the two values was obtained. When interobserver differences were greater than 5% of the greater value, the sections were reevaluated simultaneously by the same two authors, and the percentage of immunoreactivity in the sections was obtained.

Statistical analysis

Scheffe's multiple comparison test was performed to determine the statistical significance of differences in percentage of positive tumor cells. Wilcoxon rank-sum test was employed to examine the statistical difference between invasive ductal and lobular carcinoma. *P* values less than 0.05 were considered as significant. The correlation analysis between different parameters was assessed by Spearman's rank-order correlation coefficient.

Results

Immunohistochemistry

Aromatase. Aromatase immunoreactivity was observed in the cytoplasm of stromal or interstitial cells, but relatively strong immunoreactivity was observed in stromal cells around carcinomatous glands in 32 cases of carcinoma (78%) (Fig. 1). Carcinoma cells were immunohistochemically negative for the enzyme. Morphologically normal glands were observed adjacent to the carcinoma in 18 cases, and aromatase immunoreactivity comparable to that noted in the carcinoma was observed in myoepithelial and interstitial cells in 4 samples of normal breast tissue.

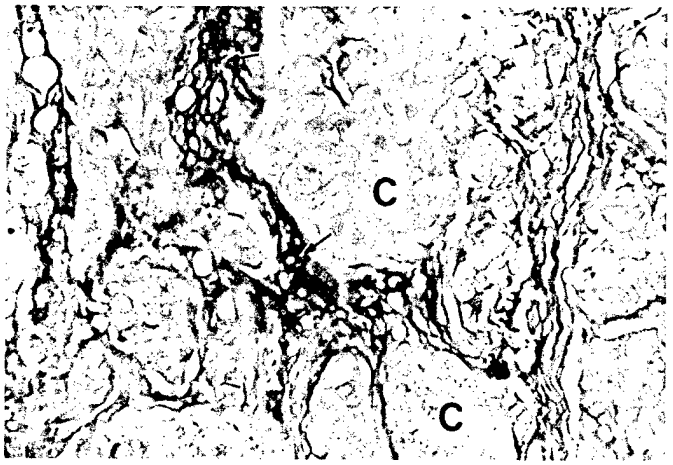

FIG. 1. Immunohistochemistry of aromatase in invasive ductal carcinoma (case 32). Immunoreactivity was observed in stromal cells (*arrows*). Carcinoma cells (C) were immunohistochemically negative for aromatase (×300).

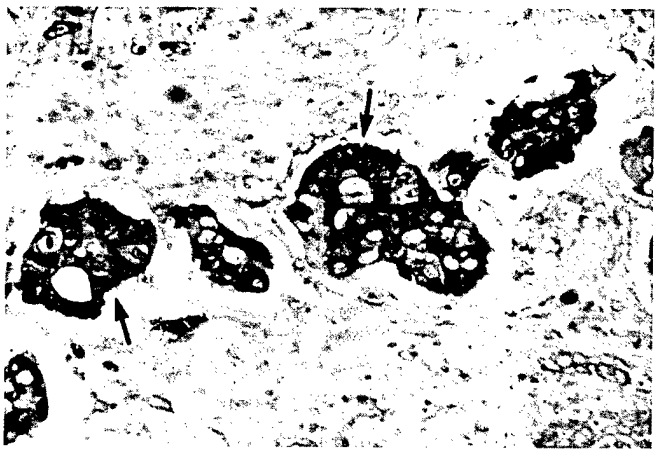

FIG. 2. Immunohistochemistry of 17β-HSD type 1 in invasive ductal carcinoma (case 25). 17β-HSD type 1 immunoreactivity was observed in carcinoma cells (*arrows*). (×300)

17β-HSD type 1. Immunoreactivity of 17β-HSD type 1 was observed in the cytoplasm of carcinoma cells in 23 of 41 cases (56%) examined (Fig. 2) and in ductules in 15 of 18 morphologically normal mammary glands present adjacent to the carcinoma. Immunoreactivity was not observed in interstitial cells. Relative immunointensity of 17β-HSD was markedly heterogenous among the cases.

Ki67 and steroid receptors. Labeling indices are provided in Table 1. ER and PR immunoreactivity was observed almost exclusively in epithelial cells including carcinoma cells and ductal cells of adjacent noncarcinomatous mammary glands. However, Ki67 immunoreactivity was observed in epithelial cells as well as in some stromal cells.

Correlation between aromatase and 17β-HSD type 1

No significant correlation of labeling indices of aromatase and 17β-HSD type 1 was detected when analyzing all 41 cases or invasive ductal carcinoma cases. In invasive lobular carcinoma, a significant correlation of labeling indices of these 2 enzymes was observed (SRCC = 0.5638, $P = 0.0119$).

Correlation between aromatase or 17β-HSD and steroid receptors

There was no significant correlation observed between labeling indices of the enzymes and steroid receptors in carcinoma cells when analyzing all 41 breast carcinomas or when considering only the invasive lobular carcinomas. In invasive ductal carcinoma, significant inverse correlation of labeling indices for aromatase and ER (SRCC = −0.4878, P = 0.0213) and for aromatase and PR (SRCC = −0.4387, P = 0.0411) was observed. No other correlation was observed in invasive ductal carcinoma.

Correlation between aromatase or 17β-HSD type 1 and Ki67, age, or clinical stage

There was no significant correlation observed between labeling indices of the enzymes and Ki67 labeling indices of carcinoma cells or age or clinical stage when all 41 carcinomas were analyzed as a group or when only the ductal or lobular carcinomas were examined.

Comparison between invasive ductal and lobular carcinoma

There were no significant differences in the labeling indices of aromatase, 17β-HSD type 1, ER, PR, and Ki67 between invasive ductal and lobular carcinoma.

Discussion

Among the factors involved in the biosynthesis of estradiol, aromatase primarily catalyzes the conversion of androstenedione to estrone, and 17β-HSD type 1 converts estrone to estradiol. Therefore, the presence of these enzymes strongly indicates site(s) of active estradiol production. In addition, within tumor tissues in estrogen-dependent human neoplasms that coexpress aromatase, 17β-HSD type 1 and ER are considered to possess a growth advantage over tumor cells, through their ability to convert C19 steroids into estradiol effectively, that do not express these enzymes and receptors. In this study, the frequency and localization of the expression of 17β-HSD type 1 and aromatase in carcinomas were consistent with the results of previous reports (3, 5, 14). However, these two enzymes involved in the *in situ* production of estradiol are not necessarily expressed in the same breast carcinoma, although the majority (53%) of invasive lobular carcinomas coexpressed these enzymes. The absence of coexpression of these two enzymes in the same carcinoma cases has also been observed in human ovarian carcinoma (15, 16). Reports of any correlation between ER and aromatase activity in human breast cancer have been inconsistent (3, 16–18). In the present study, ER labeling index of carcinoma cases did not demonstrate a logarithmic pattern but there was no correlation was observed between aromatase and 17β-HSD type 1, and an inverse correlation was observed between aromatase and ER status in invasive ductal carcinoma.

Any correlation between the clinical outcome or clinicopathological parameters and 17β-HSD type 1 expression in human breast cancer has not been reported. There have been reports of a lack of correlation of aromatase expression or activity with established clinicopathological parameters (3) and with disease free interval or survival (17) in human breast carcinoma. Estimation of the proliferative status of breast cancer is an important factor in patient management and prognosis (19). Ki67 labeling index is an effective method of evaluating cell proliferation in human tissues (13). In our study, the expression of 17β-HSD type 1 and aromatase did not correlate with Ki67 labeling index, indicating that the overexpression of these enzymes in breast cancers does not necessarily confer a growth advantage on carcinoma cells. The mechanism of regulation of aromatase and 17β-HSD expression in human breast cancer tissues has not been determined. Results of our present studies suggest that different regulatory mechanisms may be involved in the expression of aromatase and 17β-HSD type 1 in human breast cancer. Further investigation is required to clarify the mechanism of controlling local estrogen formation in human breast carcinoma. An understanding of this mechanism may contribute to improved endocrine therapy of these patients.

Among invasive carcinoma of the human breast, invasive ductal and lobular carcinomas are the two major histological types, although invasive ductal carcinomas predominate. Patients with invasive lobular carcinoma of the classical subtype have been reported in some studies to have an improved long-term prognosis compared with those with invasive ductal carcinoma (20). In addition, the incidence of bilateral carcinoma is higher in invasive lobular carcinoma than other histological subtypes (21). The differences observed in this study between these two histological subtypes of human breast carcinoma, *i.e.* the significant correlation between aromatase and 17β-HSD type 1 in invasive lobular carcinoma and the significant inverse correlation between aromatase and ER or PR in invasive ductal carcinoma may also reflect biological differences between ductal and lobular carcinoma of the human breast.

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THE AROMATASE REACTION

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I. INTRODUCTION

The biosynthesis of estrogens occurs throughout the entire vertebrate phylum including mammals, birds, reptiles, amphibians, teleost and elasmobranch fish, and Agnatha (hagfish and lampreys) (Callard, et al., 1990; Callard et al., 1980; Callard,

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1981). It has also been described in the protochordate, Amphioxus (Callard et al., 1984). In most vertebrate species that have been examined, aromatase expression occurs exclusively in the gonads and in the brain. This is true of the fish and avian species that have been examined as well as most mammals such as rodents. In many species estrogen biosynthesis in the brain has been implicated in sex-related behavior such as mating responses, and frequently a marked sexually dimorphic difference has been demonstrated. This is true for example in species of bird in which the song of the male is important in courtship behavior (Hutchinson, 1991). In the case of humans and a number of higher primates, there is a more extensive tissue distribution of estrogen biosynthesis, since this also occurs in the placenta of the developing fetus as well as in the adipose tissue of the adult (Killinger et al., 1987). The ability of the placenta to synthesize estrogen is also the property of a number of ungulate species such as cows, pigs, and horses. However, at least in cattle there is no evidence of estrogen biosynthetic capacity in adipose, whereas in rodent species such as rat and mice, as well as rabbits, neither adipose nor placenta have any ability to synthesize estrogens.

The estrogen produced in each tissue site of biosynthesis is quite specific. For example, the human ovary synthesizes primarily estradiol, whereas the placenta synthesizes estriol and adipose synthesizes estrone. This appears to reflect primarily the nature of the C_{19} steroid presented to the estrogen synthesizing enzyme complex in each tissue site. Thus, in the case of adipose, the principal source of substrate is circulating androstenedione produced by the adrenal cortex. In the case of the placenta, the principle substrate is 16α-hydroxydehydroisoandrosterone sulfate derived by the combined activities of the fetal adrenal and fetal liver. The physiological significance of estrogen biosynthesis in the placenta and adipose of humans is unclear at this time. Although the human placenta produces very large quantities of estrogen, particularly estriol, its role is not understood. This pertains because in pregnancies characterized by placental sulfatase deficiency, the placenta is essentially deprived of C_{19} substrate and hence synthesizes, relatively speaking, minute quantities of estrogen, yet such pregnancies are relatively uncomplicated (France and Liggins, 1969). At most, parturition is delayed by several days. Similarly, at this time no physiological significance has been attributed to estrogen biosynthesis by human adipose; however the latter has been implicated in a number of pathophysiological conditions. Estrogen biosynthesis by adipose not only increases as a function of body weight but as a function of age (Hemsell et al., 1974; Edman et al., 1976), and has been correlated directly with the incidence of endometrial cancer as well as with post-menopausal breast cancer. Furthermore, evidence is accumulating to suggest that the estrogen which is implicated in the development of breast cancer is that which is produced locally within the adipose tissue of the breast itself (Miller and O'Neill, 1987). On the other hand, estrogen biosynthesis in adipose may have beneficial consequences since osteoporosis is more common in small, thin women than in large, obese women. While this may be, in part, the consequence of the bones of the latter being subject to load-bearing

exercise, nonetheless it seems likely that the increased production of estrogens by the adipose of obese women is a significant factor.

II. STOICHIOMETRY OF ESTROGEN BIOSYNTHESIS

The biosynthesis of estrogens is catalyzed by an enzyme complex named aromatase (Fig. 1), comprised of aromatase cytochrome P450 (P450arom, the product of the *CYP19* gene) (Thompson and Siiteri, 1974; Mendelson et al., 1985; Nakajin et al., 1986; Kellis and Vickery, 1987, Osawa et al., 1987; Nelson et al., 1993; Simmons et al., 1985) which catalyzes the series of reactions leading to formation of the phenolic A ring characteristic of estrogens. Cytochrome P450arom is presently the sole member of gene family 19, designated CYP19. This designation is based on the fact that the C_{19} angular methyl group is the site of attack by oxygen. The aromatase reaction apparently utilizes three moles of oxygen and three moles of NADPH for every mole of C_{19} steroid metabolized (Thompson and Siiteri, 1974) (Fig. 2). Evidence is accruing that all three oxygen molecules are utilized in the oxidation of the C_{19} angular methyl group to formic acid, which occurs concomitantly with the aromatization of the A ring to give the phenolic structure characteristic of estrogens (Cole and Robinson, 1988; Akhtar et al., 1982). This is the only reaction in vertebrates capable of introducing an aromatic ring into a molecule.

III. STRUCTURAL FEATURES OF CYTOCHROME P450

Understanding the relationship of structure to function in cytochrome P450arom is of great interest because of the novelty of the complex series of reactions involved. cDNA inserts complementary to messenger RNA encoding cytochrome P450 have been isolated and characterized from a broad range of vertebrates including two species of fish, namely rainbow trout (Tanaka et al., 1992) and goldfish (GV Callard, personal communication), chicken (McPhaul et al., 1988); and several

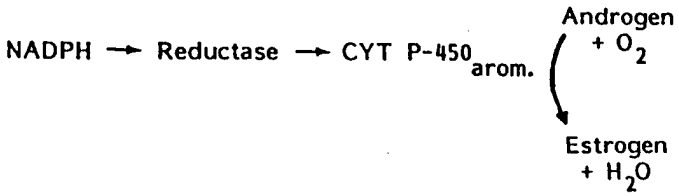

Figure 1. Diagram of the aromatase enzyme complex. Cytochrome P450arom is responsible for binding the C19 steroid substrate and modifying its A ring to the phenolic ring characteristic of estrogens. NADPH-cytochrome P450 reductase, a ubiquitous protein of the endoplasmic reticulum, is responsible for transferring reducing equivalents from NADPH to any microsomal form of cytochrome P450 with which it comes into contact.

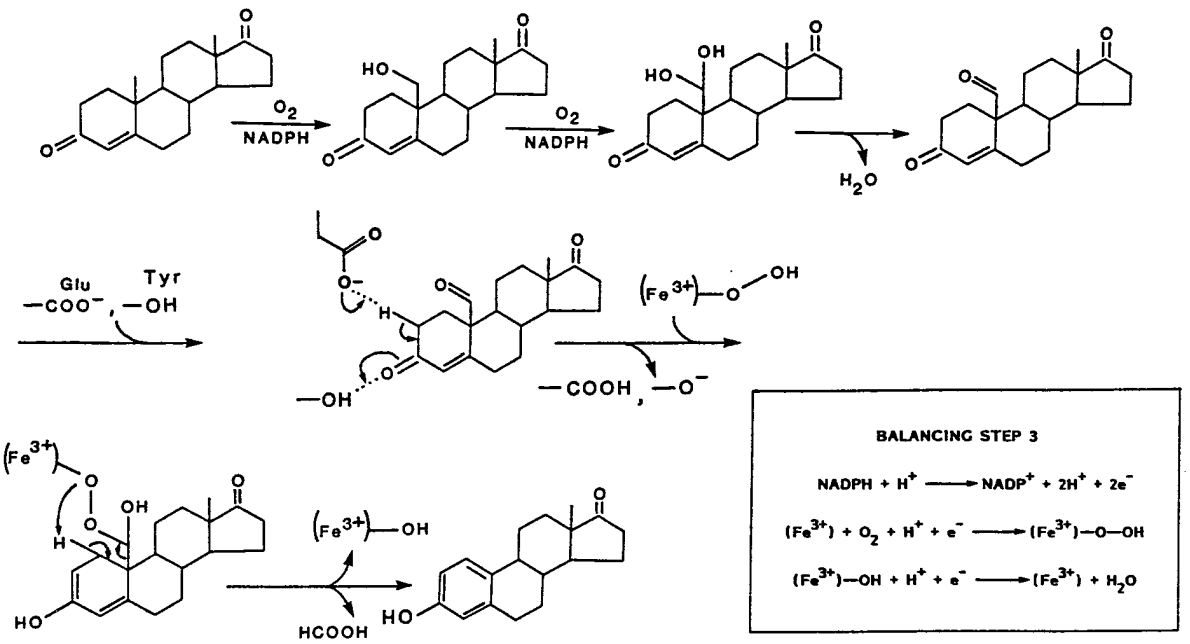

Figure 2. Model of the aromatase reaction. This is based on the proposals of Cole and Robinson (1988), and Akhtar et al. (1982). Reprinted from Graham-Lorence, 1991, with permission.

mammalian species including rat (Hickey et al., 1990), mouse (Terashima et al., 1991), human (Corbin et al., 1988; Harada, 1988; Toda et al., 1989), and most recently bovine, as indicated in Figure 3. The species showing greatest homology to the human is the bovine with an amino acid sequence identity of 86%. The identities of the derived amino acid sequences of P450arom from rat, mouse, chicken, and trout to the human are 77%, 81%, 73%, and 52% respectively. Interestingly, the variation between the two species of teliost fish, namely goldfish and trout (G.V. Callard, personal communication), is almost as great as that between the trout and human, indicating the great range of evolutionary diversity that has occurred in teliost fish since their origins.

Cytochrome P450arom shares a number of structural features common to all cytochrome P450 isoforms. Upstream of the heme-binding domain there is a region of over 20 amino acids which is totally conserved in all P450arom species from chicken to human, except that in the bovine there is the I399L replacement, a conservative change. Upstream of this there is another region of high conservation, namely the portion of the I-helix which is believed to form the substrate-binding pocket proximal to the heme prosthetic group (Graham-Lorence et al., 1991).

In a number of microsomal cytochrome P450 isoforms, the amino-terminus is characterized by a region of hydrophobic amino acids believed to comprise a membrane-anchoring domain. Curiously, in the case of P450arom, this is not apparent, since it has an extension of 20 amino acids with no obvious hydrophobic or amphipathic stretches. Rather, an N-linked glycosylation consensus sequence is present (NIT in the human), and P450arom of human placenta apparently does have attached sugar residues (Sethumadhewan et al., 1991). Instead, the region which can be described as hydrophobic lies between amino acids 20 and 40. In this context it is worth recalling that attempts over 20 years ago to define the subcellular localization of estrogen biosynthetic activity inevitably led to the conclusion that this distribution was bimodal, activity occurring both in membranes sedimenting with the microsomal fraction, as well as those sedimenting with the mitochondrial fraction, as defined by differential centrifugation. The presence of an apparent N-terminal extension containing a glycosylation consensus sequence in most species of P450arom is suggestive that at least some P450arom is present in the plasma membrane. Even more curiously, the cytochrome P450arom clone derived from the rainbow trout has a further 20 amino acid extension at the N-terminus in addition to the extra sequence which is common to all P450arom cDNAS (Tanaka et al., 1992). The importance of this further extension is also unclear at this time.

Comparison of the sequence of the P450arom cDNAS with those of other members of the cytochrome P450 superfamily has led to the conclusion that P450arom is only distantly related to other steroidogenic forms of P450 and indeed is one of the most ancient of the cytochrome P450 lineages, apparently evolving more than 1,000 Myr ago (Nelson et al., 1993). Exactly what the ancestral gene product was doing in those ancient times is unclear; presumably however it was not involved in the synthesis of estrogens. To our knowledge, aromatase has not

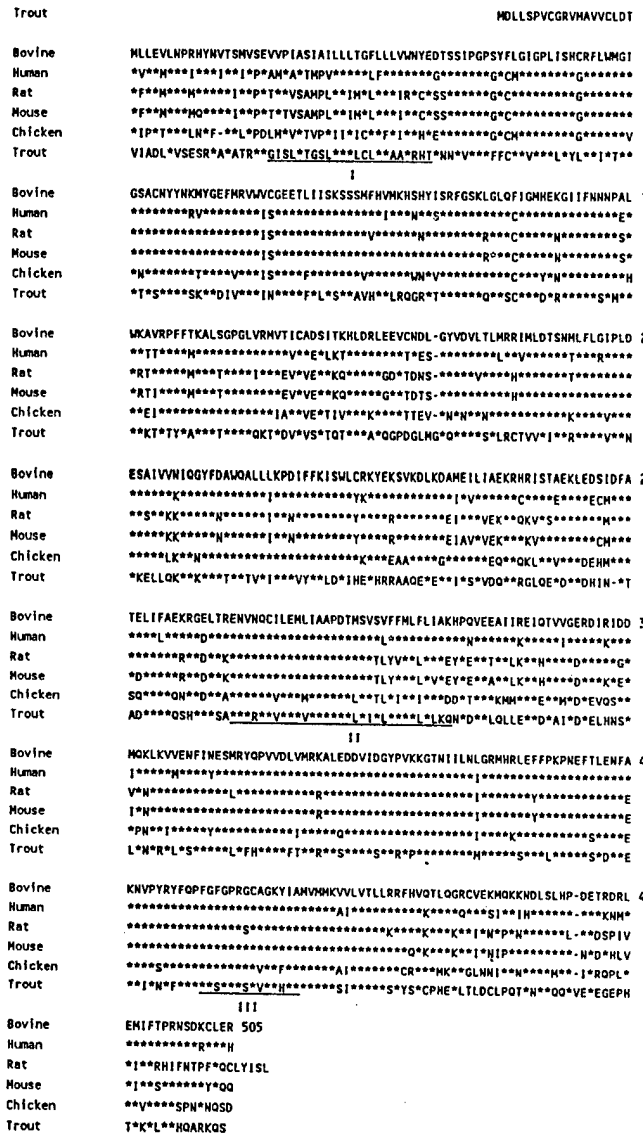

Figure 3. Sequence homologies of P450arom isoforms from a number of species including bovine, human (Corbin et al., 1988), rat (Hickey et al., 1990), mouse (Terashima et al., 1991), chicken (McPhaul et al., 1988), and rainbow trout (Tanaka et al., 1992). Regions of high homology, such as the I-helix (I), an aromatase-specific conserved region (II), and the heme-binding region (III), are indicated.

been described in invertebrates. It would be of considerable interest therefore to know what reactions the ancestral gene product catalyzes in non-vertebrate phyla.

IV. REGULATION OF *CYP19* GENE EXPRESSION IN HUMAN PLACENTA

Screening of clones isolated from human genomic libraries revealed that the open reading frame of the human *CYP19* gene comprises 9 exons, in common with other cytochrome P450 isoforms which generally have between 8 and 10 coding exons (Means et al., 1989; Harada et al., 1990; Toda et al., 1990), (Fig. 4). Also in common with most P450 species, the heme-binding region is located on the last coding exon. Additionally, in the case of human CYP19, there are two polyadenylation sites in the last coding exon downstream from the terminating stop codon. These give rise to the two species of transcript of 3.4 and 2.9 kb which encode human P450arom. Sequences approximately 140 bp upstream of the translation initiation site have homology to TATA and CAAT boxes. However, primer extension analysis using an oligonucleotide primer corresponding to the 5′ end of the first coding exon together with polyA+ RNA isolated from human placenta failed to reveal a transcription initiation site that corresponded to the use of these promoter elements. Furthermore, an oligonucleotide corresponding to sequence beginning 39 bp downstream of the putative TATA box failed to hybridize to placental messenger RNA in northern analysis (Means et al., 1989; Toda et al., 1990).

The reason for this became apparent when we sequenced two P450arom cDNA inserts isolated from a primer-extended human placental cDNA library that we had previously constructed. These contained two distinct 5′-termini which were identical downstream of a point of common divergence which was 36 bp 5′ of the translation initiation site. Sequences corresponding to these different 5′-termini were then used to screen the human genomic libraries and two other clones were isolated that contained the two alternative exons expressed in the human placental CYP19 mRNA. These we have named untranslated exons I.1 and I.2 (Fig. 4) (Means et al., 1989; Means et al., 1991; Kilgore et al., 1992). Hence we number the coding exons II through X. We have determined that the 5′-termini of P450arom mRNA transcripts in placenta are derived from the use of these two alternative untranslated exons.

When an oligonucleotide corresponding to a portion of exon I.1 was used in primer extension analysis of placental polyA+ RNA, a product was formed that indicated a transcription start site 23 bp downstream of a putative TATA box 5′ of exon I.1 (Means et al., 1991). In addition, an oligonucleotide corresponding to exon I.1 hybridized to the same mRNA species in human placental RNA as those hybridizing to probes corresponding to coding exons. In the placenta, almost all the CYP19 transcripts include sequences encoded by exon I.1. Only a minor proportion (<1%) contain sequences encoded by I.2 (Kilgore et al., 1992). Whereas exon I.2 lies 9 kb upstream of exon II, exon I.1 lies at least 35 kb upstream of exon 11, which

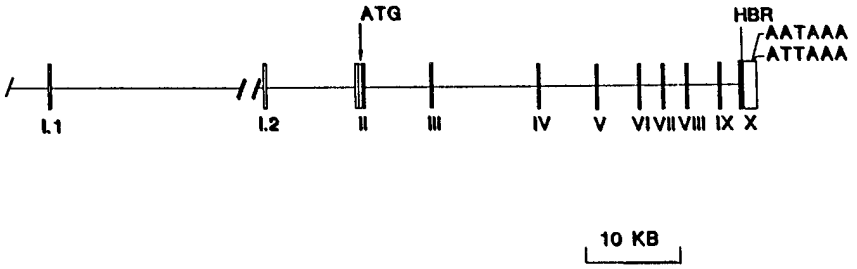

Figure 4. Schematic representation of the human *P450* gene, based on sequences transcribed in ovary and placenta. The closed bars represent translated sequences. The septum in the open bar in exon II represents the splice junction for exons I.1 and I.2, sequences to the left of the septum would be present in mature RNA only when a putative TATA box 149 bp 5′ of the ATG is utilized to promote transcription. The heme-binding region (HBR) is indicated in exon X, as are two alternative polyadenylation sites which give rise to the two species of transcript of 3.4 and 2.9 kb.

contains the translation initiation site (Fig. 4). In addition to placenta, mRNAS containing exon I.1- and I.2-specific sequences also are detected in the human choriocarcinoma cell line, JEG3. The sequence of the entire intron between exons I.1 and I.2 has not been mapped because the genomic clones which contain these exons have not yet been overlapped, so the true distance between them is not established. However, since the coding exons span a distance of some 35 kb, this means the gene is at least 70 kb long, and thus, apparently, the largest cytochrome P450 gene characterized at this time. The human *CYP19* gene has been localized to chromosome 15 (Chen et al., 1988).

V. REGULATION OF *CYP19* GENE EXPRESSION IN HUMAN OVARY

The process of maturation of ovarian follicles occurs in several stages. Small antral follicles are selectively stimulated by small increases in the concentrations of gonadophins FSH and LH such as are found at menses in humans and other primates. Selected follicles progress through a complex process of differentiation to the pre-ovulatory stage. During this stage, increasing stimulation of cAMP by FSH elevates expression of CYP19, CYP11A ($P450_{scc}$) and LH receptor in granulosa cells along with CYP17 in the cal cells (Richards, 1994). The synthesis of estradiol from cholesterol resulting from the coordinate action of these enzymes triggers the surge of LH that effects ovulation and the transition of granulosa cells to luteal cells. During this transition, CYP19 expression drops precipitously while CYP11A progresses to still higher levels independent of cAMP.

We have characterized CYP19 mRNA transcripts in human ovary (Means et al., 1991). In contrast to the placenta, northern analysis of human corpus luteum

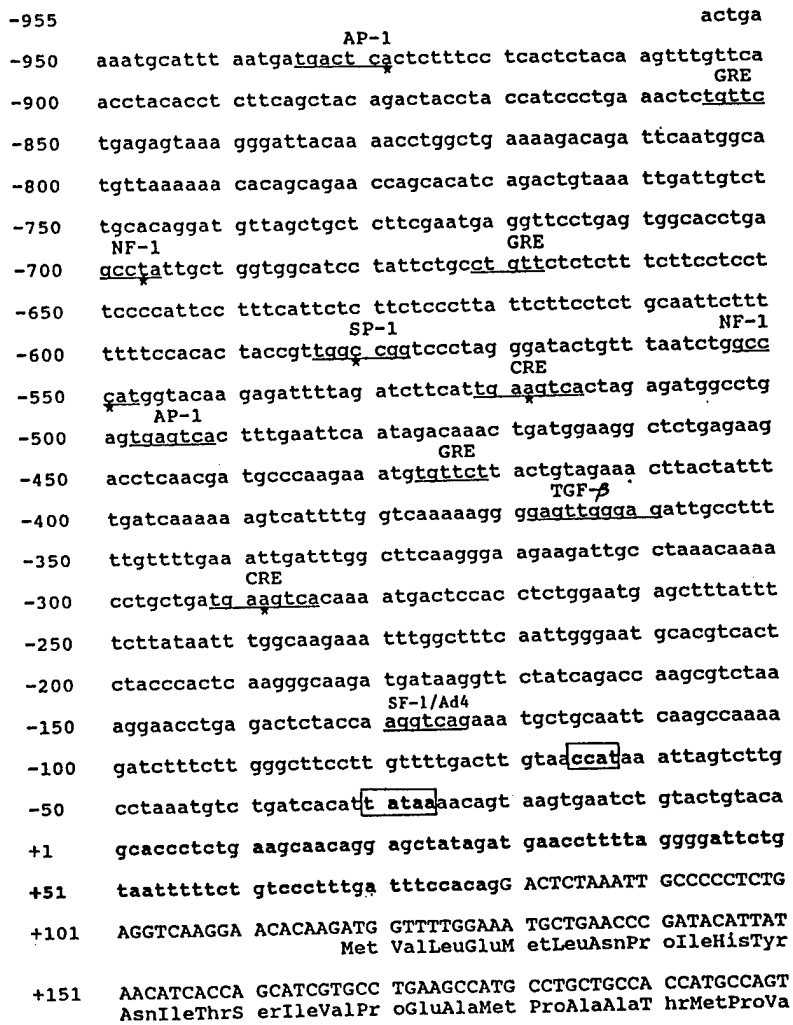

Figure 5. Sequence of the 5′ flanking DNA upstream of exon II corresponding to promoter II. Sequences with similarity to the consensus sequences for binding of known transcriptional activators are underlined, and the putative TATA and CAAT sequences are boxed. Nucleotides that diverge from the consensus sequences are indicated with an asterisk. The transition from lower case to upper case indicates the position of the untranslated exon/exon II boundary. The bases are numbered such the +1 represents the start of transcription using promoter II as defined by the primer extension and S1-nuclease protection experiments described in this manuscript.

polyA+ RNA failed to reveal any transcripts hybridizing to sequences contained in exons I.1 and I.2. To verify these results using a more sensitive approach, the technique of polymerase chain reaction (PCR) amplification was utilized in an attempt to amplify from human corpus luteum RNA exon I.1- and I.2-specific sequences. Oligonucleotides corresponding to sequences within exon II were used as a positive control. Neither of these sequences could be amplified from human ovarian RNA, whereas exon II was readily amplified, indicative that exons I.1 and I.2 are not expressed in human ovary, in contrast to the placenta. Recalling that the DNA sequence immediately upstream of exon II, which contains the translational start site, was previously found to contain sequence elements corresponding to TATA and CAAT boxes (Fig. 5), we utilized primer extension and S1 nuclease protection analyses to determine that the major CYP19 transcript in human corpus luteum was expressed from this promoter (hereafter known as promoter II) (Means et al., 1991). The predicted start site of transcription was estimated to lie some 23 bp downstream of the putative TATA element, which lies 110 bp upstream of the splice junction for exons I.1 and I.2 utilized in placenta (Fig. 5). In addition, northern analysis using corpus luteum mRNA and an oligonucleotide corresponding to sequences upstream of exon II revealed hybridizable CYP19 transcripts, whereas no such hybridizable transcripts were evident in the placental RNA. By contrast, an oligonucleotide corresponding to exon I.1 hybridized to CYP19 mRNA transcripts in placenta, however, no hybridizable transcripts were detectable in corpus luteum mRNA utilizing this probe (Means et al., 1991).

The promoter sequence immediately upstream of exon II (PII) contains several elements that are fully conserved between human and rat; notably, the cAMP responsive element (CRE) and SF1/Ad4 site. The CRE sequence has been shown to bind recombinant CRE binding protein (Richards, 1994) while the SF 1 sequence binds SF1 Zn-finger orphan receptor that is present in most ovarian cell types and is required for gonadal development (Luo et al., 1994). In experiments with bovine luteal cells, we show that cAMP stimulation elevates levels of SF1/Ad4BP together with interactions with this element in the PII promoter (Michael et al., 1995). Recent work (Yujima, unpublished) shows that SF1/Ad4BP is expressed sporadically in the granulosa and surrounding stromal cells prior to CYP19 expression while increasing in each of the major cell types during maturation of the dominant antral follicles. Evidently the selective expression in the granulosa cells of the pre-ovulatory follicle requires some additional regulatory factor.

VI. REGULATION OF *CYP19* GENE EXPRESSION IN HUMAN ADIPOSE

Given this unfolding complexity, it was clearly of great interest to determine the regulatory domains responsible for *CYP19* gene expression in human adipose tissue (Mahendroo et al., 1991). In the first instance, we sought to determine whether or not promoters I.1 or I.2 might be utilized for expression in adipose stromal cells in

culture. In order to address this issue, we attempted to amplify transcripts containing these sequences by means of PCR from polyA+ RNA extracted from adipose tissue. Such transcripts were undetectable, indicating that neither exon I.1- nor exon I.2-containing transcripts were present in adipose tissue. In this sense then, CYPI9 transcripts in adipose are similar to those in human corpus luteum and differ from those in human placenta.

In our studies of the regulation of aromatase expression in human adipose, we have utilized adipose stromal cells in culture as a model system (Ackerman et al., 1981; Simpson et al., 1989; Mendelson et al., 1986; Evans et al., 1987). These cells are the stromal elements surrounding the adipocytes and contain most of the aromatase activity present in fat tissue. In culture, they grow and develop as fibroblasts. Treatment with dexamethasone plus serum, or dibutyryl cyclic AMP plus phorbol ester in the absence of serum, give rise to increases in aromatase expression of approximately 20-fold and 150-fold respectively (Mendleson et al., 1986). Primer extension analysis of polyA+ RNA from adipose stromal cells in culture, maximally stimulated with dibutyryl cyclic AMP plus phorbol ester, indicated a transcriptional start site identical to that found in human corpus luteum, namely, 23 bp downstream from the proximal TATA sequence, i.e., promoter II. However, S1 nuclease protection assay led to a somewhat different result (Mahendroo et al., 1991). Although this analysis indicated that a population of the transcripts extended to the same position as the primer-extended product, namely 23 bp downstream from promoter II, at least 50% of the transcripts extended only to the placental intron/exon splice boundary, in other words to the identical position found with transcripts derived from placenta (Mahendroo et al., 1991). These results would indicate that at least 50% of the transcripts in the adipose stromal cells contained a 5′ end which was spliced into this junction, indicative of the presence of a 5′ untranslated exon. Since PCR analysis had ruled out the presence of exon I.1- or exon I.2-containing 5′ termini, we concluded that a third as yet unidentified 5′ untranslated exon is spliced into a number of the transcripts present in these adipose stromal cells.

In order to characterize the 5′ termini present in CYP19 transcripts in these cells, we decided to prepare primer-extended cDNA libraries by means of the RACE procedure (Mahendroo et al., 1993). Such libraries were prepared from RNA extracted from adipose tissue and adipose stromal cells cultured in the absence or presence of dexamethasone plus serum, or dibutyryl cyclic AMP plus phorbol ester in the absence of serum. Somewhat unexpectedly, sequencing of these primer-extended cDNA clones gave rise to not one, but at least four different 5′ termini Table 1. As with the other splicing events previously described, each of these termini is spliced into the common 3′-junction upstream from the translational start site. Thus once again, in each case the nucleotide sequence encoding the open reading frame is unaltered by these splicing events. In adipose tissue, sequences are present which we have called I.4, I.3, and I.3-truncate. Three different libraries were made from adipose tissue: two were from breast adipose of two different patients, the other

from thigh/calves. No I.4-containing transcripts were identified in the library made from thigh/calf tissue, whereas no I.3-truncate-containing transcripts were identified in libraries made from breast adipose tissue. The difference in distribution of 5′-ends in the three adipose tissue libraries could be due to patient to patient variation, or else could be a function of tissue localization.

What was even more intriguing was that the distribution of these 5′ ends appeared to be a function of the culture conditions under which the cells were maintained, suggesting that promoter usage may be regulated by factors present in the culture medium. In the stromal cells in culture, the choice of 5′-termini appears to be dependent on the hormonal environment of the cells. Most interestingly, in cells expressing promoter II-specific sequences, namely cells treated with cAMP and phorbol esters in the absence of serum, no I.4-specific sequences are detected. Conversely, in cells expressing I.4-specific sequences such as dexamethasone-treated cells in the presence of serum, no promoter II-specific sequences are observed. CYP19 transcripts containing exon I.3 are present in adipose tissue as well as in cells in culture under all conditions. In addition, as can be seen from Table 1, two other 5′ termini, which we have called I.4/I.2 and I.3-truncate, are observed less frequently.

Of these various termini, exon I.3 is formed as a result of a splicing event in which a region extending 100 bp upstream from the 3′-splice boundary is removed so that sequence including the promoter II TATA box and the region upstream from it are present as exonic sequence in this cDNA clone (Fig. 6). Exon I.3-truncate is formed as a consequence of a slightly larger splicing event which extends beyond the promoter II TATA box and results in the loss of a fragment 207 bp long. Untranslated exons I.3 and I.3-truncate may be derived from a second TATA box 216 bp upstream from promoter II. Of the other 5′ termini, exon I.4 represents a new sequence (Fig.

Table 1. Cellular Distribution of P450arom 5′-Termini Identified by the RACE Procedure

		5′-Sequences Identified by RACE			
Sources of Library	*PH*	*I.3*	*I.4*	*I.4/I.2*	*I.3-truncate*
Adipose Tissue:					
Breast - 1	0	0	15	0	0
Breast - 2	0	4	5	0	0
Thigh/Calves	0	16	0	0	10
Adipose Stromal Cells in Culture:					
Control – serum	1	3	2	0	1
Dex-treated + Serum	0	4	7	2	2
Bt_2cAMP + PDA treated-Serum	6	9	0	0	1

6). Exon I.4/I.2 contains this I.4 sequence, but spliced downstream of it there is 206 bp of yet another sequence, which turns out to be exon I.2.

Since the RACE procedure utilizes PCR to amplify the appropriate sequences from the transcripts, the observed distribution may not be reflective of the true quantitative distribution of these termini in the CYP19 transcripts. In order to obtain a better estimate of this distribution, we have performed northern analysis on polyA + RNA isolated from adipose stromal cells maintained under the culture conditions described above, using as probes sequences specific to the various 5′ termini which were identified using the RACE procedure. These oligonucleotides were of roughly equal length and labelled as closely as possible to the same specific activity, in order to provide a rough comparison of the band intensities. The results obtained employing exon-specific northern analysis are summarized in Table 2.

On the basis of these results, it appears that three different 5′-termini are present at significant levels in CYP19 transcripts in adipose tissue and adipose stromal cells in culture. Of these, I.4 is present in breast adipose tissue, as well as adipose stromal cells treated with dexamethasone in the presence of serum. Promoter II-specific sequences are present in cells treated with dibutyryl cAMP plus or minus phorbol ester in the absence of serum, but not in cells treated with dexamethasone in the

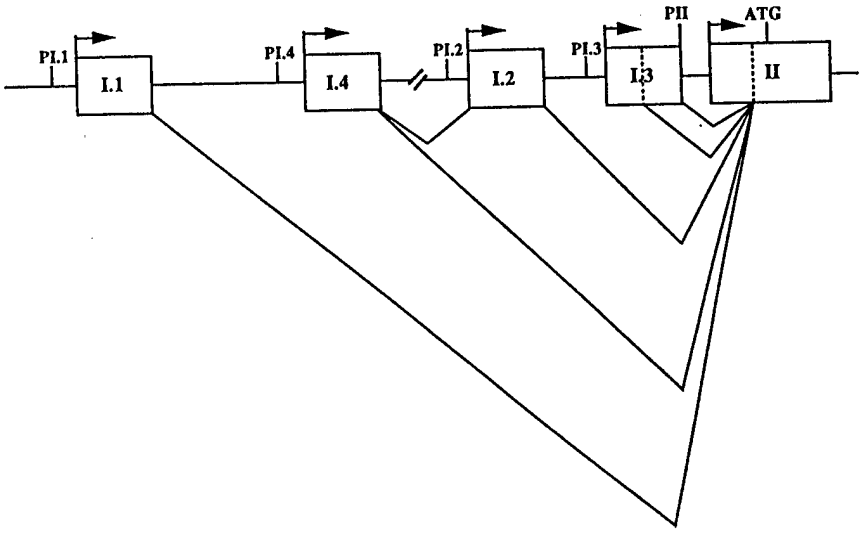

Figure 6. Structure of the human *CYP19* gene upstream of the translational start site showing alternative splicing patterns. The four untranslated exons and first coding exon (exon II) are indicated. Promoters I.1 and II and putative promoters I.4, I.2 and I.3 are also indicated. The size of the genomic region shown spans a distance at least 35 kb, but since the genomic clones containing exons I.1 and I.4 on the one hand, and exon I.2 on the other have not been overlapped, the true distance is still unknown.

Table 2. Summay of Major 5′-Termini in Adipose Cells and Tissues

Tissue/Cells	*Major 5′ Terminus*
Adipose Tissue	I.4, I.3
Adipose Stromal Cells in Culture:	
Control – Serum	I.3
Control + Serum	I.3
Dex + Serum	I.4, I.3
cAMP – Serum	PII/I.3
cAMP + PDA – Serum	PII/I.3
Ovary	PII
Placenta	I.1

presence of serum. On the other hand, I.3 is present in adipose tissue and cells maintained under all conditions.

We have recently characterized a genomic clone containing exon I.4, and find that it is upstream of exon I.2 and overlaps with the clone containing exon I.1, consistent with the finding of 5′-termini containing exon I.2 sequence fused downstream of I.4. Thus exon I.4 lies between exons I.1 and I.2, some 20 kb downstream of exon I.1. However, we still have not succeeded in overlapping the genomic clones containing I.4 and I.2, so a gap of unknown size remains in the genomic sequence, and the total size of the gene is still not established. Our current understanding of the structure of the 5′-end of the *CYP19* gene is summarized in Fig. 6.

VII. TISSUE-SPECIFIC REGULATION OF HUMAN CYP19 EXPRESSION IS ACHIEVED USING ALTERNATIVE PROMOTERS

The results of this ongoing work can he summarized as follows. Tissue-specific expression of the human *CYP19* gene appears to be regulated by tissue-specific promoters in ovary, placenta, and adipose. This conclusion is based on the presence of specific 5′-termini present in the transcripts encoding P450arom in each of these tissues. Thus, transcripts specific for proximal promoter II are found in the ovary, whereas transcripts specific for the distal promoter I.1 are found uniquely in placenta. Adipose tissue contains two species of transcripts containing I.3 and I.4-specific sequences. The latter appears to be present only in breast adipose. Other elements such as promoter I.2 appear to play at best, minor roles. On the other hand, when adipose stromal cells are placed in culture, other 5′-termini are found. Thus, whereas termini containing I.4-specific sequence are uniquely found in transcripts present in cells treated with dexamethasone in the presence of serum, in cells treated with dibutyryl cAMP (in the presence or absence of phorbol esters) in the absence

of serum, the transcripts contain 5′-termini with sequence unique to promoter II. Thus, promoter switching in these cells appears to be regulated by factors present in the culture medium of the cells. It has been reported that transcripts in skin fibroblasts contain a sequence apparently identical to I.4 (Harada, 1992). Aromatase in these cells is also stimulated by glucocorticoids (Berkovitz et al., 1989). On the other hand, aromatase expression in human granulosa cells is not stimulated by glucocorticoids, and ovarian transcripts do not contain I.4-specific sequence.

The expression of P450arom in human ovary appears to utilize a promoter, promoter II, which is proximal to the translation start site, that is to say in the "normal" location. We speculate that this ovarian promoter is the primordial promoter regulating CYP19 expression. This is consistent with results from the laboratory of JoAnne Richards (Hickey et al., 1990) indicating that in rat ovary, CYP19 expression is regulated by a promoter proximal to the translation start site. Similarly, work from the laboratory of Michael McPhaul (Matsumine et al., 1991) indicates that in the chicken ovary, a promoter proximal to the start of translation is also utilized to regulate CYP19 expression.

We speculate that when the human placenta acquired the ability to synthesize estrogens, since this capacity is very great, and since estrogen production by the placenta tends to be a function of placental size, instead of utilizing the ovarian promoter, a powerful, distal promoter was utilized instead, namely, that upstream from exon I.1. Since rat placenta does not synthesize estrogens, and chickens do not have a placenta, one would not expect utilization of tissue-specific alternative promoters in these species. It appears that human P450arom is the first cytochrome P450 to be shown to utilize alternative promoters in the regulation of tissue-specific expression. It is pertinent to ask why this is so. The answer probably relates to the unique tissue-specific distribution of estrogen biosynthesis in the human. In contrast to most other species where estrogen biosynthesis is confined to the gonads and the brain, in the human it is also present in adipose and in placenta. In both these tissues *in situ*, estrogen biosynthesis appears to be relatively unregulated, although dramatic regulation can be achieved utilizing adipose stromal cells in culture. Whereas, the expression of estrogen biosynthesis in the placenta is very great, that in adipose is rather low. It is apparent that the estrogen produced in each of these tissue sites of expression in the human subserves a different function and consequently, the regulation of this biosynthesis must be different in each tissue, and this is apparent from *in vitro* studies. Consequently, it can be hypothesized that a single promoter with a number of upstream regulatory sequences would be inadequate to permit such sophisticated complexity of tissue-specific regulation, and it is for this reason that this particular gene has resorted to the use of alternative promoters to allow for greater versatility in determining tissue-specific regulation of expression. It is worth noting that the 5′ untranslated exons which have so far been characterized are spliced into a common intron/exon boundary upstream from the translational start site. This means that the protein which is expressed in each of the various tissue-specific sites of estrogen biosynthesis is identical, in contrast

to the situation regarding a number of other genes in which alternative splicing results in differences in the aminoterminus of the protein itself.

Although no other cytochrome P450 has been shown to utilize this form of regulation of expression, a growing number of other proteins do. These include the genes for IGF-II and IGF-I, glucokinase, c-myc, c-fms, aldolase A, PTH-related peptide, GnRH, and prolactin (Battey et al., 1983; DiMattia et al., 1990). Interestingly, at least two other genes have been shown to utilize a different promoter to regulate expression in placenta or uterine tissues, as compared to their adult tissue of expression. Thus, human prolactin expression in decidua appears to be regulated by a distal promoter (DiMattia et al., 1990), as does growth hormone releasing hormone expression in placenta (Gonzalez-Crespo and Baronat, 1991). Thus, at least three genes utilize a distal promoter to regulate their expression in placenta or uterine tissue as compared to other tissues. This, however, does not appear to be a universal truth. For example, α-glycoprotein gene expression in placenta appears to utilize a unique regulatory region upstream from a single promoter rather than an alternative promoter (Steger et al., 1991).

The conclusion that tissue-specific regulation of estrogen biosynthesis is determined, in part, by the use of alternative promoters of the *CYP19* gene, has a number of important consequences. In the first place, we have observed that expression of aromatase in adipose varies with body site of tissue origin and increases dramatically with age, consistent with previous determinations of aromatase activity and *in vivo* studies (Killinger et al., 1987; Hensell et al., 1974; Edman and MacDonald, 1976). As discussed here, regional differences in adipose aromatase expression appear to be dependent on alternative promoter utilization, and so the possibility remains that the age-dependent differences may be regulated in a similar fashion. Secondly, the existence of tissue-specific promoters suggests the possibility that mutations in the 5′-upstream regions of the aromatase gene may exist which inhibit estrogen synthesis in one tissue, but not in another, e.g., in adipose but not in the ovary. Thirdly, the existence of tissue-specific promoters offers the opportunity to devise therapeutic agents which differentially inhibit *CYP19* gene expression in a tissue-specific fashion.

VIII. CYP19 REGULATION AND BREAST CANCER

This work may potentially lead to therapeutic application in the treatment of breast cancer. Estrogen production in breast adipose tissue may be a significant contributor to the etiology of breast cancer. Epidemiological data has suggested such a linkage, although the mechanism is unknown. Certainly the ability of estrogens to stimulate the generation of growth factors that stimulate the proliferation of cancer cells may be a factor. In support of a link to local estrogen production, aromatase activity is elevated in adipose tissue of the breast quadrant containing the tumor (O'Neill et al., 1988). We have used quantitative PCR to show that the highest expression of CYP19 mRNA is also located in the quadrant adjacent to the tumor (Bulun et al.,

1993). Other researchers have shown that breast tumors recruit a distinct population of stromal fibroblasts (Ronnov-Jessen et al., 1995). Disrete populations of these stromal cells probably function as pre-adipocytes. These studies support a model in which crosstalk between breast cancer cells and stromal cells is a key part of tumorigenesis (Simpson et al., 1989). This work increases the importance of the realization that adipose expression of CYP19 is regulated by promoter 1.4. Our recent studies show that this promoter is stimulated by glucocorticoids acting at a GRE element (Zhao et al., 1995a) and additionally by various cytokines (notably IL-6, IL-11, and LIF) acting at a GAS element (γ-interferon activation site) through a Jak/STAT signaling process (Zhao et al., 1995b). Interference with these mechanisms at promoter 1.4 may allow selective suppression of CYP19 in breast adipose and also the potential tumor promoting effect.

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Prostaglandin E_2 Stimulates Aromatase Expression in Endometriosis-Derived Stromal Cells*

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ABSTRACT

C_{19} steroids are converted to estrogens by aromatase P450 (P450arom). Aromatase expression in humans is regulated by use of tissue-specific promoters in the placenta (promoter I.1), adipose tissue (promoters I.4, I.3, and II), and gonads (promoter II). The use of each promoter gives rise to a population of P450arom messenger ribonucleic acid (mRNA) species with a unique untranslated 5′-terminus. Aromatase is not expressed in the endometrium of disease-free women. We demonstrated, however, the presence of P450arom mRNA in pelvic endometriotic implants and eutopic endometrial curettings of women with endometriosis. In the current report, aromatase activity and P450arom gene expression were investigated in cultured stromal cells derived from eutopic endometrium and ovarian endometriomas of women with pelvic endometriosis. We also investigated the hormonal regulation of aromatase expression and alternative promoter use in these cells. The effects of interleukin-1β (IL-1β), IL-2, IL-6, IL-11, oncostatin M, IL-15, tumor necrosis factor-α, PGE_2, estradiol, R5020, dexamethasone, and dibutyryl cAMP (Bt_2cAMP) on aromatase activity in endometriosis-derived stromal cells were assessed. We chose treatments with PGs and ILs because of the inflammatory nature of endometriosis. PGE_2 stimulated aromatase activity in endometriosis-derived stromal cells by 19- to 44-fold (37–221 pmol/mg protein·4 h), whereas Bt_2cAMP induction was 26- to 60-fold the baseline level. No stimulation was observed by estradiol or R5020 or by IL-1β, IL-2, IL-6, IL-11, IL-15, or TNFα in the presence or absence of glucocorticoids. A modest induction of aromatase activity (2-fold) was observed in dexamethasone- plus oncostatin M-treated cells. These changes in aromatase activity were accompanied by comparable changes in the levels of P450arom mRNA levels, determined by a quantitative reverse transcription-PCR method. Promoter-specific 5′-ends of P450arom transcripts in total RNA from endometriosis-derived stromal cells treated with PGE_2 and Bt_2cAMP were amplified employing a novel modified rapid amplification of cDNA5′-ends/Southern hybridization method using exon-specific oligonucleotide probes. The majority of P450arom transcripts in these cells contained the gonadal-type promoter II-specific sequences, whereas very few transcripts contained adipose-type promoter I.3- and I.4-specific sequences.

PGE_2 appears to be the most potent known stimulator of aromatase in endometriosis. Aromatase expression in PGE_2-stimulated stromal cells of endometriosis is regulated primarily by the classically located promoter II, which, in turn, is regulated by cAMP. As PGE_2 is known to increase intracellular cAMP levels, estrogen biosynthesis in endometriosis may be primarily regulated by PGE_2 that is locally produced. Consequent local estrogen production may promote the growth of endometriotic implants. (*J Clin Endocrinol Metab* **82:** 600–606, 1997)

ENDOMETRIOSIS is one of the most frequently encountered gynecological diseases in reproductive-age women, often requiring surgical or medical management. Circumstantial and laboratory evidence suggests that endometriosis is a consequence of implantation of viable endometrial tissues in the pelvis via retrograde menstruation, which is a very common event (1–6). Although surfaces of pelvic organs frequently come into contact with the contents of retrograde menstruation in most women, endometriosis is detected in only 1–5% of all women (7, 8). This is suggestive of inherent cellular and molecular differences in the eutopic endometrium of women with endometriosis, which may facilitate the implantation process. In fact, an increasing body of evidence points to biochemical differences between the eutopic endometrium of women with endometriosis and that of disease-free women (9–12). Additionally, qualitative (13, 14) and quantitative (9, 12) differences in secretory products of pelvic endometriosis and eutopic endometrium have been demonstrated. The endometriotic implants are known to elicit an inflammatory response, which is believed to be mediated by mononuclear phagocytes, macrophages, and lymphocytes (15–17). PGs, such as PGE_2 and $PGF_{2\alpha}$ (18–20), and certain cytokines, such as interleukin-6 (IL-6) (9, 12, 21) and IL-11 (12) are locally produced in eutopic endometrium and endometriotic implants. Cultured endometriosis-derived and eutopic endometrial stromal cells from patients with endometriosis, however, produce much higher quantities of IL-6 (9) than those from disease-free controls. Moreover, the endometrium from patients with endometriosis exhibits higher chemotactic activity for neutrophils and macrophages throughout the menstrual cycle compared with disease-free controls (22).

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Estrogen is believed to play important roles in the establishment and maintenance of endometriosis (23). The formation of estrogens from C_{19} steroids is catalyzed by a specific form of P450, namely aromatase P450 (P450arom; the product of the *CYP19* gene). Aromatase expression in various human cells is regulated by the use of alternative promoters in the placental syncytiotrophoblast (promoter I.1), ovarian granulosa and testicular Leydig cells (promoter II), adipose fibroblasts (promoters I.4, I.3, and II), and skin fibroblasts (promoter I.4) (24). This is accomplished by binding of specific *trans*-activating factors to defined genomic response elements upstream of the promoter used in that particular tissue (24). Tissue-specific promoter use is accomplished by alternative splicing mechanisms, which give rise to specific P450arom transcript populations with unique untranslated 5′-ends but with identical coding regions. Thus, the protein encoded is always identical regardless of the tissue site of expression, promoter use, or alternative splicing. In adipose fibroblasts in monolayer culture, aromatase expression is hormonally regulated (24). Aromatase activity and P450arom transcript levels in these cells can be markedly stimulated by cAMP analogs. This effect is potentiated by the addition of phorbol esters (25). Members of the IL-6 cytokine family [IL-6, IL-11, oncostatin-M (OSM), and leukemia inhibitory factor] (26) or serum (27) in the presence of glucocorticoids are also capable of inducing aromatase expression in adipose fibroblasts. As a further twist, the aforementioned hormones or cytokines stimulate aromatase expression in these cells by the use of alternative promoters (28). For example, serum or cytokines in the presence of glucocorticoids give rise to initiation of transcription primarily via promoter I.4. On the other hand, cAMP analogs (with or without phorbol esters) favor the use of promoters I.3 and II.

Aromatase is not expressed in endometrial or myometrial tissues of disease-free women (12, 29). Aromatase expression, however, was demonstrated in the neoplastic counterparts of these uterine tissues, namely endometrial cancer and uterine leiomyomas (30, 31). This suggests a role for estrogens formed *in situ* in the regulation of growth of these neoplasms. Previously, we showed the presence of P450arom transcripts in pelvic endometriotic implants and eutopic endometrial tissues of patients with endometriosis, whereas endometrial curettings from disease-free women and endometriosis-free peritoneal biopsies did not contain P450arom transcripts (12). In the current investigation, regulation of aromatase activity and levels of P450arom transcripts in endometriosis-derived stromal cells in culture were studied. The effects of PGE_2, dibutyryl cAMP (Bt_2cAMP), estradiol, the progesterone analog R5020, dexamethasone (DEX), tumor necrosis factor-α (TNFα), IL-1β, IL-2, IL-6, IL-11, OSM, and IL-15 on aromatase activity were determined. P450arom transcript levels under these treatment conditions also were analyzed using a quantitative reverse transcripton-PCR (RT-PCR) method. Subsequently, promoter-specific transcripts were determined by a novel modified rapid amplification of cDNA 5′-ends (5′-RACE)/exon-specific Southern hybridization method.

Materials and Methods

Tissue acquisition and processing

At the time of laparoscopy or laparotomy, the following samples were obtained from eight women: 1) ovarian endometriomas (n = 4), 2) eutopic endometrial tissues from patients with endometriosis (n = 2), and 3) eutopic endometrial tissues from disease-free patients (n = 3). All samples were histologically confirmed. Endometriotic implants were frozen in liquid nitrogen and stored at −70 C. Endometriomas and eutopic endometrial tissues were transported (in Hanks' Balanced Salt Solution with HEPES and 2% antibiotic concentration) for cell culture and were immediately processed. Written consent was obtained before surgical procedures, including a consent form and protocol approved by the institutional review board for human research of the University of Texas Southwestern Medical Center.

Cell cultures

Endometriomas and eutopic endometrial tissues were cultured using a modified protocol previously reported by Ryan *et al.* with minor modifications (32). Tissues were rinsed with sterile saline solution, minced finely, and digested with collagenase B (1 mg/mL) and deoxyribonuclease I (0.1 mg/mL) at 37 C for 30–60 min. Epithelial cells were separated from stromal cells by filtration through a 75-μm sieve. Stromal cells were then suspended in Waymouth's MB 752/1 enriched medium (Life Technologies, Grand Island, NY) containing 10% FBS. Fresh suspensions of stromal cells were plated in 35-mm culture dishes and kept in an incubator in a humidified atmosphere with 5% CO_2 at 37 C. Media were changed within 48 h and thereafter at intervals until the cells became 75% confluent. Stromal cells were then placed in serum-free Waymouth's medium. The fibroblast-like appearance of endometriosis-derived stromal cells in culture under phase contrast microscopy was identical to that of endometrial stromal cells (Fig. 1).

Treatments consisted of 1) Bt_2cAMP (0.5 mmol/L) alone; 2) Bt_2cAMP together with phorbol diacetate (PDA; 100 nm) in serum-free medium; 3) DEX (250 nmol/L) in medium containing 10% FBS; 4) DEX in serum-free medium plus one of the following cytokines: TNFα (10 ng/mL), IL-1β (1 ng/mL), IL-2 (2 ng/mL), IL-6 (2 ng/mL), IL-11 (2 ng/mL), OSM (2 ng/mL), IL-15 (2 ng/mL); 5) PGE_2 (10^{-6}-10^{-8} mol/L); 6) 17β-estradiol (10^{-7} mol/L); and 7) R5020 (5×10^{-7} mol/L). All treatments were continued for 24 h.

Determination of aromatase activity

Aromatase activity was assayed in intact stromal cells after the addition of [1β-^{3}H]androstenedione (150 nmol/L; DuPont, Boston, MA) to the medium. Endometrial stromal cells from a disease-free patient were used as negative controls. At the end of a 4-h incubation period, medium was removed, and the incorporation of tritium from [1β-^{3}H]androstenedione into [^{3}H]water was assayed as described previously (33). The cells were then scraped off the dishes, homogenized, and assayed for protein using the BCA protein assay (Pierce, Rockford, IL). Results were expressed as picomoles per mg of protein/4 h. Each *bar* in Figs. 2 and 3 represents the mean of triplicate replicates (±SEM).

Determination of P450arom transcript levels in total ribonucleic acid (RNA) using a quantitative RT-PCR method

Total RNA was isolated, using the method described by Chirgwin *et al.* (34), from frozen tissues and cells in culture. PCR amplification of a sequence in the coding region of P450arom transcripts after RT was performed as previously described (35). This procedure involved RT of 10 μg total RNA from cells or tissues using a 3′-oligonucleotide specific for coding exon IV to synthesize a single stranded complementary DNA (cDNA). These cDNA templates were amplified separately using promoter-specific 5′-oligonucleotide primers. 5′-Untranslated sequences specific for promoters II, I.4, and I.3 as well as a sequence in the common coding region were amplified, size-fractionated in 1.8% agarose gels, and transferred to a blotting nylon membrane by capillary elution. Southern hybridization with the ^{32}P-labeled oligonucleotide probes specific for unique 5′-sequences was continued overnight (Figs. 4 and 5). The sequences of these probes were published previously (12). X-Ray films

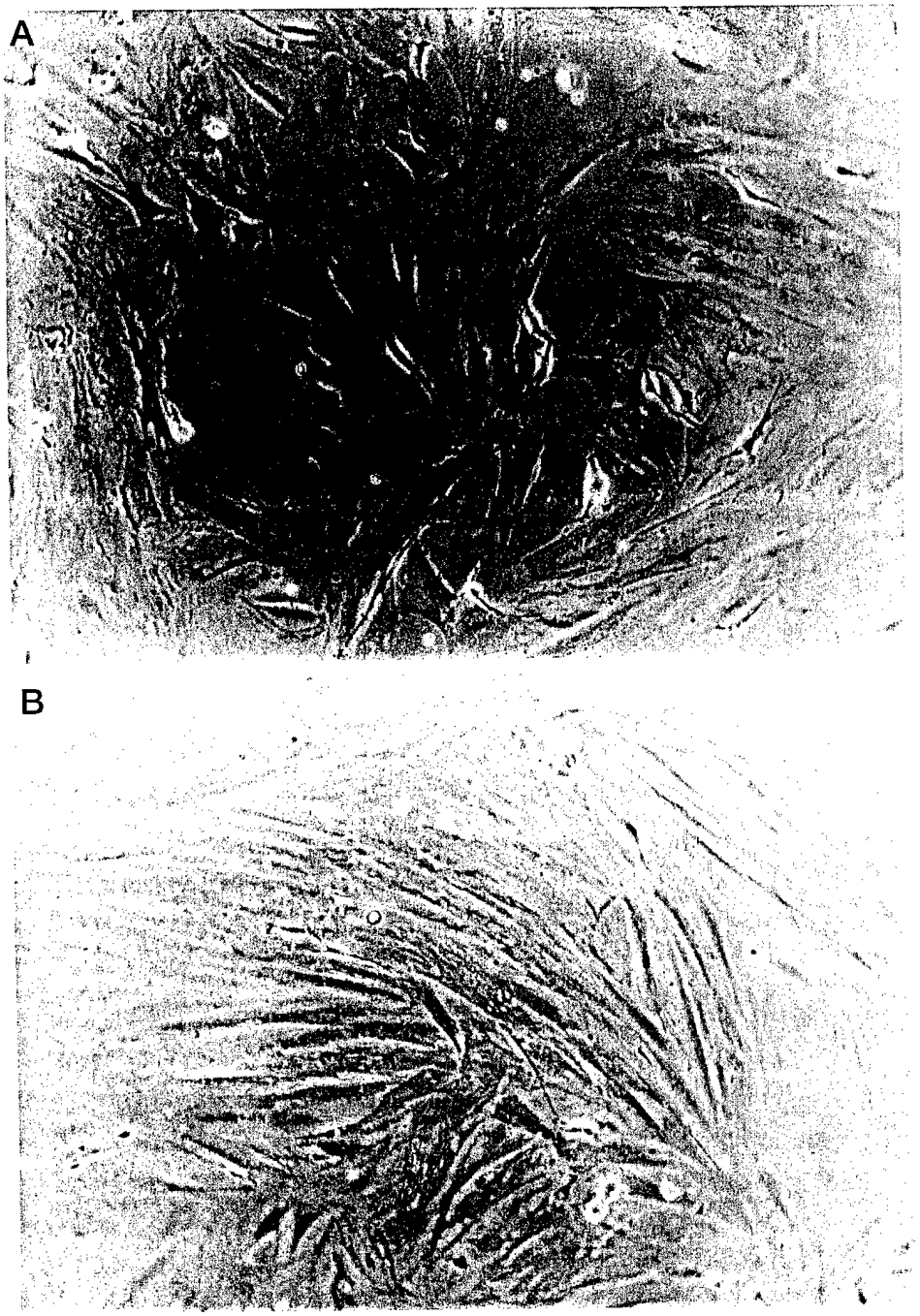

FIG. 1. Phase contrast photomicrograph of cultured stromal cells derived from the eutopic endometrium (A) and from an ovarian endometrioma (B). Magnification, ×45.

were exposed to blotting membranes for 1–16 h. Samples containing ovarian granulosa cells (positive control for promoter II-specific transcripts) and adipose stromal cells treated with Bt_2cAMP (positive control for promoters I.3-, II-, and I.4-specific transcripts) were included in this experiment. Transcripts of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), were amplified in each sample as described previously (36). This ubiquitous marker was used to normalize the quantity of RNA used. Radioactivity on blotting membranes was quantified using the PhosphorImager System (Molecular Dynamics, Sunnyvale, CA). A ratio of the value obtained from P450arom coding sequence amplification products to that from G3PDH amplification products in a total RNA sample was recorded as the arbitrary unit for the total P450arom transcript level (Fig. 4).

Modified 5′-RACE/exon-specific Southern hybridization

Construction of cDNA by 5′-RACE was performed using total RNA from PGE_2- and Bt_2cAMP-treated endometriosis-derived stromal cells in culture. 5′-RACE was performed with minor modifications, as described by Frohman *et al.* (37, 38). First strand synthesis was performed using 10 μg total RNA, 2.5 pmol antisense primer that is complementary to a sequence in exon III located in the coding region (5′-ACTTGCTGATA-ATGAGTGTT-3′), reverse transcriptase buffer, 10 mmol/L dithiothreitol, 1 mmol/L final concentration of each deoxy-NTP, and 200 U Super-Script II reverse transcriptase in a 25-μL volume (5′RACE system, Life Technologies, Gaithersburg, MD). The primer extension was carried out at 42 C for 1 h, then the single stranded cDNA was tailed at the 3′-end with poly(C) using terminal transferase. Poly(C)-tailed cDNA template

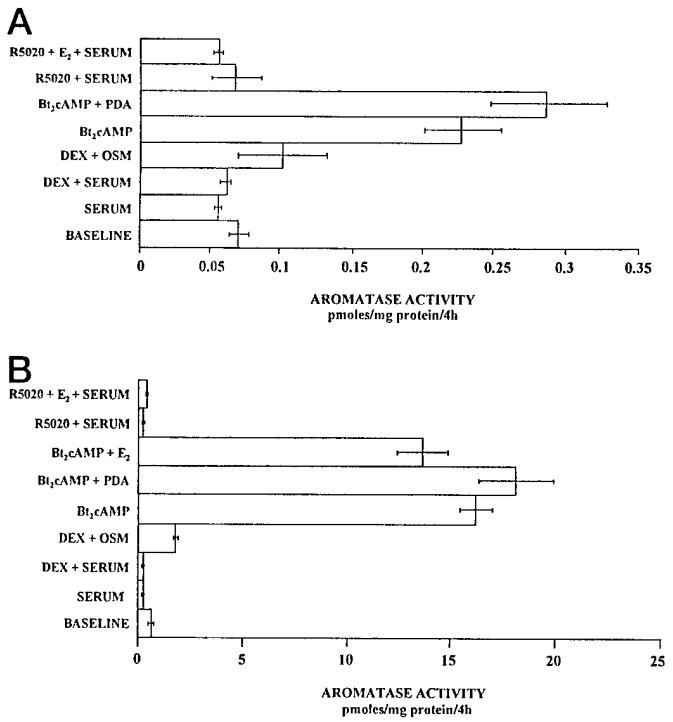

FIG. 2. A, Aromatase activity of eutopic endometrial stromal cells from a woman with endometriosis. Confluent eutopic endometrial stromal cells in primary culture were maintained for 24 h in serum-free medium. Treatments containing 1) 10% FBS (SERUM) only; 2) DEX (250 nmol/L) in medium containing serum; 3) DEX in serum-free medium plus OSM (2 ng/mL); 4) Bt_2cAMP (0.5 mmol/L) alone or together with PDA (100 mmol/L) in serum-free medium; and 5) the progesterone agonist R5020 with or without estradiol (E_2; 10^{-7} mol/L) in the presence of serum. All treatments were continued for 24 h, and aromatase activity was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 4 h. Results are expressed as picomoles of [^{3}H]water formed per mg protein/4 h and represent the mean ± SEM of triplicate replicate dishes. B, Aromatase activity of stromal cells isolated from an ovarian endometrioma that was removed from the woman represented in A. Similar treatment conditions were applied. Note that aromatase activity levels in B are 11–70 times those in A.

was then amplified by PCR using an amplification buffer system, a nested antisense primer that is complementary to an upstream sequence in coding exon III (5'ATTCCCATGCAGTAGCCAGG-3'), and a sense anchor primer that was provided in the kit. Amplification products were divided into four equal aliquots, which were separately size-fractionated in 1.8% agarose gels and transferred to blotting nylon membranes by capillary elution. Southern hybridization with ^{32}P-labeled promoter-specific oligonucleotide probes was continued overnight. The sequences of these probes were previously published (12). X-Ray films were exposed to blotting membranes for 1–16 h.

Results

Aromatase activity

Eutopic endometrial stromal cells from disease-free women (n = 3). These cells were used as negative controls. No detectable baseline or hormone-inducible aromatase activity was demonstrated (data not shown).

Eutopic endometrial stromal cells from women (n = 2) with endometriosis. Although it was detectable, baseline aromatase activity in eutopic endometrial stromal cells from a patient with endometriosis (0.06 pmol/mg protein·4 h; Fig. 2A) was markedly lower than that in endometriosis-derived stromal cells (Fig. 2B). Bt_2cAMP, in the absence of serum, stimulated aromatase activity by 5-fold. The addition of PDA neither potentiated nor suppressed Bt_2cAMP stimulation. DEX in the presence of serum or cytokines failed to stimulate aromatase activity, although a marginal stimulation was noted in DEX-plus OSM-treated cells. The progesterone agonist R5020 in the absence or presence of estradiol or serum did not affect aromatase activity. Similar results were observed in cultured endometrial cells from another subject with endometriosis.

Endometriosis-derived stromal cells (n = 4). In a representative experiment, the mean baseline aromatase activity of endometriosis-derived stromal cells (0.65 pmol/mg protein·4 h; Fig. 2B) was approximately 11 times that of eutopic endometrial stromal cells (Fig. 2A) from the same woman. In these cells, which were derived from an endometrioma, Bt_2cAMP treatment gave rise to a 26-fold increase over baseline activity. Again, the addition of PDA did not modify Bt_2cAMP stimulation. DEX induced aromatase activity (2-fold over baseline) only in the presence of OSM, but not in the presence of other cytokines or serum. No stimulation was observed in R5020- or estradiol-treated cells. In cells from another patient (Fig. 3), PGE_2 (10^{-8} mol/L) stimulated aromatase activity by 19-fold over the baseline value, whereas Bt_2cAMP induction was 32-fold. Moreover, increasing concentrations of PGE_2 showed stimulation in a dose-dependent manner (data not shown). No stimulation was observed with IL-1β, IL-2, or IL-15 (Fig. 3). Additionally, TNFα, IL-6, or IL-11 treatment in the presence or absence of DEX had no effects on aromatase activity (data not shown). PGE_2 and Bt_2cAMP inductions of aromatase activity in endometriosis-derived cells from two other women were up to 44-fold and 60-fold, respectively (data not shown).

Determination of P450arom transcript levels in endometriosis-derived and eutopic endometrial stromal cells (Fig. 4)

We compared P450arom transcript levels in total RNA from eutopic endometrial stromal cells and endometrioma-derived stromal cells, which were treated with Bt_2cAMP and DEX plus OSM. Total P450arom transcript levels in RNA samples were determined by RT-PCR amplification of a com-

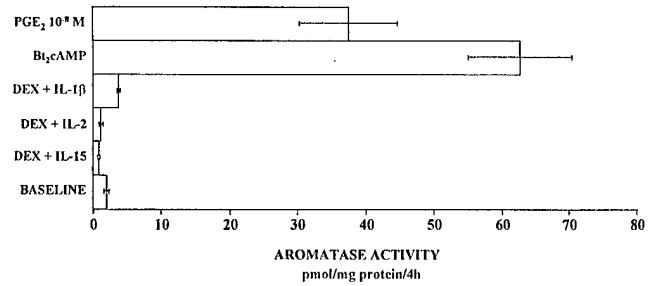

FIG. 3. Aromatase activity of endometriosis-derived stromal cells. Confluent stromal cells in primary culture were maintained for 24 h in serum-free medium. Treatments consisted of 1) DEX (250 nmol/L) in serum-free medium plus one of the following cytokines: IL-1β (1 ng/mL), IL-2 (2 ng/mL), or IL-15 (2 ng/mL); 2) Bt_2cAMP (0.5 mmol/L) in serum-free medium; and 3) PGE_2 (10^{-8} mol/L). All treatments were continued for 24 h.

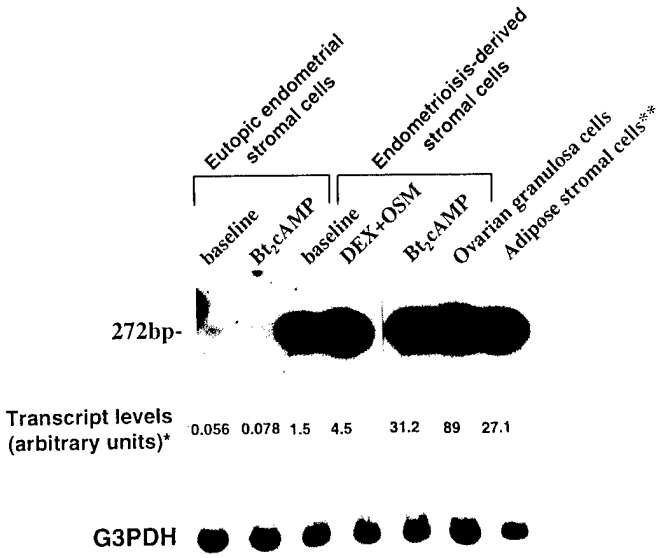

FIG. 4. Determination of total P450arom transcript levels using quantitative RT-PCR. We compared total P450arom transcript levels in eutopic endometrial stromal cells (lanes 1 and 2) and endometriosis-derived stromal cells (lanes 3–5) from the same women. Total RNA was isolated from cells incubated with Bt_2cAMP (0.5 mmol/L) and DEX (250 nmol/L) plus OSM (2 ng/mL). Samples containing ovarian granulosa cells and adipose stromal cells treated with Bt_2cAMP were used as controls. PCR amplification of a sequence in the coding region of P450arom cDNA was performed after RT of 10 mg total RNA. PCR products were size-fractionated in 1.8% agarose gel and transferred to a nylon membrane. Southern hybridization was performed using a ^{32}P-labeled oligonucleotide probe complementary to the coding region of P450arom cDNA. G3PDH transcripts in the same RNA samples were amplified as an internal standard. Endometriosis-derived stromal cells treated with Bt_2cAMP, ovarian granulosa cells, and adipose stromal cells contained abundant P450arom transcripts. *, Arbitrary units represent a ratio of the radioactivity value of the target sequence (P450arom transcripts) divided by the radioactivity value of G3PDH amplification products for that particular sample. **, Adipose stromal cells treated with Bt_2cAMP.

mon coding sequence. Changes in transcript levels were associated with comparable changes in aromatase activity (Fig. 4, lanes 3–5) in endometriosis-derived cells. Stromal cells originated from eutopic endometrium of the same woman (lanes 1 and 2, Fig. 4) contained detectable, but very low, levels of P450arom transcripts, which remained below the linear range of the quantitative RT-PCR assay (36). RNA samples of ovarian granulosa cells and Bt_2cAMP-treated adipose fibroblasts were included as controls (lanes 6 and 7, Fig. 4).

Detection of promoter-specific P450arom transcripts in endometriosis-derived and eutopic endometrial stromal cells (Fig. 5)

Total RNA samples shown in Fig. 4 were subjected to RT-PCR followed by exon-specific Southern hybridization using ^{32}P-labeled oligonucleotide probes specific for sequences associated with transcription via promoters II, I.4, and I.3 (Fig. 5). Bt_2cAMP treatment was associated with promoter II- and I.3-specific transcripts, whereas promoters II, I.4, and I.3 were used in DEX- plus OSM-treated cells. It should be pointed out here that as the amplification products

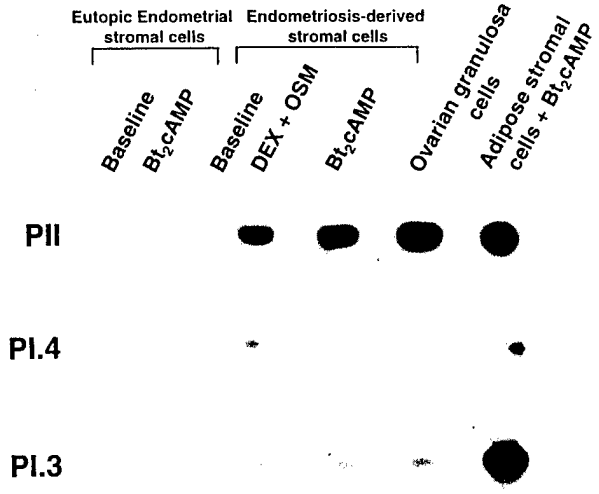

FIG. 5. RT-PCR/exon-specific Southern hybridization analysis of untranslated 5′-terminals of P450arom transcripts in the same RNA samples as those in Fig. 4. After total RNA isolation, primer extension by RT of 10 mg total RNA from cells was performed by use of a 3′-oligonucleotide primer complementary to coding exon IV to synthesize cDNA. Specific oligonucleotides were used as primers and probes to amplify exon I.3, exon I.4, and promoter II-specific sequences. (Amplification of the common coding region in these samples was shown in Fig. 4.) Ovarian granulosa cells (positive control for promoter II-specific transcripts) and adipose stromal cells treated with Bt_2cAMP (positive control for promoters II, I.3, and I.4) were included in this experiment. As G3PDH transcripts were amplified as an internal standard in these samples (see Fig. 4), the intensities of the bands represent relative quantities of promoter-specific P450arom transcripts. Promoter II appears to be primarily used in untreated, as well as in Bt_2cAMP- and DEX- plus OSM-treated cells.

of promoter-specific sequences (Fig. 5) were also normalized to amplification products of G3PDH transcripts (Fig. 4), the intensity of the bands in Fig. 5 can be interpreted as relative quantities of promoter-specific transcripts. It was apparent that, under all incubation conditions, promoter II-specific transcripts comprised the majority. In eutopic endometrial stromal cells from a woman with endometriosis, amplified products of promoter-specific 5′-ends were not detected (Fig. 5, lanes 1 and 2), and in untreated endometriosis-derived stromal cells (Fig. 5, lane 3), a faint band was detected only for promoter II-specific amplification products with prolonged exposure of the autoradiograph. The most likely explanation is the presence of low messenger RNA (mRNA) copy numbers in these three samples. [A sequence in the coding region of P450arom transcripts in all of these cells was detected using RT-PCR (Fig. 4, lanes 1–3).] Additionally, a novel modified RACE/exon-specific Southern hybridization method was used to analyze 5′-ends of P450arom transcripts in PGE_2- and Bt_2cAMP-treated endometriosis-derived stromal cells. Almost all P450arom transcripts in Bt_2cAMP- and PGE_2-treated cells contained promoter II-specific 5′-ends, whereas very few promoter I.3- and I.4-specific transcripts were found (Fig. 6). Although some I.3- and I.4-specific bands cannot be seen in Fig. 6, they were detected by longer exposure of the autoradiograph. PDA did not potentiate or suppress Bt_2cAMP or PGE_2 stimulation of P450arom gene expression. This was consistent with aromatase activity determinations after similar treatments (Figs. 2 and 3).

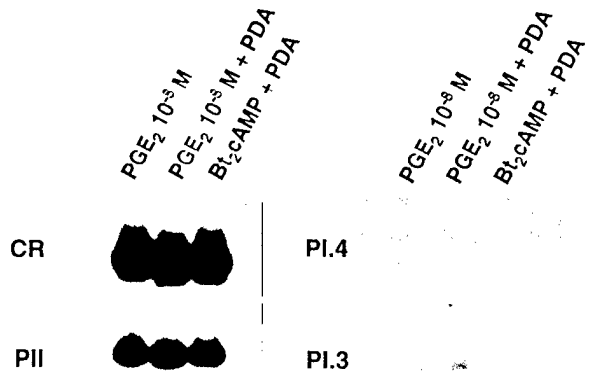

FIG. 6. A modified 5′-RACE/exon-specific Southern hybridization method was employed to analyze 5′-ends of P450arom transcripts in PGE_2 and Bt_2cAMP-treated endometriosis-derived stromal cells. 5′-RACE cDNAs were constructed using 10 mg total RNA. Amplification products were divided into four equal aliquots, which were separately size-fractionated in 1.8% agarose gels and transferred to separate membranes. Southern hybridization with ^{32}P-labeled exon-specific oligonucleotide probes was continued overnight. Aromatase expression in PGE_2- and Bt_2cAMP-treated cells appears to be primarily directed via promoter II. Promoter I.3- and I.4-specific bands were detected only after prolonged exposures of the autoradiograph. PDA does not appear to modify effects of PGE_2 or Bt_2cAMP on P450arom transcript levels. CR: Coding region.

P450arom transcript levels and the pattern of promoter use were similar in endometriosis-derived stromal cells from all patients studied (n = 4).

Discussion

Aromatase expression was demonstrated previously in pelvic endometriotic implants and eutopic endometrial tissues of women with endometriosis (12). In the current study, we investigated the regulation of aromatase activity and P450arom transcript levels in stromal cells in primary culture that were isolated from these tissues. Aromatase activity detected in endometriosis-derived stromal cells was comparable to that measured in adipose fibroblasts (33) and was much higher than that seen in eutopic endometrial stromal cells from the same woman. Bt_2cAMP in the absence of serum markedly stimulated aromatase activity in both groups of cells. PDA did not potentiate the stimulatory effect of this cAMP analog. PDA was previously shown to potentiate the action of Bt_2cAMP to stimulate aromatase activity in adipose fibroblasts (25), whereas in ovarian granulosa cells, PDA suppressed the stimulatory effects of Bt_2cAMP (39). Therefore, the patterns of response of endometriosis-derived stromal cells to Bt_2cAMP and PDA treatments have been consistently distinct from those observed in ovarian granulosa cells or adipose fibroblasts. It should also be pointed out that aromatase expression in endometriosis-derived cells could not be stimulated by DEX plus serum, and a blunted induction (compared to adipose fibroblasts) was observed in response to DEX plus OSM treatments. This pattern was also clearly distinct from that observed in adipose fibroblasts (26). Changes in total P450arom transcript levels induced by Bt_2cAMP and DEX plus OSM treatments were associated with comparable changes in aromatase activity, suggesting that aromatase activity in these cells is regulated primarily by changes in the level of mRNA encoding P450arom. Analysis of promoter-specific 5′-ends in endometriosis-derived stromal cells indicated use of primarily the gonadal-type promoter II in association with Bt_2cAMP, PGE_2, and DEX plus OSM treatments and under basal conditions. It appears that these factors are capable of regulating aromatase expression in endometriotic stromal cells, but unlike in adipose fibroblasts, promoter use is not switched under varying hormonal conditions. In other words, I.4- and I.3-specific transcripts were not detected in quantities ordinarily found in adipose fibroblasts in response to DEX-OSM or Bt_2cAMP treatments (28).

PGE_2 is the most potent known (patho)physiological stimulator of estrogen biosynthesis in endometriosis. This prostanoid was capable of inducing aromatase activity up to 44-fold the baseline level. PGE_2 can cause a diverse range of actions that are mainly determined by the subtype of receptor used in that tissue. It was observed that various prostanoids, whether natural or synthetic, showed different effects on a variety of isolated tissues (40, 41). For example, when applied to preparations of guinea pig trachea, PGE_2 can cause smooth muscle contraction, relaxation, or both depending on the use of selective receptor antagonists (42). These actions were later explained by the discovery of different PGE (EP) receptor subtypes (EP_1, EP_2, EP_3, and EP_4) that, in turn, are linked to different signal transduction pathways. PGE_2 interacts with several receptor subtypes, one of which, EP_2, is coupled to stimulation of adenylate cyclase, whereas another, EP_1, is coupled to calcium uptake, inositol triphosphate formation, and protein kinase C activation (41, 43). In endometriosis-derived stromal cells, it appears that the stimulation of aromatase activity by PGE_2 may be mediated by a cAMP-dependent pathway (EP_2 receptor binding), as Bt_2cAMP also induces aromatase expression in these cells. PGE_2 treatment of endometriosis-derived stromal cells in culture was initially carried out based on our observations involving incubation of adipose fibroblasts with various prostanoids. PGE_2 and PGD_2 were capable of markedly inducing aromatase activity of adipose fibroblasts, whereas $PGF_{2\alpha}$, PGI_2, and PGJ_2 treatments failed to elicit a response (our unpublished observations). Studies regarding the effects of prostanoids other than PGE_2 and their synthetic analogs and determination of the receptor types in endometriotic stromal cells are currently underway. One may also envision that differential expression of other genes besides P450arom would take place in endometrial tissues of women with pelvic endometriosis compared with those of disease-free women (9, 10, 12). In fact, expression (or lack of expression) of some of these genes may render endometriosis-derived stromal cells cAMP responsive and give rise to activation of the gonadal-type P450arom promoter II.

The basis for the 11–70-fold difference between levels of aromatase expression in eutopic endometrium and pelvic endometriosis may be due to the transformation of endometrial stromal cells after implantation in pelvis in response to paracrine factors produced by the ovary and pelvic peritoneum. The aromatization capability of eutopic endometrial cells from women with endometriosis may facilitate their implantation in pelvic peritoneum and may promote the growth of these implants. Cytokines produced by blood-borne monocytes or local macrophages may induce PG synthesis in certain cell types of endometriosis, such as stromal, glandular,

or vascular endothelial cells. This, in turn, may markedly stimulate estrogen production in pelvic endometriotic implants. The failure of GnRH analog treatment in a certain number of cases of pelvic endometriosis may be explained in part by local estrogen biosynthesis in these tissues. The final question is whether pathophysiologically sufficient quantities of estradiol are produced to sustain the maintenance and growth of these implants. This is dependent on the availability of the type of C_{19} substrate (testosterone *vs.* androstenedione), the presence of the reductive types of 17β-hydroxysteroid dehydrogenase enzyme, and the ready availability of produced estradiol to estrogen receptors within the same cell type (intracrine effect). It should be emphasized here that aromatase activity in stimulated endometriosis-derived cells is extremely high and is comparable with that in placental trophoblasts or ovarian granulosa cells in culture. Thus, it is likely that local estrogen production makes an impact on the development and growth of pelvic endometriosis.

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Alternatively Spliced Transcripts of the Aromatase Cytochrome P450 (*CYP19*) Gene in Adipose Tissue of Women*

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ABSTRACT

Estrogen biosynthesis in adipose tissue has assumed great significance in terms of a number of estrogen-related diseases. The biosynthesis of estrogens from C_{19} steroids is catalyzed by a specific form of cytochrome P450, namely aromatase cytochrome P450 (P450arom; the product of the *CYP19* gene). The human *CYP19* gene comprises nine coding exons, II–X, and its transcripts are expressed in the ovary, placenta, testes, adipose tissue, and brain. Tissue-specific expression of the *CYP19* gene is determined at least in part by the use of tissue-specific promoters, which give rise to transcripts with unique 5′-noncoding termini. Thus, the distal promoter I.1 is responsible for expression uniquely in placenta. On the other hand, the proximal promoter II, which regulates expression via a cAMP-dependent signaling pathway, is responsible for expression in the gonads. Transcripts in breast adipose tissue contain 5′-termini corresponding to expression derived from promoters I.4, II, and I.3, with I.4-specific termini predominating. The latter are derived from promoter I.4, which contains a glucocorticoid response element and an interferon-γ activation site element and is responsible for expression in the presence of glucocorticoids and members of the class I cytokine family. The object of the present study was to determine the distribution of these various transcripts in adipose tissue from abdomen, buttocks, and thighs of women, as this would provide important clues to the factors regulating aromatase expression in these sites. To achieve this, we employed competitive reverse transcription-PCR to amplify unique 5′-ends of each of the transcripts of the *CYP19* gene that are expressed in adipose tissue as well as for the coding region to evaluate total *CYP19* gene (P450arom) transcript levels. We observed that exon I.4-specific transcripts were predominantly present in adipose tissue samples obtained from women regardless of the tissue site or the age of the individual. In these tissues, promoter II- and exon I.3-specific transcripts were present in lower copy numbers. We also demonstrated that in these sites total or exon-specific P450arom transcripts levels increased in direct proportion to advancing age and that transcript levels were the highest in buttocks, followed by thighs, and lowest in the abdomen. These results suggest that in normal human adipose tissue, aromatase expression is mainly under local control by a number of cytokines via paracrine and autocrine mechanisms in the presence of systemic glucocorticoids. (*J Clin Endocrinol Metab* **82:** 70–74, 1997)

ESTROGENS have diverse actions at different body sites of women. Estradiol is produced in the ovarian granulosa cells of premenopausal women, whereas estriol and estradiol are secreted by the placenta (1). In both women and men, estrone is produced in the adipose tissue (2–4), and a substantial fraction of this estrone is further converted to estradiol in the periphery (5). Adipose tissue is the major site of estrogen biosynthesis in postmenopausal women (4, 6). Increased estrogen production in elderly obese women is believed to play a role in the pathogenesis of endometrial cancer (7). Furthermore, estrogen produced by adipose tissue within the breast may act locally to promote the growth of breast carcinomas (8, 9).

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Estrogen biosynthesis is catalyzed by an enzyme known as aromatase cytochrome P450 (P450arom; the product of the *CYP19* gene) (10–12). In the human, aromatase expression occurs in a number of human tissues and cell types, including the syncytiotrophoblast of the placenta (13), ovarian granulosa cells (14, 15), testicular Leydig cells (16–18), various sites in the brain (19, 20), as well as adipose tissue (3, 6, 21–23). Hemsell and co-workers first addressed the significance of human adipose tissue as a major source of estrogen production and demonstrated that in both women and men, there is a progressive increase in the efficiency with which circulating androstenedione is converted to estrone with advancing age (2). Subsequently, we have shown that with aging, there is an increase in the specific activity of the aromatase enzyme in adipose stromal cells, and we concluded that this may result in increased estrone production associated with aging (24, 25). Recently, we determined that this age-related increase in aromatase activity in adipose tissue is a result of increased levels of P450arom transcripts in various body sites of women, including buttocks, thighs, and abdomen (26). Moreover, expression was highest in buttocks, with lower levels of expression in thighs and abdomen.

The coding region of the *CYP19* gene spans nine exons beginning with exon II, but 5′-termini (exon I) of aromatase

transcripts differ from one another in a tissue-specific fashion (27). These 5′-termini correspond to untranslated exons spliced into the P450arom transcripts due to the use of tissue-specific promoters.

Analysis of P450arom transcripts in samples of breast adipose tissue revealed that in addition to exon I.4-specific transcripts, promoter II (PII)-specific as well as exon I.3 (I.3)-specific transcripts are present, albeit in lower copy number (28, 29). Interestingly, when adipose fibroblasts are placed in culture, the proportions of these exon-specific transcripts depend on the stimulatory factors present in the culture medium (28). Thus, when aromatase expression is stimulated by members of the class I cytokine family, such as IL-6 or IL-11, in the presence of glucocorticoids, the transcripts that are present are those derived from promoter I.4 (30). On the other hand, when expression is stimulated by dibutyryl cAMP in the presence or absence of phorbol esters, the transcripts that are present are those specific for PII and I.3 (28).

An important question then arises as to the nature of the 5′-termini (exon I) of the aromatase transcripts present in body sites other than the breast, as aromatase expression varies with age and in a site-specific fashion. In the present study we report determination of the various exon-specific aromatase transcripts in adipose tissue of buttocks, thighs, and abdomen obtained from normal women of various ages.

Materials and Methods

Tissue acquisition and processing

Our studies were carried out in 11 healthy women who ranged in age from 23–61 yr and in body weight from 50–84 kg. Body mass index [defined as body weight (kilograms)/height (meters)^2] varied from 18–31 (Table 1). The average adult has a body mass index of 25. Subcutaneous fat samples (n = 33) from buttocks, thighs, and abdomen of these women were obtained by needle aspiration biopsies, as previously described (26). The samples (wet weight ranging from 0.3–0.4 g) were immediately placed in guanidinium thiocyanate solution, and RNA was isolated within 2 h (31). Written consent was obtained before all surgical procedures, employing a consent form and protocol approved by the institutional review board for human research of the University of Texas Southwestern Medical Center.

Reverse transcription-PCR (RT-PCR)

RT-PCR was performed according to a recently standardized competitive RT-PCR method that we developed for this purpose (32). RNA was initially treated with deoxyribonuclease I to remove any contaminating DNA. Total RNA was then reverse transcribed using random hexamers. Complementary DNA was used in subsequent PCR amplifications for 25 cycles. Specific sense 5′-end primers were used to amplify the various 5′-termini. The 3′-end primer was identical in all samples (32). A trace amount of [^{32}P]deoxy-CTP was added to each sample. The reaction products were analyzed on 4% nondenaturing polyacrylamide gels, and radioactivity on the gels was visualized by exposure to x-ray film. To check the integrity and comparative quantity of RNA used in amplification of P450arom transcripts, transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, were amplified by the RT-PCR method as described previously (32). Specific transcript levels were expressed as arbitrary units normalized by GAPDH.

TABLE 1. Clinical parameters of 11 normal women involved in this study

Patient No.	Age (yr)	BW (kg)	BMI	Waist/hip ratio
1	23	52.7	20.0	0.775
2	24	50.0	22.0	0.775
3	24	51.8	20.0	0.681
4	36	53.2	21.0	0.735
5	43	63.6	27.0	0.743
6	44	62.7	27.0	0.733
7	46	52.3	18.0	0.697
8	53	76.0	31.0	0.774
9	55	84.0	30.5	0.753
10	57	66.0	27.0	0.863
11	61	81.8	30.0	0.770

BMI, Body mass index.

Statistical analyses

Data were analyzed on a VAX-8800 computer with the UTSTAT program (ACS Data Group, Dallas, TX). Regression analysis was performed to calculate simple correlation coefficient (Pearson's R) to determine whether adipose tissue P450arom transcript levels correlated with the ages of the subjects. A two-way parametric repeated measures ANOVA was applied to determine whether there were significant differences between levels of specific transcripts (PII specific, I.3, and I.4) at each body site and between different body sites (buttocks, thighs, and abdomen). A Newman-Keuls multiple comparisons test was then applied to evaluate levels of significance for differences between individual groups.

Results

Figure 1 depicts a representative experiment employing three samples (buttocks, thighs, and abdomen) from one individual and shows the amplification of the coding region (total transcripts) and transcripts with three unique 5′-ter-

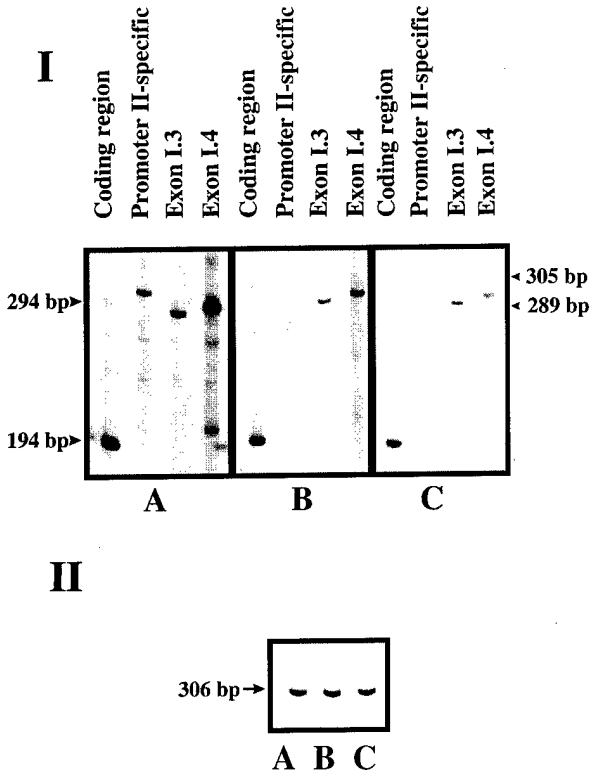

FIG. 1. I) Amplification of coding region and specific 5′-termini of P450arom transcripts in RNA isolated from adipose tissue of women. Complementary DNA from 1 μg RNA was used for each reaction. The experiment shown is representative of several. A, Buttocks; B, thighs; C, abdomen. II) GAPDH amplification.

mini: PII, I.3, and I.4. The amplification products were of the expected size (coding, 194 bp; PII, 305 bp; I.3, 289 bp; I.4, 294 bp). The value in arbitrary units for each P450arom transcript level was obtained from the quantified radioactivity of the amplification products (Fig. 2). These values were normalized to total RNA quantity and to GAPDH amplification products in each sample. With advancing age, there was a progressive increase in total P450arom transcript levels in samples obtained from the buttocks, thighs, and abdomen. This age-dependent increase was statistically significant in the buttocks and thighs (correlation coefficients: Pearson's $R = 0.889$; $P < 0.002$ and $R = 0.817$; $P < 0.05$ respectively; Fig. 2, A and B), whereas this linear trend ($R = 0.704$) did not reach statistical significance ($P < 0.10$) in the abdomen (Fig. 2C). The P450arom transcript levels were highest in the buttocks, followed by the thighs, and lowest in the abdomen. Statistically significant differences were found among these three body sites using parametric repeated measures ANOVA [$p(F) < 0.001$]. This was followed by a Newman-Keuls multiple comparisons test, which showed statistically significant differences between buttocks and thighs ($P < 0.005$) and buttocks and abdomen ($P < 0.005$). Although transcript levels in the thighs were higher than those in the abdomen, this difference did not reach a level of statistical significance.

When the levels of the exon-specific transcripts (PII, I.3, and I.4) were added together, the sum was approximately equal to the measured quantity of total P450arom transcript levels (*i.e.* common coding region) in each case. This indicates that these three species accounted for essentially all of the P450arom transcripts present in the RNA (Fig. 2). I.4-specific transcripts comprised the majority of P450arom mRNA in all adipose tissue samples regardless of age and body site, followed by I.3-specific transcripts, and PII-specific transcripts were present in lowest quantities. A repeated measures parametric ANOVA revealed statistically significant differences among these three types of transcripts at each body site [$p(F) < 0.001$, 0.005, and 0.001 in buttocks, thighs, and abdomen, respectively]. A Newman-Keuls multiple comparisons test revealed statistically significant differences between I.4 and PII ($P < 0.001$, 0.025, and 0.001 in buttocks, thighs, and abdomen, respectively). The differences between I.4 and I.3 were significant ($P < 0.005$) in buttocks and abdomen, but not in thighs. Significant differences were also observed between PII and I.3 in all three body sites ($P < 0.001$, 0.025, and 0.001 in buttocks, thighs, and abdomen, respectively). With advancing age, there was a progressive increase in exon-specific transcripts in samples obtained from the buttocks, thighs, and abdomen (Fig. 2). The age-dependent increase was statistically significant for I.4- and I.3-specific transcripts in samples obtained from buttocks and thighs (Fig. 2, A and B). This linear trend, however, did not reach statistical significance for any exon-specific transcript in the abdomen (Fig. 2C). This may be due to the presence of low levels of P450arom transcripts in abdominal adipose tissue.

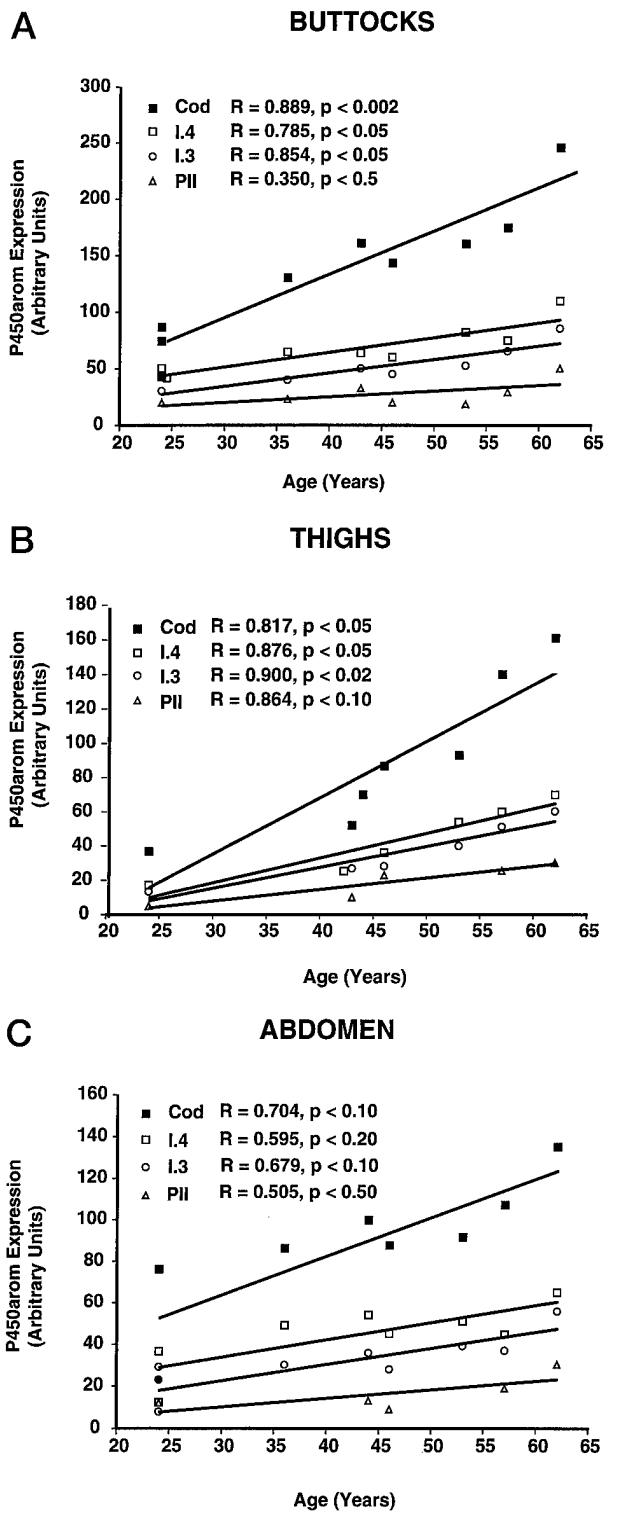

FIG. 2. Amplification of specific 5′-termini of P450arom transcripts in complementary DNA from 1 μg RNA isolated from different body sites of normal women. A, Buttocks; B, thighs; C, abdomen. Data are normalized to GAPDH transcripts. PII, PII-specific transcripts; I.3, I.3-specific transcripts; I.4, I.4-specific transcripts.

Discussion

Recognition of the importance of adipose tissue as a source of estrogens in postmenopausal women came from the pioneering studies of MacDonald and co-workers in the 1970s, who demonstrated that the fractional conversion of androstenedione to estrone in humans increases as a function of

obesity and aging (2, 4, 33). This parameter was shown to be correlated positively with excess body weight in both pre- and postmenopausal women and may be increased as much as 10-fold in morbidly obese postmenopausal women. Furthermore, the fractional conversion of androstenedione to estrone is also increased with aging. This increase with both obesity and aging bears a striking relationship to the incidence of endometrial cancer, which is a disease primarily of elderly obese women. It is now generally accepted that the continuous production of estrogen by the adipose tissue in such women is a major causative factor in the etiology of this condition. Evidence is also accumulating suggesting a role of estrogen produced by adipose tissue in the pathogenesis of breast cancer. A number of studies have attempted to relate tumor site to either aromatase activity or expression (8, 9, 29, 34–37) in breast adipose tissue. In most cases (8, 9, 29, 34, 36), a direct relationship has been found between the presence of a tumor and aromatase expression in the tumor-bearing quadrant.

Previously, we have shown that the positive effect of aging on estrogen biosynthesis is due to a progressive increase in adipose tissue P450arom transcript levels in different body sites (buttocks, thighs, and abdomen) of normal women (26). Additionally, P450arom transcript levels were highest in the buttocks, followed by the thighs, and lowest in the abdomen. To gain insight into the factors that stimulate aromatase expression in adipose tissue, we recently studied the expression of the various exon-specific transcripts of P450arom in breast adipose tissue of cancer-free reduction mammoplasty patients and patients with breast cancer (29). We found that in breast adipose tissue of cancer-free patients, I.4-specific transcripts were present predominantly, whereas I.3-specific and PII-specific transcripts were present in lower copy numbers. Interestingly, in the breast adipose tissue of patients with cancer, promoter switching takes place. PII- and I.3-specific transcripts were present in high copy numbers compared to I.4-specific transcripts. In the present study, we observed that I.4-specific transcripts were present in highest copy numbers in adipose tissue of buttocks, abdomen, and thighs, whereas I.3-specific and PII-specific transcripts were present in low copy numbers. There was no difference in the expression pattern of exon-specific transcripts of P450arom with advancing age. Thus, it seems likely that the same promoters are being used for the expression of P450arom transcripts in adipose tissue regardless of the body site. This suggests that similar mechanisms of transcriptional regulation of the *CYP19* gene are involved in its expression in adipose tissue of the buttocks, thighs, abdomen, and breast of women as well as in the age-dependent increase in expression.

It is well established that a number of cytokines are produced by adipose fibroblasts, such as interleukin-6, leukemia inhibitory factor, and interleukin-11 (38). Furthermore, tumor necrosis factor-α is believed to be produced by adipocytes (39, 40). These factors have been shown to stimulate aromatase expression via promoter I.4 in the presence of glucocorticoids in adipose fibroblasts (30) Thus, when adipose fibroblasts are placed in culture in the presence of one of these stimulatory factors plus dexamethasone, most of the P450arom transcripts expressed contain I.4 at the 5′-terminus. On the other hand, when expression is stimulated by dibutyryl cAMP in the presence or absence of phorbol esters or by PGE_2, the transcripts present are those specific for PII and I.3. In fact, circulating IL-6 levels were shown to increase with advancing age (41). This explains at least in part why adipose tissue aromatase activity (25), P450arom transcript levels (26), and promoter I.4-specific transcripts (current study) also increase with age. We conclude that in normal human adipose tissue aromatase expression is mainly under local control by a number of cytokines via paracrine and autocrine mechanisms in the presence of systemic glucocorticoids.

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RECENT PROGRESS IN HORMONE RESEARCH, VOL. 52

Aromatase Expression in Health and Disease

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ABSTRACT

Family 19 of the P450 superfamily is responsible for the conversion of C_{19} androgenic steroids to the corresponding estrogens, a reaction known as aromatization, since it involves conversion of the Δ^4-3-one A-ring of the androgens to the corresponding phenolic A-ring characteristic of estrogens. Its members occur throughout the entire vertebrate phylum. The reaction mechanism of aromatase is very interesting from a chemical point of view and has been studied extensively; however, a detailed examination of structure-function relationships has not been possible due to lack of a crystal structure. Recent attempts to model the three-dimensional structure of aromatase have permitted a model that accounts for the reaction mechanism and predicts the location of aromatase inhibitors. The gene encoding human aromatase has been cloned and characterized and shown to be unusual compared to genes encoding other P450 enzymes, since there are a number of untranslated first exons that occur in aromatase transcripts in a tissue-specific fashion, due to differential splicing as a consequence of the use of tissue-specific promoters. Thus, expression in ovary utilizes a proximal promoter that is regulated primarily by cAMP. On the other hand, expression in placenta utilizes a distal promoter that is located at least 40 kb upstream of the start of transcription and that is regulated by retinoids. Other promoters are employed in brain and adipose tissue. In the latter case, class I cytokines such as IL-6 and IL-11 as well as TNFα are important regulatory factors. PGE_2 is also an important regulator of aromatase expression in adipose mesenchymal cells via cAMP and PGE_2 appears to be a major factor produced by breast tumors that stimulates estrogen biosynthesis in local mesenchymal sites. In all of the splicing events involved in the use of these various promoters, a common 3′-splice junction is employed that is located upstream of the start of translation; thus, the coding regions of the transcripts—and hence the protein—are identical regardless of the tissue site of expression; what differ in a tissue-specific fashion are the 5′-ends of the transcripts. This pattern of expression has great significance both from a phylogenetic and ontogenetic standpoint as well as for the physiology and pathophysiology of estrogen formation. Recently, a number of mutations of the aromatase gene have been described, which give rise to complete estrogen deficiency. In females this results in virilization *in utero* and primary amenorrhea with hypergonadotropic hypogonadism at the time of puberty. In men the most striking feature is continued linear bone growth beyond the time of puberty, delayed bone age, and failure of epiphyseal closure, thus indicating an important role of estrogens in bone metabolism in men. In both sexes the symptoms can be alleviated by estrogen administration.

1. Introduction

Estrogen biosynthesis is catalyzed by an enzyme known as aromatase P450 (Thompson and Siiteri, 1974; Mendelsohn *et al.,* 1985; Nakajin *et al.,* 1986; Kellis and Vickery, 1987). The biosynthesis of estrogens appears to occur throughout the entire vertebrate phylum including mammals, birds, reptiles, amphibians, teleost and elasmobranch fish, and *Agnatha* (hagfish and lampreys) (Callard *et al.,* 1978,1980; Callard, 1981). It has also been described in the protochordate *Amphioxus* (Callard *et al.,* 1984). To our knowledge, estrogen biosynthesis has not been reported in nonchordate animal phyla, although the aromatase P450 gene family appears to be an ancient lineage of P450 gene products, diverging as much as 10^9 years ago (Nelson *et al.,* 1993). In most vertebrate species that have been examined, aromatase expression occurs in the gonads and in the brain. This is true of the fish and avian species that have been examined as well as most mammals such as rodents. In many species, estrogen biosynthesis in the brain has been implicated in sex-related behavior such as mating responses and frequently a marked sexually dimorphic difference has been demonstrated. This is true, for example, in avian species in which the song of the male is important in courtship behavior (Hutchinson, 1991). In the case of humans and a number of higher primates, there is a more extensive tissue distribution of estrogen biosynthesis, since this also occurs in the placenta of the developing fetus as well as in the adipose tissue of the adult. The ability of the placenta to synthesize estrogen is also the property of a number of ungulate species such as cows, pigs, and horses. However, at least in cattle, there is no evidence of estrogen biosynthetic capacity in adipose, whereas in rodent species such as rats and mice, as well as in rabbits, neither adipose nor placenta has any ability to synthesize estrogens.

The physiological significance of estrogen biosynthesis in the placenta and adipose of humans is unclear at this time. The C_{18} steroid produced in each tissue site of biosynthesis is quite tissue specific. For example, the human ovary synthesizes primarily estradiol, whereas the placenta synthesizes estriol and adipose synthesizes estrone. This appears to reflect primarily the nature of the C_{19} steroid presented to the estrogen-synthesizing enzyme in each tissue site. Thus, in the case of adipose tissue, the principal source of substrate is circulating androstenedione produced by the adrenal cortex. In the case of the placenta, the major precursor is 16α-hydroxydehydroisoandrosterone sulfate derived as a consequence of the combined activities of the fetal adrenal and liver. Although the human placenta produces very large quantities of C_{18} steroids, particularly estriol, the physiological importance of this activity is unclear. This pertains because in pregnancies characterized by placental sulfatase deficiency, the placenta is essentially deprived of C_{19} substrate and hence synthesizes, relatively speaking, minute quantities of estrogen, yet such pregnancies are quite uncomplicated (France and

Liggins, 1969). At most, parturition is delayed by several days. Similarly, at this time no physiological significance has been attributed to estrogen biosynthesis by human adipose; however, the latter has been implicated in a number of pathophysiological conditions. Estrogen biosynthesis by adipose tissue not only increases as a function of body weight but as a function of age (Hemsell *et al.,* 1974; Edman and MacDonald, 1976) and has been correlated with the incidence of endometrial cancer as well as with postmenopausal breast cancer. Furthermore, evidence is accumulating to suggest that the estrogen implicated in the development of breast cancer is that which is produced locally within the adipose tissue of the breast itself (Miller and O'Neill, 1987). On the other hand, estrogen biosynthesis in adipose tissue may have beneficial consequences, since osteoporosis is more common in small, thin women than in large, obese women. While this may be, in part, the consequence of the bones of the latter being subject to load-bearing exercise, nonetheless it seems likely that the increased production of estrogens by the adipose of obese women is a significant factor.

II. Enzymology of Estrogen Biosynthesis

A. STRUCTURE-FUNCTION RELATIONSHIPS

As indicated above, the biosynthesis of estrogens is catalyzed by a microsomal member of the superfamily of enzymes known collectively as cytochrome P450, namely, aromatase P450 (P450arom, the product of the *CYP19* gene) (Thompson and Siiteri, 1974; Mendelson *et al.,* 1985; Nakajin *et al.,* 1986; Kellis and Vickery, 1987; Nelson *et al.,* 1993; Osawa *et al.,* 1987) (Fig. 1). This heme protein is responsible for binding the C_{19} steroid substrate and catalyzing the series of reactions leading to formation of the phenolic A ring characteristic of estrogens. Associated with the P450arom is a flavoprotein, NADPH-cytochrome P450 reductase (Simmons *et al.,* 1985), which is an essentially ubiquitous protein in the endoplasmic reticulum of most cell types and is responsible for transferring reducing equivalents from NADPH to any microsomal form of cytochrome P450 with which it comes into contact. Whereas the reductase may be the product of a single gene, cytochrome P450arom is a member of a flourishing superfamily of genes, namely the cytochrome P450 family, which contains at the present time over 300 characterized members belonging to 36 gene families (Nelson *et al.,* 1993). Within this, cytochrome P450arom is presently the sole member of gene family 19, designated *CYP19.* This designation is based on the fact that the C19 angular methyl group is the site of attack by oxygen.

Understanding the relationship of structure to function in cytochrome P450arom is of great interest not only because of the novelty of the complex series of reactions involved but also because of the importance of developing

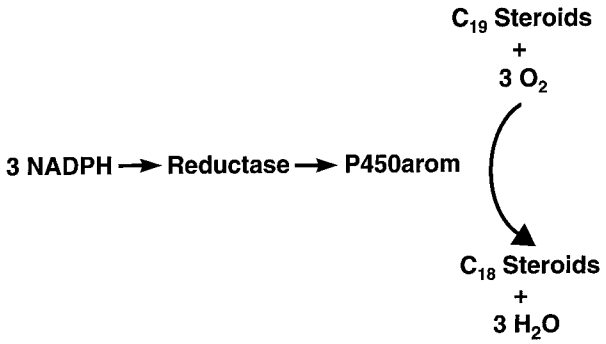

FIG. 1. The aromatase enzyme complex as it occurs in the endoplasmic reticulum of cells in which it is expressed. The aromatase reaction is catalyzed by aromatase P450 (P450arom), a member of the cytochrome P450 superfamily. Associated with it is a flavoprotein, NADPH-cytochrome P450 reductase, which is an essentially ubiquitous protein in the endoplasmic reticulum of most cell types and is responsible for transferring reducing equivalents from NADPH to any microsomal form of cytochrome P450 with which it comes into contact.

potent specific inhibitors of this enzyme for use in the management of breast cancer and other estrogen-related diseases. cDNA inserts complementary to messenger RNA encoding cytochrome P450 have been isolated and characterized from a broad range of vertebrates including several species of fish, namely rainbow trout (Tanaka *et al.,* 1992), catfish (Trant, 1994), and goldfish (Callard and Tchoudakova, 1996); an avian species, namely chicken (McPhaul *et al.,* 1988); and several mammalian species, including rat (Hickey *et al.,* 1990), mouse (Terashima *et al.,* 1991), human (Corbin *et al.,* 1988; Harada, 1988; Toda *et al.,* 1989; Pompon *et al.,* 1988), and bovine (Hinshelwood *et al.,* 1993). As indicated in Figure 2, the species showing greatest homology to the human is the bovine, with an amino acid sequence identity of 86%. The identities of the derived amino acid sequences of P450arom from rat, mouse, chicken, and trout to the human are 77%, 81%, 73%, and 52%, respectively. Interestingly, the variation between two species of teleost fish, namely goldfish and trout (GV Callard, personal communication), is almost as great as that between the trout and human, indicating the great range of evolutionary diversity that has occurred in teleost fish since their origins.

Cytochrome P450arom shares a number of structural features common to all P450 isoforms. Most notably, towards the carboxy terminus there is the heme-binding region that contains a totally conserved cysteine residue that serves as the fifth coordinating ligand of the heme iron. The fact that a thiolate ion is present at this site instead of the nitrogenous base most commonly found in other b-type

```
Trout                                                                MDLLSPVCGRVMAVVCLDT

Human       MVLEMLNPIHYNITSIVPEAMPAATMPVLLLTGLFLLVWNYEGTSSIPGPGYCMGIGPLISHGRFLWMGI 70
Bovine      *L**V***R***V**M*S*VV*I*SIAI*****FL******D******S*PL*******C*******
Rat         *F******M***V*IM***TV*VSA**L**IM**L**IR*C*SS*********L***************
Mouse       *F******MQ**V*IM***TVTVSA**L**IM**L**I**C*SS*********L***************
Chicken     *IP*T***LN*F-**L**DL**V**V*III*ICFLF*I**H*E************************V
Trout       VIADL*VSESR*A*ATRS*GISL**GSL***LC*L*AA*RHTDNN*V***FF*L*V***L*YL**I*T**

Human       GSACNYYNRVYGEFMRVWISGEETLIISKSSSMFHIMKHNHYSSRFGSKLGLQCIGMHEKGIIPNNNPEL 140
Bovine      ********KM*********VC***************V***S**I**********F**************A*
Rat         ********KM************************V*V***SN*I******R*********N********S*
Mouse       ********KM************************V***S**I******R*********N********S*
Chicken     *N******KT****V*********F********V**V***WN*V**************Y*N********AH
Trout       *T*S****SK**DIV****N****F*L*S**AVH*VLRQGR*T******Q**S****D*R*****S*MA*

Human       WKTTRPFFMKALSGPGLVRMVTVCAESLKTHLDRLEEVTNES-GYVDVLTLLRRVMLDTSNTLFLRIPLD 210
Bovine      **AV****T*************I**D*ITK********C*DL-*********M**I******M***G****
Rat         *R*V********T****I***E**V**I*Q*****GD**DN*-*****V**M*HI**********G****
Mouse       *R*I********T********E**V**I*Q*****G***DT*-*********M*HI******M***G****
Chicken     **EI****T***********IAI*V**TIV***K*****T*V-*N*N**N*M**I******K***GV***
Trout       **K**TY*A***T****QKT*D**VS*TQ****A*QGPDGLMG*Q****S**RCTVV*I**R***GV**N

Human       ESAIVVKIQGYFDAWQALLIKPDIFFKISWLYKKYEKSVKDLKDAIEVLIAEKRCRISTEEKLEECMDFA 280
Bovine      ******N***********L**********CR***********M*I******H****A****DSI***
Rat         **S**K******N*********N*********R***R*******E**I*VEK**QKV*SA****DS****
Mouse       *****K******N*********N*********R***R*******E*A**VEK**HKV**A****D*****
Chicken     *****L***N*********L**********C****EAA****G*M*I**EQ**QKL**V***D*H****
Trout       *KELLQ***K***T**TV*****VY**LD*IHE*HRRAAQE*E****S*VDQ**RGLQEAD**DHIN-*T

Human       TELILAEKRGDLTRENVNQCILEMLIAAPDTMSVSLFFMLFLIAKHPNVEEAIIKEIQTVIGERDIKIDD 350
Bovine      ****F*****E**********************V**********Q******R*****V****R***
Rat         ****F**R*****K*********************T*YV**L***EY*E**T**L***H**V*D***R*G*
Mouse       *D**F**R*****K*********************T*Y***L*V*EY*E**A**L***H**V*D*****E*
Chicken     SQ**F*QN*****A******V***M******L**T**I**I***DD*T***KMMR**E**M*D*EVQS**
Trout       AD**F*QSH*E*SA***R**V***V******L*I******L*LKQN*D**LQLLE**D*A**D*ELHNS*
                                             I

Human       IQKLKVMENFIYESMRYQPVVDLVMRKALEDDVIDGYPVKKGTNIILNIGRMHRLEFFPKPNEFTLENFA 420
Bovine      M*****V****N**********************************L*********************
Rat         V*N***V****N**L**********R**************************Y***********E
Mouse       **N***V****N*************R**************************Y***********E
Chicken     MPN**IV***************I*****Q**********************K**********S****E
Trout       L*N*R*L*S**N**L*FH****FT**R**S****S**R*P********M*****S***L*****S*D**E
                                                    II

Human       KNVPYRYFQPFGFGPRGCAGKYIAMVMMKAILVTLLRRFHVKTLQGQCVESIQKIHDLSLHP-DETKNML 490
Bovine      ****************************VV**********Q****R***KM**KN******-***RDR*
Rat         ***************S***********VV*****K********KR*I*NMP*NN*****L-**DSPIV
Mouse       ****************************VV********Q*****KR*I*N*P*KN******-N*DRHLV
Chicken     ****S*************V**F****************CR*Q*MK*RGLNN***NN***M**-I*RQPL*
Trout       **I*N*F*****S***S*V**H*******S******S*YS*CPHE*LTLDCLPQTNN**QQ*VE*EGEPH
                              III

Human       EMIFTPRNSDRCLEH 505
Bovine      **********K***R
Rat         *I**RHIFNTPF*QCLYISL
Mouse       *I**S*****KY*QQ
Chicken     **V****SPNKNQSD
Trout       T*K*L**HQA*KQS
```

FIG. 2. A comparison of the predicted amino acid sequences of P450arom from various species. The sequences of human (Corbin *et al.,* 1988), rat (Hickey *et al.,* 1990), mouse (Terashima *et al.,* 1991), chicken (McPhaul *et al.,* 1988), trout (Tanaka *et al.,* 1992), and bovine (Hinshelwood *et al.,* 1993) P450arom were derived as indicated. The *underlined sequences* are the putative membrane-spanning region (I), I-helix region (II), and heme-binding region (III). The numbering system for the amino acids (aa) begins at 1 for bP450arom. bP450arom is 503 aa in length, similar to human, mouse, and chicken; however, with two gaps for better alignment with the trout sequence, the numbering ends at 505 aa. The trout sequence has an additional 20 aa at the N-terminal.

cytochromes[1] is responsible for the unique spectrophotometric as well as catalytic properties of this family of heme proteins. Upstream of this domain there is a region of over 20 amino acids that is totally conserved in all P450arom species from chicken to human, except that in the bovine there is the replacement of isoleucine 399 with a leucine, a conservative change. Upstream of this there is another region of high conservation, namely, the portion of the I-helix that is believed to form the substrate-binding pocket proximal to the heme prosthetic group (Graham-Lorence *et al.,* 1991).

In a number of microsomal cytochrome P450 isoforms, the amino-terminus is characterized by a region of hydrophobic amino acids believed to comprise a membrane-anchoring domain. Curiously, in the case of P450arom, this is not apparent. The first 20 amino acids have no obvious extensive hydrophobic stretches. Rather, an N-linked glycosylation consensus sequence is present (NIT in the human) and P450arom of human placenta has been shown to have attached sugar residues (Sethumadhavan *et al.,* 1991; Shimozawa *et al.,* 1993; Chen *et al.,* 1993), although removal of these or mutation of the consensus sequence has little effect on activity (Sethumadhavan *et al.,* 1991; Amarneh *et al.,* 1993). Instead, the region that can be described as hydrophobic lies between amino acids 20 and 40. It seems likely that this region serves as the membrane-spanning domain and that the extra 20 amino acid extension containing the sugar residues projects into the luminal space (Shimozawa *et al.,* 1993; Amarneh *et al.,* 1993). The cytochrome P450arom clone derived from the rainbow trout has an extra 20 amino acid extension at the N-terminus upstream of the sequence that is common to all P450arom cDNAs (Tanaka *et al.,* 1992; Trant, 1994; Callard and Tchoudakova, 1996). The importance of this further extension is also unclear at this time. It should be noted that whereas removal of the first 10 amino acids of human P450arom has little effect on aromatase activity, removal of the first 20 amino acids causes greater than 95% activity loss (Amarneh *et al.,* 1993), implying that the region between residues 10 and 20 is in some way critical for conformational integrity. Curiously however, removal of the next 20 amino acids results in full restoration of activity (Amarneh *et al.,* 1993; Amarneh and Simpson, 1995), although further deletion results in permanent loss of activity.

For a number of years the only known three-dimensional structure of a cytochrome P450 was that of P450cam, the camphor-metabolizing enzyme of *P. putida,* whose structure was solved by Poulos and colleagues (1987) using x-ray diffraction. Application of this methodology to determining the structures of eu-

[1] Cytochromes were initially classified by Keilin into three groups designated a, b, and c, based on the relative positions of their characteristic α, β, and γ absorption bands. Additionally, b-type cytochromes have as their prosthetic group protoporphyrin IX, which is noncovalently bound to the protein.

karyotic P450s has been blocked because of the difficulty of solubilizing these proteins in the absence of detergent and their intransigence to crystallization. Since P450arom is no exception, structure-function studies of this enzyme were limited, until recently, to attempts to align putative structural domains with the corresponding regions of P450cam and using these alignments to predict residues that might be present in the substrate-binding pocket or otherwise implicated in the reaction mechanism.

The task of molecular modeling of P450arom has now been greatly facilitated by the solution of the three-dimensional structures of two more P450 cytochromes, namely P450BM3 (Ravichandran *et al.,* 1993) and P450terp (Hasemann *et al.,* 1994). While both of these proteins are bacterial, P450BM3 is the first member of class II whose structure is known (i.e., one that interacts directly with the flavoprotein NADPH-cytochrome P450 reductase, similar to P450arom) (Ravichandran *et al.,* 1993). Knowledge of this structure together with that of P450arom has allowed us to create new linear alignments of P450arom (Amarneh *et al.,* 1993; Graham-Lorence *et al.,* 1995), which we believe reflect quite accurately the boundaries of the various helical, β-sheet, and loop regions of P450arom. These alignments have, in turn, permitted the construction of a three-dimensional model of P450arom (Fig. 3). This model of P450arom was built

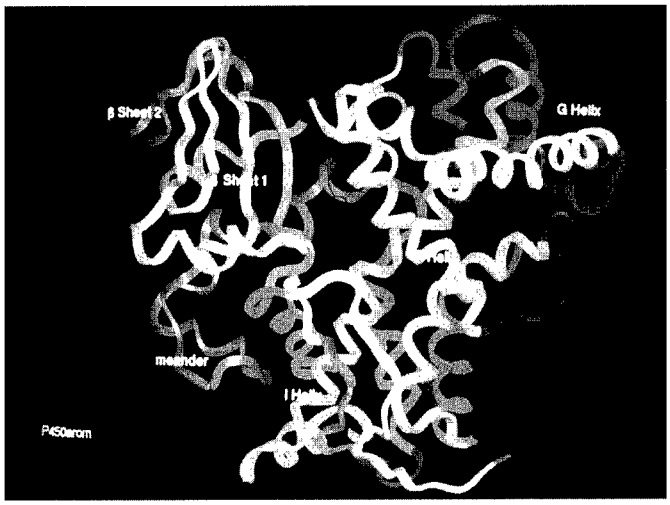

FIG. 3. The modeled structure of P450arom viewed from the side distal to the redox partner binding region. [Reprinted with permission from Graham-Lorence, S., Amarneh, B., White, R.E., Peterson, J.A., and Simpson, E.R. *Protein Sci.* **4,** 1065–1080, 1995.]

based on a "core structure" identified from the structures of the soluble, bacterial P450s rather than by molecular replacement, after which the less-conserved elements and loops were added in a rational fashion. Minimization and dynamic simulations were used to optimize the model and the reasonableness of the structure was evaluated. From this model we have postulated a membrane-associated hydrophobic region of aliphatic and aromatic residues involved in substrate recognition, a redox-partner binding region that may be unique compared to other P450s, as well as residues involved in active site orientation of substrates and inhibitors of aromatase (Fig. 4). Ultimately, however, such models are no substitute for solution of the actual structures. While this may be a long way from realization, several groups are presently seeking to express recombinant forms of P450arom as a first step to the isolation of large quantities of the protein.

B. STOICHIOMETRY AND REACTION MECHANISM

The aromatase reaction apparently utilizes three moles of oxygen and three moles of NADPH for every mole of C_{19} steroid metabolized (Thompson and Siiteri, 1974) (Fig. 5). Evidence is accruing that all three oxygen molecules are

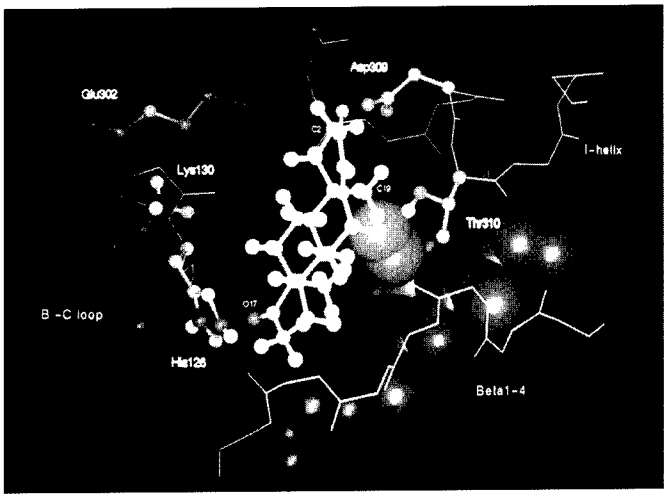

FIG. 4. Active site model of P450arom after 6 psec of dynamic simulation at 300K in vacuum followed by minimization. In this model, the heme is in a CPK representation. The conserved T310 along with D309 and E302 are indicated on the I-helix and H128 and K130 on the B′-C loop in ball-and-stick representations. [Reprinted with permission from Graham-Lorence, S., Amarneh, B., White, R.E., Peterson, J.A., and Simpson, E.R. *Protein Sci.* **4,** 1065–1080, 1995.]

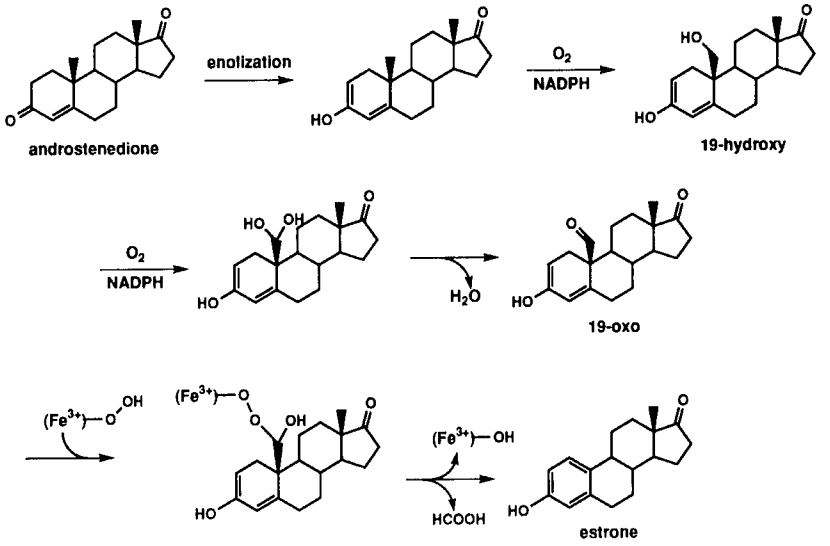

FIG. 5. An overall view of the reaction mechanism of aromatase showing the conversion of androstenedione to estrone.

utilized in the oxidation of the C_{19} angular methyl group to formic acid, which occurs concomitantly with the aromatization of the A ring to give the phenolic structure characteristic of estrogens (Cole and Robinson, 1988; Akhtar *et al.,* 1982).

There is general agreement that the first two oxygen molecules are utilized to oxidize the C_{19} angular methyl group by standard hydroxylation mechanisms (Fig. 5). However, work from several laboratories suggests that the most satisfactory mechanism to explain the third oxidative reaction is to postulate a peroxidative attack on the C19 angular methyl group (Cole and Robinson, 1988; Akhtar *et al.,* 1982), as indicated in Figures 5–7. This is predicated on the suggestion of Akhtar (Cole and Robinson, 1988; Aktar *et al.,* 1993) that when the substrate contains a carbonyl residue, then the peroxide intermediate of the cytochrome P450 is likely to be trapped by the substrate at the active site (Fig. 5) instead of breaking down to the iron-oxo intermediate characteristic of normal hydroxylation reactions: the theoretical basis for the peroxidative mechanism has been validated by Korzekwa and colleagues (1993), based on molecular orbital calculations and computer modeling. Thus, the steps necessary for converting the A-ring into a phenol derivative are complete.

As mentioned above, recently we constructed a molecular model of P450arom based on alignments with known bacterial P450 structures (Graham-Lorence *et al.,* 1995), and believe that it can explain the events in the catalytic

cycle of P450arom (Fig. 5). As shown in Figure 6, the first two hydroxylations at the C19 methyl group are as any other P450-catalyzed hydroxylation. In reaction [1] molecular oxygen is bound by the reduced heme iron forming a peroxide. It is believed that the conserved threonine (T310) aids in splitting the oxygen-oxygen bond, thus "activating" the oxygen by forming an iron-oxo intermediate [2] (Imai *et al.,* 1989). The threonine is immediately reprotonated by the neighboring acidic amino acid (D309) in this scheme. The activated oxygen on the iron will then abstract a hydrogen atom from a proximal carbon on the substrate, forming a carbon radical. Finally, the iron-bound hydroxyl radical will recombine with the carbon radical resulting in a hydroxylation at that carbon [3] (Gerber and Sligar, 1994). This occurs twice at the C19 methyl, first forming 19-OH, then 19-oxo androstenedione [4], with a gem-diol as an intermediate (Fig. 6).

After this sequence of reactions, enolization occurs at the 3-keto group as shown in Figure 7 (reaction 5). This involves the abstraction from the β-face of androstenedione (and either the α-or β-faces of testosterone) of the C2 hydrogen

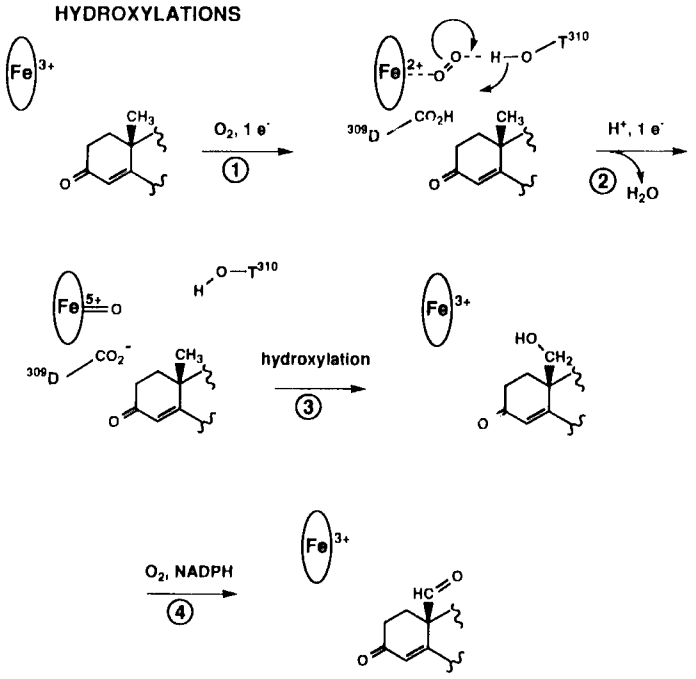

FIG. 6. Proposed mechanism of the aromatase reaction: the hydroxylation steps. This scheme shows the first and second hydroxylation steps demonstrating the involvement of D309 and T310 in oxygen activation.

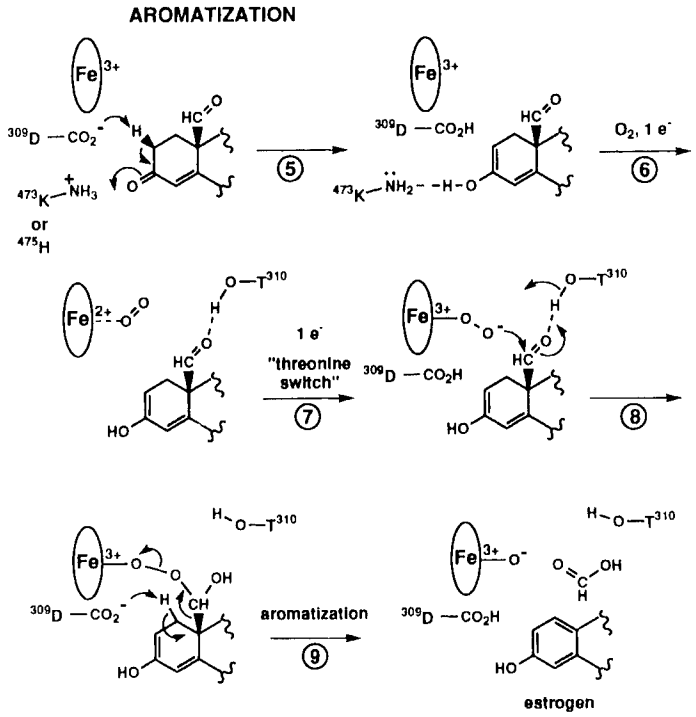

FIG. 7. Proposed mechanism of the aromatase reaction: the aromatization step and the "threonine switch." This scheme shows the enolization reaction with D309 abstracting the 2β-hydrogen and K473 or H475 as possible candidates for proton donation to the 3-keto. The aromatization reaction of the A ring of androstenedione is also shown in which the "threonine switch" promotes the peroxidative attack on C19, resulting in deformylation.

and the donation of a proton to the 3-keto group. Originally, we believed that the abstraction of the C2 hydrogen was accomplished by E302 (Graham-Lorence *et al.,* 1991); however, in the present model, it became obvious that E302 is too far from the heme-iron by at least one helical turn. We therefore suggest that this abstraction is accomplished by D309. We also suggest that either K473 or H475 from β-sheet 4 donates a proton to the 3-keto group, with K473 being the more likely candidate since it is highly conserved across species in P450arom.

The third oxygen activation step is unusual in that rather than being a hydroxylation, it is a peroxidative attack. The question is why? As shown in reactions [6] and [7] of Figure 7, we propose a "threonine switch" in which the threonine hydrogen bonds to the aldehyde polarizing the carbonyl double bond, decreasing electron density on the carbon atom and promoting attack of the peroxide anion. Additionally, since the Fe-O-O$^-$ was not protonated by T310, it is

more nucleophilic than it would have been as Fe-O-OH and thus more likely to attack the carbonyl group. Finally, during the peroxidative attack of the C19-oxo group, the adjacent acidic residue D309 abstracts a proton from the C1 position in [8] and [9], thus initiating deformylation of C19 and aromatization of the A ring. Thus the steps necessary for converting the A-ring into a phenolic derivative are complete. This is the only reaction in vertebrates capable of introducing an aromatic ring into a molecule.

III. The Human *CYP19* Gene

Some years ago, we and others cloned and characterized the *CYP19* gene that encodes human P450arom (Means *et al.,* 1989; Harada *et al.,* 1990; Toda *et al.,* 1990) (Fig. 8). The coding region spans nine exons beginning with exon II. Sequencing of RACE-generated cDNA clones derived from P450arom transcripts present in the various tissue sites of expression revealed that the 5′-termini of these transcripts differ from one another in a tissue-specific fashion upstream of a common site in the 5′-untranslated region (Means *et al.,* 1991; Kilgore *et al.,* 1992; Toda and Shizuta, 1993; Jenkins *et al.,* 1993). Using these sequences as probes to screen genomic libraries, it was found that these 5′-termini correspond to untranslated exons that are spliced into the P450arom transcripts in a tissue-specific fashion, due to the use of tissue-specific promoters. Placental transcripts contain at their 5′-ends untranslated exon I.1, which is located at least 40 kb upstream from the start of translation in exon II (Means *et al.,* 1991; Mahendroo *et al.,* 1991). This is because placental expression is driven from a powerful distal placental promoter, I.1, upstream of untranslated exon I.1. On the other hand,

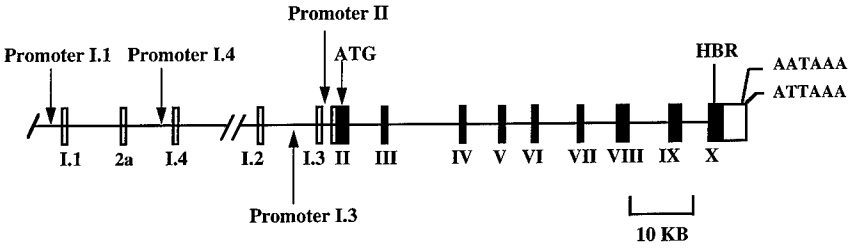

FIG. 8. Schematic representation of the human *CYP19* gene. The closed bars represent translated sequences. The septum in the open bar in exon II represents the 3′-acceptor splice junction for the untranslated exons. The sequence immediately to the left of the septum is that present in mature transcripts whose expression is driven by promoter II. The five untranslated exons, I.1, 2a, I.2, I.3, and I.4, are indicated in their approximate locations. Also shown are promoters I.1 and II and putative promoters I.2, I.3, and I.4. The heme-binding region (HBR) is indicated in exon X, as are two alternative polyadenylation signals that give rise to the two species of P450arom transcript of 3.4 and 2.9 kb. The genomic region shown spans a distance of at least 75 kb; however, as the gap between exons I.4 and I.2 has never been bridged, the true size is unknown.

transcripts in the ovary contain sequence at their 5′-ends that is immediately upstream of the start of translation. This is because expression of the gene in the ovary utilizes a proximal promoter, promoter II (Jenkins *et al.,* 1993). By contrast, transcripts in adipose tissue contain yet another distal untranslated exon, I.4, which is located in the gene 20 kb downstream from exon I.1 (Mahendroo *et al.,* 1993). Additionally, adipose tissue contains transcripts specific for promoter II as well as those containing exon I.3, which contains promoter II as part of the exonic sequence and shares common regulatory elements with promoter II. A number of other untranslated exons have been characterized by ourselves and others (Harada *et al.,* 1993; Toda *et al.,* 1994), including one specific for brain (Honda *et al.,* 1994). Splicing of these untranslated exons to form the mature transcripts occurs at a common 3′-splice junction that is upstream of the start of translation. This means that although transcripts in different tissues have different 5′-termini, the protein encoded by these transcripts is always the same, regardless of the tissue-site of expression; thus, there is only one human P450arom enzyme encoded by a single-copy gene.

IV. Expression of Aromatase in Human Ovary

As indicated previously, aromatase P450 is expressed in the pre-ovulatory follicles and corpora lutea of ovulatory women by means of a promoter proximal to the start of translation (PII) (Means *et al.,* 1991; Jenkins *et al.,* 1993). Aromatase expression in the granulosa cells of the ovary is primarily under the control of the gonadotropin FSH, whose action is mediated by cAMP. To understand how this transcription is controlled by cAMP, we constructed chimeric constructs containing deletion mutations of the proximal promoter 5′-flanking DNA fused to the rabbit β-globin reporter gene (Michael *et al.,* 1994). Assay of reporter gene transcription in transfected bovine granulosa and luteal cells revealed that basal and cAMP-stimulated transcription was lost upon deletion from −278 to −100 bp, indicating the presence of a functional response element in this region; however, no classical cAMP-responsive element was found. Mutation of a CAAGGTCA motif located at −130 bp revealed that this element is crucial for basal and cAMP-stimulated reporter gene transcription. When a single copy of this element was placed upstream of a heterologous promoter, it could act as a weak cAMP-response element. Electrophoretic mobility shift assay in the presence of specific antibodies and u/v-cross-linking established that Ad4BP/SF-1 binds to this hexameric element. SF-1 (Steroidogenic Factor-1) is an orphan member of the steroid hormone receptor gene superfamily that has been shown to be a critical developmental factor for the gonads as well as the adrenals (Luo *et al.,* 1994; Lala *et al.,* 1992). However deletion mutation analysis revealed the presence of another sequence upstream of the SF-1 site that was also critical for cAMP-responsiveness. This sequence, at −211/−202 bp, is TGCACGTCA,

identical to a canonical CRE save for the extra C. A similar element is present in the rat gene (Fitzpatrick and Richards, 1994). In human and rat genes, electrophoretic gel mobility shift and antibody super-shift analysis revealed that CREB binds to this site (Michael and Simpson, 1996; Fitzpatrick and Richards, 1994). These results are summarized in Figure 9. However, it is apparent that much remains to be elucidated regarding the mechanism whereby FSH and cAMP regulate aromatase expression in the ovary.

V. Expression of Aromatase in Human Placenta

As indicated previously, placental expression of aromatase in the human is driven from a powerful distal placental promoter I.1 upstream of untranslated exon I.1 that is located at least 40 kb upstream from the start of translation in exon II (Means *et al.*, 1991). At this time, the true distance is unknown since the genomic clones containing exon I.1 on the one hand and exon II on the other have never been overlapped. Employing various deletion mutations of the upstream flanking region of exon I.1, we and others have examined several putative regulatory sequences within this region and the proteins that interact with these sequences to regulate expression of aromatase in choriocarcinoma cells. Thus,

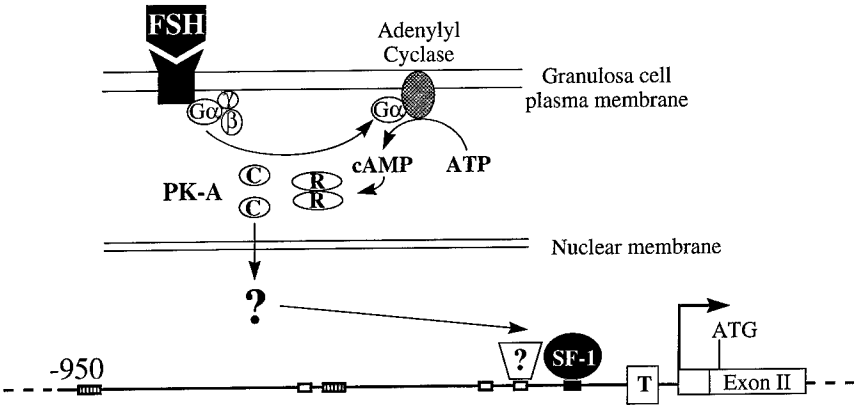

FIG. 9. Schematic of second-messenger signalling pathways whereby human aromatase gene expression is regulated in the ovary. FSH binds to its seven transmembrane domain receptor, which results in an activation of adenylyl cyclase via a $G_{\alpha s}$ activation pathway. This, in turn, results in activation of protein kinase A (PKA). Downstream of this events are unclear but the classical cAMP response element-binding protein (CREB) does appear to be involved. Steroidogenic Factor 1 (SF-1) and CREB and possibly other unknown proteins that bind to the CRE-like element (CLS) upstream of the SF-1 binding site act together to mediate basal and cAMP-responsiveness of *CYP19* gene expression from promoter II.

Toda and colleagues (1992) have identified a binding site for C/EBP-β that is located between −2141 and −2115 bp relative to the start of transcription in exon I.1. They further identified an element located between −238 and −200 bp that appears to synergize with the C/EBP-β element upstream. Yamada *et al.* (1995) identified two elements within −300 bp upstream of exon I.1 that recognize the same trans-acting factor that binds to the trophoblast-specific element previously located in the enhancer region of the human glycoprotein hormone α-subunit gene.

We have identified an imperfect palindromic sequence 5′-AGGTCATGCCCC-3′ located at −183 to −172 bp that is responsible for stimulation of aromatase expression by retinoic acids (Sun *et al.,* 1996). This does not function as a binding site for SF-1, since SF-1 is not expressed in placenta or in the cells; however, it does appear to bind a heterodimer comprising RXRα and VDR. It was reported recently that levels of RXR and RAR receptor expression increased during the process of cytotrophoblast differentiation into syncytiotrophoblasts in the placenta (Stephanon *et al.,* 1994). This is coincident with the increase in aromatase expression. These results suggests that retinoids may indeed play an important role in developmental regulation of aromatase gene expression in the placenta.

VI. Expression of Aromatase in Adipose Tissue

We have found that aromatase expression does not occur in adipocytes but rather in the stromal cells that surround the adipocytes and which may themselves be preadipocytes (Price *et al.,* 1992). These stromal cells grow in culture as fibroblasts. Consequently, we have employed these cells in primary culture as a model system to study the regulation of estrogen biosynthesis in adipose tissue (Ackerman *et al.,* 1981). When serum is present in the culture medium, expression is stimulated by glucocorticoids, including dexamethasone (Simpson *et al.,* 1981). Under these conditions, P450arom transcripts contain primarily untranslated exon I.4 at their 5′-ends (Mahendroo *et al.,* 1993; Zhao *et al.,* 1995a). We subsequently have characterized the region of the *CYP19* gene upstream of exon I.4 (Fig. 10) and have found it to contain a TATA-less promoter as well as an upstream GRE and an Sp1 sequence within the untranslated exon, both of which are required for expression of reporter gene constructs in the presence of serum and glucocorticoids (Zhao *et al.,* 1995a). Additionally, we found this region to contain a GAS (interferon-γ activating sequence) element. Such sequences are known to bind transcription factors of the STAT family (Darnell *et al.,* 1994; Schindler *et al.,* 1992; Zhong *et al.,* 1994).

We have studied aromatase expression in samples of adipose tissue obtained from women of various ages using competitive RT-PCR with an internal standard

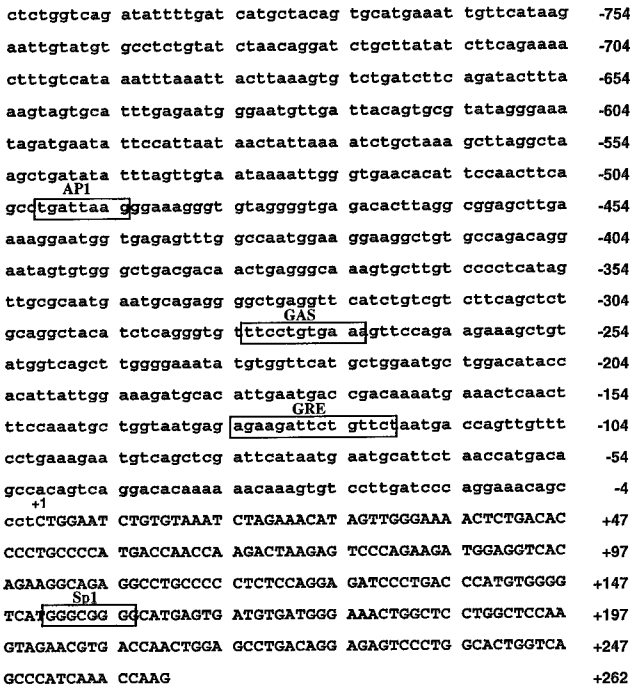

FIG. 10. Sequence of the *CYP19* gene upstream of and including exon I.4. The start of transcription, determined by primer extension and S1 nuclease analysis, is indicated as +1. Consensus binding sites, namely AP1, GAS, GRE, and Sp1, are shown in boxes. The nucleotides comprising exon I.4 are shown in capital letters.

and have found a marked increase in the specific content of P450arom transcripts in adipose tissue with increasing age (Bulun and Simpson, 1994), thus providing a molecular basis for the previous observation that the fractional conversion of circulating androstenedione to estrone increases with age (Hemsell *et al.,* 1974; Edman and MacDonald, 1976). Furthermore, there are marked regional variations in aromatase expression, with highest values being found in adipose from buttocks and thighs as compared with abdomen and breast (Michael and Simpson, 1996; Killinger *et al.,* 1987).

We also used this RT-PCR technique to examine regional variations in aromatase expression in breast adipose tissue and have found that highest expression occurs in adipose tissue proximal to a tumor, as compared to that distal to a tumor (Bulun *et al.,* 1993; Agarwal *et al.,* 1996). This is in agreement with previous observations regarding the regional distribution of aromatase activity within breast adipose (O'Neill *et al.,* 1988; Reed *et al.,* 1993) as well as an immunocytochemical study (Sasano *et al.,* 1994). These results suggest there is crosstalk

between a breast tumor and the surrounding adipose cells in terms of the ability of the latter to synthesize estrogens and that factors produced by developing breast tumors may set up local gradients of estrogen biosynthesis in the surrounding fat via paracrine mechanisms (Agarwal *et al.,* 1996; Simpson *et al.,* 1994).

VII. Cytokines That Stimulate Aromatase Expression in Adipose Tissue

Recently, we observed for the first time that the effect of serum to stimulate aromatase expression in human adipose stromal cells (in the presence of glucocorticoids) can be mimicked by specific factors, namely, members of the class I cytokine family, which includes interleukin-11 (IL-11), IL-6, oncostatin-M (OSM), and leukemia inhibitory factor (LIF) (Stahl *et al.,* 1994; Narazaki *et al.,* 1994). Members of this cytokine family employ a receptor system involving two different Jak-associated components, gp 130 and LIFRβ or a related β-component (Stahl and Yancopoulos, 1993). However, the IL-6 receptor complex includes a component whose cytoplasmic domain is apparently not involved in signalling (Stahl and Yancopoulos, 1993) and that can exist in a soluble form (Kishimoto *et al.,* 1992). Recently, an α-subunit of the IL-11 receptor complex has been cloned (Hilton *et al.,* 1994), although this does not apparently exist in a soluble form. The concentration dependence of the stimulation of aromatase by IL-6, IL-11, LIF, and OSM is indicative of high-affinity receptor binding.

Addition of class I cytokines to adipose stromal cells resulted in a rapid phosphorylation of Jak1 kinase (Zhao *et al.,* 1995b). By contrast, Jak3 kinase was not phosphorylated under these conditions to any significant extent, whereas Jak2 kinase was phosphorylated to an equal extent both in the presence or absence of IL-11. As indicated by blotting with an antiphosphotyrosine antibody and by inhibition in the presence of herbimycin A, this phosphorylation occurred on tyrosine residues present in the Jak1 kinase. Both gp130 and LIFRβ can associate with and activate at least three members of the Jak family, Jak1, Jak2, and Tyk2, but utilize different combinations of these in different cells (Stahl *et al.,* 1994); however, it is apparent that Jak1 is the kinase of choice in human adipose stromal cells.

This action results in the rapid phosphorylation of STAT3 on tyrosine residues but this was not the case for STAT1. Recently, it has been shown that STAT3 is the substrate of choice for the IL-6/LIF/OSM cytokine receptor family and that the specificity of STAT phosphorylation is based not upon which Jak kinase is activated (Zhong *et al.,* 1994; Stahl *et al.,* 1994; Boulton *et al.,* 1994) but rather is determined by specific tyrosine-based motifs in the receptor components, namely, gp130 and LIFRβ, shared by these cytokines (Stahl *et al.,* 1995). Finally, gel shift analysis indicated that STAT3 can interact with the GAS element present in the promoter I.4 region of the P450arom gene upon addition of IL-11 to these cells. This interaction, in turn, results in activation of expression, as indicated by

transfection experiments employing chimeric constructs in which the region -330/+170 bp of the I.4 promoter region was fused upstream of the CAT reporter gene. The results indicate that both deletion of the GAS sequence as well as mutagenesis of this sequence resulted in complete loss of IL-11- and serum-stimulated expression in the presence of glucocorticoids.

Activation of this pathway of expression by these cytokines is absolutely dependent on the presence of glucocorticoids. This action of glucocorticoids is mediated by the GRE element downstream of the GAS element (Zhao *et al.,* 1995a). Additionally, the Sp1-like element present within untranslated exon I.4 also is required, at least for expression of the −330/+170 bp construct (Zhao *et al.,* 1995a). These sequences, while present within a 400 bp region of the gene, are not contiguous and the nature of the interaction between STAT3, the glucocorticoid receptor, and Sp1 to regulate expression of the P450arom gene via the distal promoter I.4 remains to be determined. Our present understanding of the regulation of expression of aromatase in adipose tissue is summarized in Figure 11.

Recently, we have found that TNFα also stimulates aromatase expression in adipose stromal cells in the presence of dexamethasone. This action of TNFα is mimicked by ceramide, indicative that sphingomyelinase activity is involved in the TNFα response. This action of TNFα appears to involve promoter I.4, specifically, an AP1 site upstream of the GAS element that binds a c-jun/fos heterodimer upon activation by TNFα (Zhao *et al.,* 1997).

VIII. Mesenchymal-Epithelial Interactions in Regulation of Aromatase Expression in Adipose Tissue

As indicated previously, adipose tissue is the major site of estrogen biosynthesis in elderly women and men. The fact that this expression is confined to the stromal cells rather than the adipocytes themselves is consistent with the known actions of IL-6, IL-11, and TNFα to inhibit the differentiation of 3T3 L1 fibroblasts into adipocytes (Keller *et al.,*1993). Thus, aromatase is a marker for the undifferentiated fibroblast state. As indicated previously, aromatase expression in adipose increases dramatically with age (Bulun and Simpson, 1994; Grodin *et al.,* 1973). There is also a marked regional distribution with expression being greatest in buttock and thigh regions as compared to abdomen and breast (Bulun and Simpson, 1994; Price *et al.,* 1992). However, within the breast, there is also a marked regional variation with expression being highest at sites proximal to a tumor as compared to those distal to a tumor (Bulun *et al.,* 1993; Agarwal *et al.,* 1996).

Recently, we developed a competitive RT-PCR technique to measure the levels of the various P450arom transcripts in adipose tissue. We found that in breast, abdomen, buttocks, and thighs of healthy subjects, I.4-containing tran-

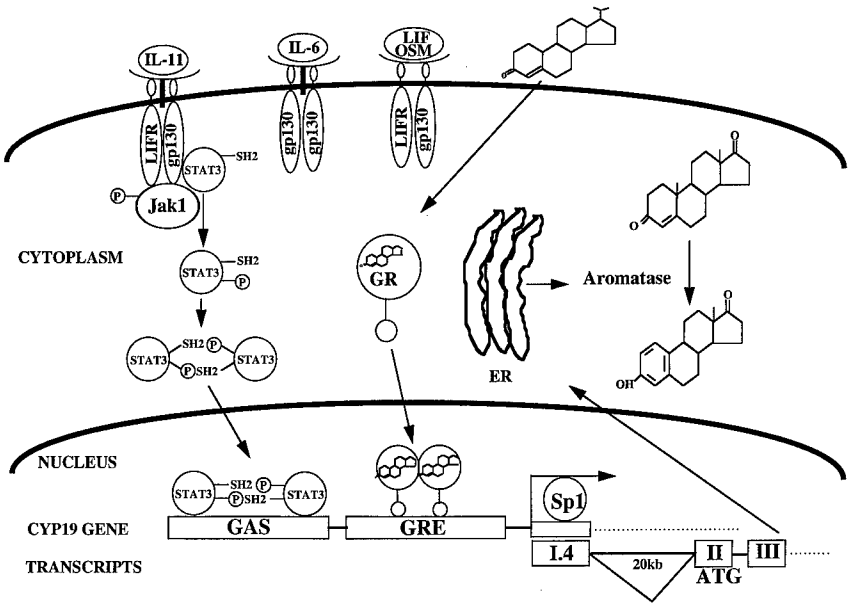

FIG. 11. Schematic of second-messenger signalling pathways whereby class I cytokines stimulate aromatase gene expression in human adipose stromal cells. Jak1 kinase is bound to the common receptor subunit gp 130 and activated following ligand binding and receptor dimerization, as a consequence of phosphorylation on tyrosine residues. STAT3 is recruited to binding sites on gp130 and is phosphorylated on tyrosine residues by Jak1. These phosphotyrosine residues are recognized by SH2-homology domains on STAT3, resulting in dimerization followed by translocation to the nucleus and binding to the GAS element of promoter I.4 of the aromatase gene. Following binding of glucocorticoid receptors to the GRE and Sp1 to its site on untranslated exon I.4, activation of transcription of the aromatase gene from promoter I.4 is initiated. Splicing of the initial transcript results in formation of mature mRNA, which translocates to the ribosomes and is translated to give rise to aromatase protein.

scripts predominated, with I.3- and II-specific transcripts in much lower abundance (Agarwal *et al.,* 1997). Based on these findings, we suggest that aromatase expression in adipose tissue may be under tonic control by circulating glucocorticoids and that regional and age-dependent variations may be the consequence of paracrine and autocrine secretion of stimulatory cytokines such as IL-6 and TNFα, the levels of which have been shown to increase with age (Wei *et al.,* 1992; Daynes *et al.,* 1993).

To our surprise, however, we found that the increase in aromatase expression in a tumor-containing breast was not due to an increase in I.4-specific transcripts but rather of transcripts specific for promoters II and I.3 (Agarwal *et al.,* 1996). Similar results have been obtained by Harada and colleagues (1993). Since ex-

pression from these promoters is regulated by cAMP, these results strongly suggest that breast tumors secrete a factor(s) that stimulates aromatase expression in the surrounding stroma by increasing adenylate cyclase. Our recent evidence suggests that this factor is PGE_2 (Zhao *et al.,* 1996). PGE_2 is a powerful stimulator of aromatase expression via both the PKA and PKC pathways. Moreover PGE_2 is known to be produced by breast tumor fibroblasts and epithelium as well as by macrophages recruited to the tumor site (Schrey and Patel, 1995).

Such local paracrine mechanisms could be important in the stimulation of breast cancer growth by estrogens. Commonly, breast tumors produce a desmoplastic reaction whereby there is local proliferation of stromal cells surrounding the tumor, strongly indicative of the production of growth factors by the tumor. These proliferating stromal cells express aromatase, as indicated by immunocytochemistry (Sasano *et al.,* 1994). It is possible then to propose a positive feedback loop whereby adipose stromal cells surrounding a developing tumor produce estrogens that stimulate the tumor to produce prostanoids, growth factors, and cytokines (Dickson and Lippman, 1987). Some of these act to stimulate the further growth and development of the tumor in a paracrine and autocrine fashion. Additionally, these or other factors act to stimulate proliferation of the surrounding stromal cells and expression of aromatase within these cells. Thus, a positive feedback loop is established by paracrine and autocrine mechanisms, which leads to the continuing growth and development of the tumor (Fig. 12).

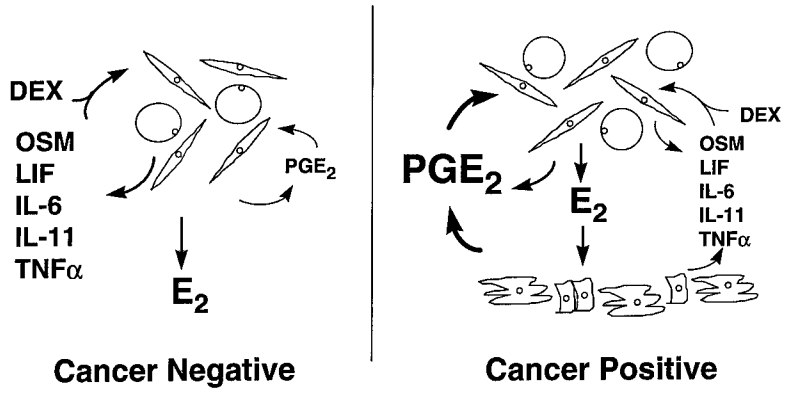

FIG. 12. Proposed regulation of aromatase gene expression in breast adipose tissue from cancer-free individuals and from those with breast cancer. In the former case, expression is stimulated primarily by class I cytokines or TNFα produced locally, in the presence of systemic glucocorticoids. As a consequence, promoter I.4-specific transcripts of aromatase predominate. In the latter case, PGE_2 produced by the tumorous epithelium, tumors fibroblasts, and/or macrophages recruited to the tumor site is the major factor stimulating aromatase expression, as evidenced by the predominance of promoter II-specific transcripts of aromatase.

IX. Aromatase Expression in Other Tissues

Stimulation of aromatase expression by serum in the presence of glucocorticoids is not confined to cells present in adipose tissue but also has been reported in skin fibroblasts (Berkovitz *et al.,* 1989) and in hepatocytes derived from fetal liver (Lanoux *et al.,* 1985). In each of these cell types, the P450arom transcripts contain exon I.4 as their 5′-terminus (Toda *et al.,* 1994; Harada, 1992); however, the factors that mimic the action of serum to stimulate aromatase expression in these cell types have as yet to be elucidated. Clones from intestine also contained predominantly exon I.4, as did a few from brain. Harada *et al.* (1993) also reported that a sequence identical to the one we call I.4 was present in transcripts from fetal liver and brain. Sequence-specific northern and PCR analysis of fetal testes and ovary and Sertoli cell tumors obtained from patients with Peutz-Jegher Syndrome, a condition characterized by the presence of estrogen- producing bilateral multifocal sex cord tumors, revealed mainly promoter II-specific sequence, similar to the situation in adult ovary (Bulun *et al.,* 1993). Interestingly, in an estrogen-secreting hepatocellular carcinoma, aromatase expression was driven by promoter II in contrast to the situation in fetal liver or in adult liver where expression is undetectable (S.E. Bulun, unpublished observations). No exon I.1-specific sequences (the predominant sequence in placenta) were present in any clones isolated from these libraries.

A major finding in the last few years was the discovery of a new exonic sequence expressed in the brain of rat, monkey, and human (Honda *et al.,* 1994; Mouri *et al.,* 1995). This "brain-specific" sequence is the major 5′-terminus of transcripts in rat amygdala and is also present in transcripts in the hypothalamus-preoptic area (HPOA) (Kato *et al.,* 1996). Regulation of aromatase in brain differs from that in other tissues in that expression appears to be increased by androgens and either suppressed or not affected by cAMP (Lephart *et al.,* 1992). In cultured cells derived from mouse embryonic hypothalamus, aromatase expression is elevated by α1-adrenergic agonists but not those selective for α2- or β-adrenergic receptors. Substance P, cholecystokinin, neurotensin, and brain natriuretic peptide as well as phorbol esters and Bt_2 cGMP all increased aromatase expression, suggesting a major role of PKC and PKG pathways in this regulation, which is presumably mediated via the brain-specific promoter (Abe-Dohmae *et al.,* 1996).

However, promoter II-specific transcripts have also been detected in amygdala and HPOA regions (Kato *et al.,* 1996) and as mentioned above, I.4-specific transcripts have also been detected in brain (Toda *et al.,* 1994). So it may be that different promoters are employed in the various brain loci of expression and that consequently the regulation is quite different in different brain sites. It should also be noted that transcripts derived from the brain-specific promoter have been detected by RT-PCR in other nonneural cells, namely, ovary, placenta, and THP-

1 cells (M. Shozu, unpublished observations), although these were present in low abundance in these sites.

X. Mutations of the Human *CYP19* Gene

Given the size and complexity of the human *CYP19* gene, it is surprising that, until recently, no mutations of the gene had been identified, although deficiencies of most of the other steroidogenic forms of cytochrome P450 have been well characterized. The possibility was considered that a complete absence of aromatase activity could be lethal, perhaps because of a need for estrogens at the time of implantation of the blastocyst. However, in spite of this, a number of definitively characterized cases of aromatase deficiency have recently been described. In 1991, Shozu *et al.* reported a case of female pseudohermaphroditism secondary to aromatase deficiency in a Japanese infant and biochemical and molecular genetics studies were conducted by Harada *et al.* (1992a) to characterize the molecular basis of this deficiency. They found that the consensus 5′-splice acceptor sequence in intron 6 was mutated from GT to GC, resulting in the use of a cryptic splice acceptor site further upstream in intron 6. This resulted in the incorporation of 87 bases from intron 6 that were translated into an additional 29 amino acids inserted into the middle of the mature polypeptide. The patient was homozygous for this condition and the parents were obligate heterozygotes (Harada *et al.,* 1992b). The second case was an 18-year-old American woman who presented with primary amenorrhea and sexual infantalism and cystic ovaries (Ito *et al.,* 1993). At birth she was noted to have ambiguous external genitalia. This individual turned out to be a compound heterozygote with a different single-base change on each allele, bp 1303 (C-T) and bp 1310 (G-A), both in exon X, which resulted in codon changes of R435C and C437Y, respectively. Both these residues are in the heme binding region, cysteine-437 being the conserved cysteine comprising the fifth coordinating ligand of the heme iron and arginine-435 being a highly conserved residue, two amino acids upstream of this cysteine, which our modelling studies (Graham-Lorence *et al.,* 1995) predict to interact with one of the propionic acid residues of the heme (Ito *et al.,* 1993).

Recently, two more women and two men with aromatase deficiency have been described (Morishima *et al.,* 1995; Qin *et al.,* 1996; Forest *et al.,* 1996). The women, one American and one French, have symptoms similar to those described above, which can now be regarded as typical for the condition. In the case of the men, one of whom was American (the brother of the second American woman) and one Italian, their development was unremarkable through puberty. However, cessation of linear growth never occurred and by the time they were over 25, they were well over 6 feet in height. This was a consequence of failure of epiphyseal fusion and both had marked osteopenia and lack of bone mineralization. These symptoms are very similar to those described in a male subject

with estrogen receptor deficiency (Smith *et al.,* 1994) and point to a previously unrecognized role of estrogen in bone mineralization in men. They also had high circulating gonadotropins and testosterone, suggesting that estrogens (presumably arising in the brain due to conversion of circulating testosterone) have an important role in negative regulation of gonadotropin release.

Thus, it is apparent that individuals with mutations in P450arom do exist and estrogens do not apparently have a critical role to play in fetal development in the human; however, they do have some unsuspected roles in postnatal development. Based on the structure of the gene, it is possible to predict not only defects due to mutations in the coding region but also tissue-specific defects caused by mutations in specific promoter regions as well as in splice junctions. Thus, for example, a mutation in the 3′-splice site upstream of the start of translation should permit normal expression in the ovary but prevent placental expression. It remains to be determined if, in fact, such conditions are a reality.

XI. Ontogeny and Phylogeny of Aromatase Expression

Comparison of the sequences of the P450arom cDNAs with those of other members of the cytochrome P450 superfamily has led to the conclusion that P450arom is only distantly related to other steroidogenic forms of P450 and indeed is one of the most ancient of the cytochrome P450 lineages, apparently evolving more than 1,000 Myr ago (Nelson *et al.,* 1993). Certainly, aromatase is present throughout the entire vertebrate phylum but to our knowledge has not been described in invertebrates. It would be of considerable interest therefore to know what reactions the ancestral gene product catalyzes in nonvertebrate phyla.

Inactivation of the SF-1 gene in mice by means of homologous recombination has indicated that this transcription factor is a critical developmental factor for the gonads as well as the adrenals, since these organs fail to develop in such animals (Luo *et al.,* 1994). In the normal embryonic mouse, SF-1 expression is first detected in the genital ridge around embryonic day 9 (Ikeda *et al.,* 1994). It subsequently is expressed in the developing adrenal cortex. In genotypic males, testicular development is initiated following a burst of SRY expression on embryonic day 11.5 and subsequently the testes express SF-1 in both the Leydig cells and in the Sertoli cells. In females, SF-1 is subsequently expressed in the developing ovary. This developmental expression of SF-1 in the embryonic mouse correlates well with the expression of steroidogenic enzymes as well as of anti-müllerian hormone in the case of the male. In the mouse embryo, SF-1 is also expressed in the cells that give rise to the hypothalamus around embryonic day 11. As the hypothalamus develops, SF-1 is expressed in the ventral medial region (VMH). Subsequent to this, aromatase expression in the hypothalamus is detectable and increases with embryonic development, reaching a maximum just prior to the onset of gestation (Ikeda *et al.,* 1995).

An interesting question then arises as to the role of SF-1 in regulating the developmental expression of aromatase in the brain. The brain-specific promoter of the human aromatase gene contains an SF-1-like sequence in the intron immediately downstream from the brain-specific 5′-untranslated exon (Honda *et al.,* 1994). It remains to be determined if this element plays an important role in the expression of aromatase in this region of the brain. Aromatase is also expressed in other regions of the brain, including the amygdala and preoptic nucleus. It is not clear whether the brain-specific promoter is responsible for expression of aromatase in all of these regions of the brain, since promoter II- and I.4-specific transcripts have also been detected in various brain sites (Toda *et al.,* 1994; Kato *et al.,* 1996).

Although little is known about expression of the aromatase gene in lower vertebrates, recently the sequence of an aromatase gene from the rainbow trout was published (Tanaka *et al.,* 1995). In this case, the sequence immediately upstream from the start of transcription contained two sites with close homology to the mammalian SF-1 site, probably indicating that this is the promoter region for aromatase in the gonads of fish. Thus, not surprisingly, this proximal promoter is a primordial promoter of aromatase throughout the entire vertebrate phylum. In lower vertebrates such as fish, aromatase is also expressed to a very high level in the brain (Callard *et al.,* 1978, 1980; Callard, 1981). Recently, it has been shown that in the goldfish, two transcripts for aromatase exist, one in ovary and one in brain, that differ significantly throughout the entire coding region, indicative of the presence of two separate genes (Callard and Tchoudakova, 1996). Thus, the question of the evolution of the human gene with a single coding region but with multiple untranslated first exons becomes a very interesting and complex issue. Another variant on this theme occurs in the pig (Corbin *et al.,* 1995), in which placental transcripts differ from those in ovary at several regions in the coding region. This is consistent with one gene with alternatively spliced coding exons. Thus, the phylogenetic evolution of the aromatase gene will likely turn out to be very complex.

It is clear that in most vertebrates aromatase expression is confined to the brain and the gonads. However, as indicated previously, in a number of mammals—including primates and some ungulate species such as cow, pig, and horse—expression also occurs in the placenta. In all of these species, it appears that a unique placental-specific distal promoter is employed (Hinshelwood *et al.,* 1995). However, there appears to be little sequence homology between the placental promoter regions of the human and bovine genes, making it difficult to propose that these arose from a common ancestral sequence. Furthermore, the creation of transgenic mice expressing a reporter gene downstream of the human promoter I.1 sequence has shown that expression of the reporter occurs in the mouse placenta and only at that site (Graves *et al.,* 1996). Since mouse placenta does not express endogenous aromatase activity, this means that, nevertheless,

the requisite regulatory proteins and transcription factors are present in the mouse placenta.

Since SF-1 is apparently not expressed in placenta, this may explain why the proximal promoter of aromatase is not employed in this fetal tissue but rather a distal promoter is used that is regulated by mechanisms totally independent from those involving SF-1. Thus, whereas SF-1 may play a critical role in both the phylogenetic and ontogenetic regulation of aromatase expression, the diversity of promoters of aromatase found in a number of mammalian species may reflect, in part, the need for alternative means of regulating aromatase gene expression in tissues where SF-1 is not present or else is not functional.

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DISCUSSION

Etienne Baulieu: IL-6 is increasing in the blood of aging people. You indicated correlation of aromatase activity in four tissues and with age and mentioned IL-6's effect on aromatase function. Is there any *in vivo* correlation of IL-6 level and aromatase activity in the four tissues?

Evan Simpson: Certainly there is evidence that IL-6 levels increase with age. In that sense there is a correlation with the age-dependent changes in aromatase.

John Funder: In the patient you showed with aromatase reactivity, the spermatic vein levels of E_2 were 720 but presumably the gonadotropin levels were affected by the very high peripheral E_2. In that case there needs to be increased testicular conversion, as well as peripheral, to account for the difference between the spermatic vein and general peripheral levels, so that the defect may be general, rather than merely peripheral?

Evan Simpson: At this time we cannot rule out the possibility that the problem may be more general than simply occurring in fibroblasts. But equally the spermatic vein levels can reflect testicular secretion together with the circulating levels.

Yoav Gothilf: Are the different aromatase transcripts translated at the same rate?

Evan Simpson: We have not addressed this issue but it should be done, as the rates of translation could well be different.

Jerome Strauss: Since the fat expresses leptin receptors and also produces leptin and the leptin receptor appears to use an IL-6 receptor-like signaling system, it is intriguing to consider the possibility that leptin is a regulator of adipose tissue aromatase. Have you looked at this?

Evan Simpson: We considered this and obtained leptin from Amgen to test this but unfortunately leptin had little action on aromatase expression in adipose stromal cells, whether or not glucocorticoids were present.

Jerome Strauss: The hormonal profiles in pregnant women bearing an aromatase-deficient fetus are relatively unique in that estrogens are low and androgens are elevated. Did the pregnancy yielding the aromatase-deficient subjects end at birth with normal spontaneous labor and delivery?

Evan Simpson: As in the case with placental sulfatase deficiency, the progress of the pregnancies was relatively unremarkable.

Cynthia Bethea: Nursing decreases incidence of breast cancer, so does prolactin play a role in aromatase activity?

Evan Simpson: No evidence has been seen for a direct effect of prolactin on aromatase expression of adipose stromal cells.

Susan Leeman: Was there any evidence of vascular proliferation surrounding the breast tumor tissue that you studied?

Evan Simpson: I am not aware that vascular proliferation occurred but I am afraid I can't give you a definitive answer.

Aidan McElduff: Your data showed that aromatase activity increased with increasing obesity and that the activity was predominantly on the tumor. Can you comment on what this tells us about fat cell recruitment with acquired obesity?

Evan Simpson: It would imply that the number of stromal cells increases with obesity.

Bert O'Malley: Tell us more about bone aromatase and its contribution to conversion of androgen to estrogen in men.

Evan Simpson: Aromatase activity has been reported in cells with osteoblastic and osteoclastic properties. Given the role of estrogen in bone mineralization in men, this may be an important site of synthesis.

Bert O'Malley: Why do some recent reports of aromatase inhibitors reveal minimal effects on bone density of animals?

Evan Simpson: I am not sure I know the studies to which you refer. Certainly the estrogen receptor knockout mouse displays problems with bone mineralization.

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Endocrine Disorders Associated with Inappropriately High Aromatase Expression

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Aromatase P450 ($P450_{arom}$) is responsible for conversion of C_{19} steroids to estrogens in a number of human tissues, such as the placenta, gonads, adipose tissue, skin and the brain. Aromatase expression in human tissues is regulated by use of alternative promoters in the placenta (promoter I.1), adipose tissue (promoters I.4, I.3 and II) and gonads (promoter II). Aromatase expression is absent in the disease-free adult liver, adrenal and uterine tissues. Excessive or inappropriate aromatase expression in adipose fibroblasts and endometriosis-derived stromal cells, as well as in testicular, hepatic, adrenal and uterine tumors, is associated with abnormally high circulating estrogen levels and/or with increased local estrogen concentrations in these tissues. Whether systemically delivered or locally produced, elevated estrogen levels will in turn promote the growth of hormone-responsive tissues. We recently studied aromatase expression in testicular tumor and adipose tissue samples from prepubertal boys with gynecomastia, in hepatocellular cancer and adrenocortical tumor samples from adult men with gynecomastia, in breast adipose tissue samples proximal to breast tumors, and in endometrial cancer, leiomyoma and endometriosis tissues. Excessive aromatase activity and $P450_{arom}$ transcript levels were found in these tissue samples or in cultured cells derived from these tissues. In these neoplastic or non-neoplastic tissues or cells, the regulation of aromatase expression was studied in terms of alternative promoter use, both *in vivo* and in response to various hormonal stimuli. Our results were suggestive of a common metabolic abnormality associated with activation of a cyclic AMP-dependent signalling pathway that gives rise to transcriptional transactivation of aromatase expression via promoters I.3 and II in all of the above tissues. This article describes the common pathophysiological and molecular features of excessive aromatase expression in these disease states. © **1997 Elsevier Science Ltd**

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INTRODUCTION

Estrogen has been implicated in the pathophysiology of a number of disease states, including breast tumors, endometrial cancer, uterine leiomyomas, endometriosis and gynecomastia [1]. Whether estrogen plays a role in carcinogenesis itself is uncertain. However, therapeutic strategies designed to block estrogen production or its action on the target tissues have been reasonably successful in controlling tissue growth in the above conditions. Estrogens are synthesized in a number of human cells and tissues, such as ovarian granulosa cells, the placental syncytiotrophoblast, adipose and skin fibroblasts and the brain [2]. The C_{19} steroids, androstenedione, testosterone and 16α-hydroxyandrostenedione are converted to estrone, estradiol-17β and estriol by aromatase P450 ($P450_{arom}$), the product of the *CYP19* gene [2]. Tissue-specific promoters regulate aromatase expression in the ovary (promoter II) and placenta (promoter I.1) via alternative splicing mechanisms (Fig. 1). Promoter use in adipose fibroblasts in culture, on the other hand, appears to be a function of various hormonal treatments [2]. For example, cyclic AMP ana-

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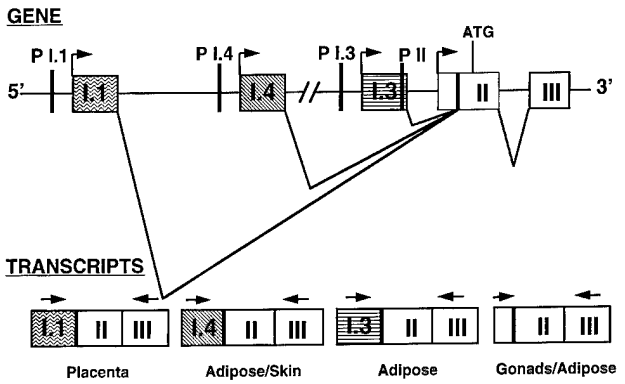

Fig. 1. Structure of the human *CYP19* ($P450_{arom}$) gene upstream of the translation start site. The region encoding the $P450_{arom}$ protein contains nine exons (II–X), of which only exons II and III are shown. Exon II contains the translation start site (ATG). A number of promoters (I.1, I.4, I.3 and II) direct aromatase expression in a tissue-specific fashion. Untranslated first exons immediately downstream of these promoter regions are expressed and encode the 5′-untranslated terminus of $P450_{arom}$ mRNA in placenta (I.1) and adipose tissue (I.4, I.3 and II). These are spliced into the identical site upstream of the translation initiation site. In the ovary, promoter II is used, and transcription is started 120 bp upstream of the translation initiation site in exon II. Please note that the coding region is identical in each transcript regardless of the promoter used or splicing pattern. Therefore, the encoded protein is identical in each case.

logues give rise to the use of promoters I.3 and II, whereas class I cytokines in the presence of glucocorticoids switch promoter use to I.4. Use of each promoter gives rise to a population of $P450_{arom}$ mRNA species with a unique untranslated 5′-terminus (Fig. 1). In the following examples of excessive aromatase expression in a number of neoplastic and non-neoplastic tissues, promoter use was analysed using various techniques such as reverse transcriptase–polymerase chain reaction (RT–PCR)/Southern hybridization and exon-specific northern blotting.

We have recently studied the origin of estrogen in a number of endocrinopathies associated with excessive estrogen production. Aromatase expression and its regulation were characterized in Sertoli cell tumors of Peutz–Jeghers syndrome, in adipose and skin fibroblasts of a boy with excessive peripheral aromatization, in samples of feminizing hepatocellular and adrenocortical tumors, in adipose fibroblasts proximal to a breast cancer, in the upper and outer regions of cancer-free breasts, in endometrial cancer, in smooth muscle cells of uterine leiomyomas and in stromal cells of endometriosis. First, these studies will be described briefly. Then, common molecular and cellular mechanisms of estrogen biosynthesis in these disorders will be discussed.

PREPUBERTAL GYNECOMASTIA

Prepubertal gynecomastia is a rare disorder. The largest group of publications that describe a defined etiology of this disorder is that of estrogen-secreting testicular Sertoli cell tumors associated with Peutz–Jeghers syndrome [3–5]. Other prepubertal boys have been described in which estrogen excess was caused by increased conversion of C_{19}-steroids (primarily androstenedione) to estrone or estrone sulfate in extraglandular tissues [6, 7]. We recently studied gonadal sex cord tumors from several patients with Peutz–Jeghers syndrome, and adipose biopsy samples from a boy with excessive peripheral aromatization:

Sertoli cell tumors of Peutz–Jeghers syndrome

Testicular and ovarian sex cord tumors associated with gynecomastia in prepubertal boys and isosexual precocity in girls have been reported in Peutz–Jeghers syndrome (P–JS) [5, 8]. Aromatase activity in a Sertoli cell tumor was previously demonstrated [4]. We studied the regulation of aromatase expression in samples of Sertoli cell tumors from four prepubertal boys and a girl with P–JS, and an ovarian granulosa cell tumor from an adult woman; as well as in healthy fetal and adult testicular and ovarian tissues. In Sertoli cell tumors, immunoreactive (IR)-$P450_{arom}$ was detected only in the cytoplasm of neoplastic cells, whereas the normal-appearing sex cords did not contain any IR-$P450_{arom}$. On the other hand, IR-$P450_{arom}$ is not detectable in prepubertal testes and is limited to Leydig cells in adult testicular tissues [9]. Using RT–PCR and northern blotting, we determined the tissue-specific utilization of various $P450_{arom}$ promoters by analysing specific 5′-termini from mRNA templates. We found extremely high levels of $P450_{arom}$ transcripts in gonadal sex cord tumors comparable to those in the placenta or corpus luteum, whereas barely detectable levels were found in fetal gonads or in adult testes. Results also indicated that a universal gonadal promoter (PII) directs $P450_{arom}$ gene expression in healthy fetal and adult ovaries and testes, as well as in sex cord tumors of the P–JS and an adult ovarian granulosa cell tumor. These results were interpreted to mean that the utilization of PII in the human ovary and testis is preserved from the fetal period into adult life, as well as in neoplastic Sertoli and granulosa cells. The markedly increased aromatase expression in neoplastic Sertoli cells is thus not a consequence of switching promoters. Rather it appears to involve the activation (or failure of inhibition) of the upstream regulatory elements of the same promoter which is normally functional in all gonadal tissues, namely the proximal PII.

Excessive peripheral aromatization

Excessive peripheral aromatization [10] is an endocrinopathy characterized by gynecomastia of prepubertal onset, contrasexual precocity and hypogonadism in boys. Additionally, it may give rise to isosexual precocity in girls. This disorder was first described by MacDonald *et al.* [6]. Subsequently,

familial forms have been reported [7]. We recently described the clinical course of this endocrinopathy from childhood into adulthood, the cell types responsible for excessive estrogen biosynthesis (undifferentiated fibroblasts in adipose tissue and skin), molecular characterization of aromatase expression in adipose tissue, and treatment of this disorder using an aromatase inhibitor. A 17-year-old boy with gynecomastia of prepubertal onset was found to have extremely high circulating estrone and estradiol levels, whereas testosterone and gonadotropin levels were below the normal range. Extensive clinical work-up ruled out an estrogen-secreting tumor in the testis, adrenal or any other site. He underwent bilateral mastectomy at 15 years of age. The constant of conversion of androstenedione to estrone in the periphery was 55%, which was 50 times that of normal young men. $P450_{arom}$ mRNA levels in buttock and thigh adipose tissue biopsies were 14–21 times those of a normal adolescent boy. Aromatase expression was found to be primarily regulated by the cAMP inducible $P450_{arom}$ promoters, I.3 and II. A genomic Southern hybridization and direct sequencing of the $P450_{arom}$ gene ruled out any defects in the promoter or coding regions. We described, for the first time, that excessive peripheral aromatization in this disorder occurs in the adipose tissue as a result of a metabolic defect possibly associated with activation of a cAMP-dependent signalling pathway.

GYNECOMASTIA IN YOUNG ADULT MEN

Breast enlargement that develops in young adult men can result from a deficiency of testosterone production or action (primary or secondary testicular failure, androgen resistance), drugs (estrogens, marijuana), or an increase in estrogen production. A primary increase in estrogen production may result from a variety of causes. Increased testicular estrogen secretion may result from aberrant production of chorionic gonadotropin or by rare testicular tumors. In congenital adrenal hyperplasia, the increased availability of androstenedione for extraglandular conversion; or in liver failure, the decreased metabolism of estrogens are some of the suggested mechanisms. Estrogen secretion by tumors of the adrenal or liver is another cause of gynecomastia. Aromatase expression is normally absent in the adult liver and adrenal. On the other hand, the liver is an important site of aromatase expression in the fetus [11, 12]. Aromatase expression in the liver becomes undetectable in postnatal life. In the following two cases, aromatase expression in feminizing tumors of the liver and adrenal will be described.

Aromatase expression in hepatocellular carcinoma

An 18-year-old man presented with marked gynecomastia and hepatomegaly that developed over 1 year. After removal of a 1.5 kg hepatocellular carcinoma, gynecomastia partially regressed [13]. Preoperative hormone levels were: estradiol (E_2), 312 pg/ml; estrone (E_1), 1200 pg/ml; testosterone (T), 1.53 ng/ml; FSH/LH, 1.3/2.8 mIU/ml; β-hCG, <5 mIU/ml. Levels 7 months after tumor resection were: E_2, <50 pg/ml; E_1, 74 pg/ml; T, 6.85 ng/ml; FSH/LH, 6.3/3.7 mIU/ml. As mentioned earlier, tissue-specific promoters are used to regulate $P450_{arom}$ gene transcription in human tissues, e.g. promoters I.3, I.4 and II in adipose fibroblasts, and promoter II in the gonads. On the other hand the human fetal liver exclusively uses promoter I.4 to express markedly high levels of $P450_{arom}$. Postnatal hepatic $P450_{arom}$ expression, however, decreases and becomes undetectable in the adult. Total RNA samples were isolated from the hepatocellular carcinoma and adjacent normal liver. Northern analysis indicated the presence of $P450_{arom}$ transcripts in the hepatocellular cancer, but not in the adjacent liver nor in the disease-free adult liver samples. RT–PCR/Southern hybridization showed that promoters I.3 and II were used for $P450_{arom}$ gene transcription in the hepatocellular cancer, whereas only promoter I.4-specific transcripts were detected in the fetal liver, as expected. Because aromatase is not normally expressed in the adult liver, the presence of extremely high levels of aromatase expression in this hepatocellular carcinoma tissue is intriguing, particularly because there is preferential use of $P450_{arom}$ promoters I.3 and II by the tumor, instead of fetal liver-type promoter I.4.

Aromatase expression in an adrenocortical tumor

A 29-year-old man with advanced bilateral gynecomastia that developed over 1 year was evaluated [14]. His circulating estrone (750 pg/ml) and estradiol (510 pg/ml) levels were found to be extremely high. After surgical removal of a 210 g left adrenocortical tumor, estrogen levels returned to normal values. Very high levels of $P450_{arom}$ transcripts comparable to those in the placenta or corpus luteum were found in the tumor tissue using northern analysis or RT–PCR, whereas transcripts were not detectable in disease-free adult adrenals. Only promoter II-specific transcripts were detected by RT–PCR, whereas I.3 or I.4-specific transcripts were not found.

AROMATASE EXPRESSION IN THE BREAST

The breast stroma is largely composed of adipose tissue, which is a blend of various proportions of mature adipocytes and undifferentiated fibroblasts. Vascular and neural tissues comprise very small portions of the breast stroma. As mentioned earlier, aromatase expression in the breast almost exclusively resides in the fibroblast component of the adipose tissue [15, 16]. The fibroblast to adipocyte ratio in adipose tissue displays large variations from one region

to another within the same breast or from one individual to another [17]. These variations in the distribution of fibroblasts determine local estrogen biosynthesis in the breast [17, 18]. The following studies describe molecular and cellular mechanisms that regulate aromatase expression in the breast.

The highest levels of adipose tissue $P450_{arom}$ transcripts in mastectomy specimens were found in the quadrants bearing tumors in 67% of patients

Local estrogen biosynthesis in breast adipose tissue may influence the growth of breast tumors. O'Neill *et al.* [19], in a study of 12 mastectomy patients, have demonstrated that the breast quadrant displaying the highest level of aromatase activity is most frequently the one containing the tumor. The findings of this study are suggestive that breast cancers develop preferentially in anatomical sites in which there is the highest aromatase activity; but these findings were disputed by another group of investigators who did not find such a correlation between adipose tissue aromatase activity and the breast tumor location [20]. In order to use a more sensitive method to address this question, we devised a specific competitive RT–PCR to determine $P450_{arom}$ mRNA levels in breast adipose tissue [15, 18]. In 10 out of 15 patients (67%), the highest $P450_{arom}$ transcript levels localized to the quadrants bearing tumors. This correlation was statistically significant ($P = 0.0006$). We also quantified by computerized morphometry the histological components of the adipose tissue samples from each quadrant in mastectomy specimens. The distribution of fibroblast to adipocyte ratios significantly correlated with the distribution of $P450_{arom}$ transcript levels, in that quadrants containing the highest proportions of fibroblasts matched to the highest transcript levels ($P = 0.007$). It follows therefore, that quadrants bearing tumors contain the highest numbers of fibroblasts.

In disease-free breasts, the highest fibroblast to adipocyte ratios and $P450_{arom}$ transcript levels were found in the outer regions

We determined distribution of $P450_{arom}$ mRNA levels and fibroblast to adipocyte ratios in disease-free breasts of premenopausal women between the ages of 19 and 42 [21]. Adipose tissue samples were collected from three regions of both breasts in 13 women undergoing reduction mammoplasty. Samples were taken from midportions of the outer and inner regions, as well as from the midline above the nipple (designated as upper). The mode of sampling was dictated by the technique of surgery. Total RNA was isolated ($n = 11$), and a hematoxylin and eosin-stained section was prepared ($n = 12$) from the same sample from each region of both breasts. Overall, 67% of the highest fibroblast to adipocyte ratios and 64% of the highest $P450_{arom}$ transcript levels were detected in an outer breast region, whereas in only one patient, the highest values were detected in an inner region. Parametric analysis of variance (ANOVA) showed significant differences between the fibroblast content of the three regions ($P[F] = 0.037$). This distribution pattern directly correlates with the most common or the least common sites of carcinoma in the breast in large series, which are the outer and inner regions, respectively. Moreover, a direct relationship was demonstrated between adipose fibroblasts and $P450_{arom}$ transcripts within the breast, in that regions with the highest fibroblast to adipocyte ratios contained the highest $P450_{arom}$ transcript levels (ANOVA of contrast variables, $P = 0.0009$). This suggests that, similar to our previous findings in the breast bearing a tumor, adipose tissue aromatase expression in the disease-free breast is also determined by the local ratio of fibroblasts to adipocytes. We further conclude that because breast cancer occurs in regions of the breast with the highest levels of aromatase expression, the presence of high fibroblast content and $P450_{arom}$ transcript levels in the outer region of the disease-free breast may be of pathophysiological significance in the development of breast cancer.

Switching of alternative promoter use from I.4 in the disease-free breast to promoter I.3 and II in the breast adipose tissue bearing a carcinoma

As indicated above, tissue-specific expression of the $P450_{arom}$ gene is determined, at least in part, by the use of tissue-specific promoters, which give rise to transcripts with unique 5′-non-coding termini. Transcripts in adipose tissue contain 5′-termini derived from specific untranslated exons, corresponding to expression derived from the proximal promoters II and I.3, as well as a distal promoter, I.4. We determined the distribution of these various exon-specific transcripts in breast adipose tissues from cancer-free women undergoing reduction mammoplasty and from patients with breast cancer, because this would provide important clues as to the nature of the factors regulating aromatase expression in these sites. In order to achieve this, we employed competitive RT–PCR, utilizing an internal standard for each exon-specific transcript of the $P450_{arom}$ gene, as well as for the coding region in order to evaluate total $P450_{arom}$ transcripts. In patients with cancer ($n = 18$), total $P450_{arom}$ transcript levels were significantly higher in adipose tissue proximal to a tumor in comparison to adipose tissue distal to a tumor, in agreement with previous findings. Moreover, total transcript levels were higher in breast adipose tissue of cancer patients in comparison to those of cancer-free individuals ($n = 9$), even when the adipose tissue from the cancer patient was taken from a quadrant with no detectable tumor. We observed that promoter I.4-specific transcripts were predominant in breast adipose obtained from cancer-free women. In this tissue, promoter-II- and I.3-specific transcripts were present in low copy

numbers. On the other hand, in breast cancer patients, $P450_{arom}$ mRNA from breast adipose tissue had primarily promoter-II- and I.3-specific transcripts, whereas comparatively few transcripts had I.4-specific sequence at the 5′-terminus. We conclude that $P450_{arom}$ gene transcription in breast adipose tissue of cancer-free individuals preferably utilizes promoter I.4, implicating a role of glucocorticoids and members of the IL-6 cytokine family in the regulation of this expression. On the other hand, the increased expression in breast adipose tissue bearing a carcinoma results from expression from promoters II and I.3, which are regulated by unknown factors acting via increased cAMP formation, which are presumably secreted by the tumor or associated cells. We recently showed that one of these factors may be prostaglandin $(PG)E_2$, because PGE_2 is a very potent stimulator of aromatase expression in adipose fibroblasts via promoter II (our unpublished observations).

AROMATASE EXPRESSION IN UTERINE DISEASE

Aromatase expression is absent in the disease-free endometrium, decidua and myometrium [22]. On the other hand, aromatase activity and $P450_{arom}$ mRNA have been demonstrated in the following disease states:

Aromatase expression in endometrial cancer

Aromatase activity has previously been demonstrated in endometrial tumors [23]. We investigated $P450_{arom}$ gene expression and its regulation in endometrial tumor samples ($n = 9$). Using RT–PCR, varying levels of $P450_{arom}$ transcripts were detected in all endometrial adenocarcinomas ($n = 8$) and one mixed Müllerian tumor studied. No correlations were observed between $P450_{arom}$ transcript levels and the histological type of the tumor, grade, myometrial invasion, stage of the disease, or the patient's age. RT–PCR/Southern hybridization indicated that promoters II and I.3 were primarily used for $P450_{arom}$ expression in adenocarcinomas. On the other hand, the distribution of transcripts specific for I.4, I.3 and promoter II in one mixed Müllerian tumor was uniform. Because $P450_{arom}$ transcripts were detected in all endometrial malignancies studied, whereas they were not demonstrable in the disease-free endometrium, the activation or failure of the inhibition of aromatase expression in these tumors may serve to promote neoplastic proliferation.

Aromatase expression in uterine leiomyoma tissues and smooth muscle cells

Previously, conversion of radiolabelled androstenedione to estrone has been demonstrated in uterine leiomyomas [24]. By the use of competitive RT–PCR, we detected and quantified $P450_{arom}$ transcripts in total RNA isolated from 32 of the 35 leiomyomas (91%) and from 18 of the 24 adjacent myometrial (75%) tissue samples from 26 women. $P450_{arom}$ transcripts were not detectable in myometrial tissues from disease-free uteri ($n = 8$). $P450_{arom}$ transcript levels in leiomyomas were similar to those in adipose tissue and were 1.5- to 25-fold higher than in adjacent myometrial tissues. We did not find any correlation between $P450_{arom}$ levels and leiomyoma size, histopathology, uterine weight, or patient's age. In leiomyoma smooth muscle cells in culture ($n = 4$) and tissue explants ($n = 4$), aromatase activity was stimulated by dibutyryl cAMP and this effect was potentiated by a phorbol ester. These increases in aromatase expression were accompanied by comparable increases in the levels of translatable $P450_{arom}$ mRNA. Treatment with dexamethasone or platelet-derived growth factor did not stimulate aromatase expression. Consistently higher levels of aromatase activity and $P450_{arom}$ transcripts were found in the leiomyoma tissues than in smooth muscle cells in culture (two- to 20-fold). RT–PCR analysis of untranslated 5′-termini of mRNA species in leiomyomas revealed the use primarily of promoter II (the ovarian-type promoter) for $P450_{arom}$ gene transcription. We concluded that aromatase expression in leiomyomas is regulated by the rate of $P450_{arom}$ gene transcription, which is, in turn, regulated by the use of promoter II. These findings are consistent with the hypothesis that localized estrogen biosynthesis may be of pathological significance in the promotion of leiomyoma growth.

Aromatase expression in pelvic endometriotic implants and endometrioma-derived stromal cells

As indicated above, aromatase is not expressed in the endometrium of disease-free women [22, 25]. We demonstrated, however, the presence of $P450_{arom}$ mRNA in pelvic endometriotic implants and in eutopic endometrial curettings of women with endometriosis [26]. We then investigated aromatase activity and $P450_{arom}$ gene expression in cultured stromal cells derived from eutopic endometrium and ovarian endometriomas of women with pelvic endometriosis. We also investigated the hormonal regulation of aromatase expression and alternative promoter use in these cells. The effects of IL-1β, IL-2, IL-6, IL-11, oncostatin M (OSM), IL-15, TNF-α, prostaglandin E_2 (PGF_2), estradiol-17β, R5020, dexamethasone (DEX) and dibutyryl cyclic AMP (Bt_2cAMP) on aromatase activity in endometriosis-derived stromal cells were assessed. We chose treatments with prostaglandins and interleukins because of the inflammatory nature of endometriosis. Ovarian endometriomas were obtained from four women at the time of laparoscopy. Eutopic endometrial curettings were obtained from two of the above subjects and from two disease-free women (normal controls). PGE_2 stimulated aromatase activity in endometriosis-derived stromal cells by

approximately 30-fold (35–221 pmol/mg protein/4 h), whereas Bt_2cAMP induction was 26- to 60-fold compared with baseline levels. No stimulation was observed by R5020 or estradiol-17β, or by IL-1β, IL-2, IL-6, IL-11, IL-15 or TNF-α in the presence or absence of DEX. A modest induction of aromatase activity (three-fold) was observed in DEX + OSM-treated cells. These changes in aromatase activity were accompanied by comparable changes in the levels of $P450_{arom}$ transcripts. The majority of $P450_{arom}$ transcripts in cells treated with PGE_2 and Bt_2 cAMP contained promoter II-specific sequences, whereas very few transcripts contained promoter I.3- and I.4-specific sequences. PGE_2 appears to be the most potent known (patho)physiological stimulator of aromatase in endometriosis. Aromatase expression in PGE_2-stimulated stromal cells of endometriosis is primarily regulated by promoter II, which in turn is regulated by cAMP. Because PGE_2 is known to increase intracellular cAMP levels, estrogen biosynthesis in endometriosis may be primarily regulated by PGE_2 that is locally produced. Consequent local estrogen production may promote the growth of endometriotic implants.

DISCUSSION

$P450_{arom}$ is the only enzyme that is capable of converting C_{19} steroids to estrogens in a variety of human tissues [2]. As indicated earlier, alternative promoters are responsible for the regulation of aromatase expression in these tissues (Fig. 1). Studies of aromatase expression in tissue samples from subjects with various endocrinopathies or estrogen-responsive tumors are summarized in Table 1. The following common features are readily apparent. First, varying levels of aromatase expression were detected in endometrial tumors, leiomyomas, adrenocortical tumors, hepatocellular carcinomas and Sertoli cell tumors. On the other hand, the disease-free tissue counterparts of these neoplasia do not express aromatase, e.g. disease-free endometrium, myometrium, adrenal, liver, and Sertoli cells. Second, aromatase expression in undifferentiated mesenchymal cells in adipose tissue can be stimulated by the presence of a proximal breast carcinoma or by an unknown metabolic disorder that gives rise to prepubertal gynecomastia (excessive peripheral aromatization). Third, aromatase expression is detected in endometrial stromal cells of women with endometriosis, suggesting an inherent metabolic defect possibly predisposing these women to the development of endometriosis. Fourth, the cAMP-inducible promoters I.3 and II appear to direct excessive aromatase expression in all of these neoplastic or non-neoplastic tissues. This is suggestive of a common metabolic abnormality giving rise to excessive activation of the cyclic AMP-dependent signalling pathway in affected cells. In adipose fibroblasts and endometriotic stromal cells, excessive PGE_2 production or the aberrant expression of certain PGE_2 receptor types may be the responsible mechanisms. This is currently under investigation.

Aromatase expression in tumors of the liver, adrenal and Sertoli cells, and in adipose fibroblasts of individuals with excessive peripheral aromatization can give rise to abnormally high circulating estradiol levels and gynecomastia. On the other hand, increased aromatase expression in breast cancer, endometrial cancer, leiomyomas and endometriosis does not give rise to elevated estrogen levels in the peripheral blood. Therefore, the pathophysiological role of increased locally produced estrogen in tissue growth is not readily apparent in these conditions. Aromatase activity in leiomyoma-derived smooth muscle cells,

Table 1. List of human tissues that display excessive aromatase expression

Aromatase expression		
Absent	Present	$P450_{arom}$ promoter(s)
Testicular Sertoli cell	Sertoli cell tumor	II
Liver	Hepatocellular cancer	I.3, II
Adrenal	Adrenocortical tumor	II
Endometrium	Endometrial cancer	II
	Endometriotic implant	II
	Eutopic endometrium of women with endometriosis	?
Myometrium	Leiomyoma	II
	Myometrium from leiomyomatous uterus	?
Low	High	
Adipose and skin fibroblasts in disease-free individuals	Fibroblasts in patients with excessive peripheral aromatization	I.3, II
Adipose tissue in disease-free breast	Adipose tissue proximal to breast carcinoma	I.3, II
Adipose tissue in medial breast region (disease-free breast)	Adipose tissue in lateral breast region (disease-free breast)	I.4

endometriosis-derived fibroblasts, and adipose fibroblasts surrounding breast tumors is quite high (within the pmol range) to suggest the presence of significant quantities of locally produced estrogens in these tissues. Additionally, aromatase expression in these cells can be regulated by various hormones and cytokines. These indirect observations lend credence to the concept of possible growth-stimulatory roles of locally produced estrogens in hormone-responsive neoplasia.

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Molecular Basis of Severe Gynecomastia Associated with Aromatase Expression in a Fibrolamellar Hepatocellular Carcinoma*

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ABSTRACT

This report represents the first study in the literature linking development of severe gynecomastia, in a 17½-yr-old boy, to high levels of aromatase expression in a large fibrolamellar hepatocellular carcinoma, which gave rise to extremely elevated serum levels of estrone (1200 pg/mL) and estradiol-17β (312 pg/mL) that suppressed FSH and LH (1.3 and 2.8 IU/L, respectively), and consequently testosterone (1.53 ng/mL). After removal of a 1.5-kg hepatocellular carcinoma, gynecomastia partially regressed, and essentially, normal hormone levels were restored (estradiol-17β, <50 pg/mL; estrone, 74 pg/mL; testosterone, 6.85 ng/mL; and FSH/LH, 6.3/3.7 mIU/mL). Conversion of C_{19} steroids to estrogens occurs in a number of human tissues and is catalyzed by aromatase P450 (P450arom), the product of the *CYP19* gene in a number of human tissues. Tissue-specific promoters are used to regulate P450arom gene transcription in adult human tissues, *e.g.* promoters I.4 and I.3 in adipose fibroblasts, and promoter II in the gonads. Human fetal liver uses promoter I.4 to express markedly high levels of P450arom, whereas hepatic P450arom expression normally becomes undetectable in postnatal life. Using immunohistochemistry, diffuse intracytoplasmic aromatase expression was detected in the liver cancer cells from this severely feminized boy. Northern analysis indicated the presence of P450arom transcripts in total RNA from the hepatocellular cancer but not in the adjacent liver nor in disease-free adult liver samples. Promoter use for aromatase expression was determined by a specific RT-PCR method. Promoters I.3 and II were used for P450arom gene expression in the hepatocellular cancer tissue. Because aromatase is not expressed in the disease-free adult liver, the presence of extremely high levels of aromatase expression in this fibrolamellar hepatocellular carcinoma tissue is intriguing, particularly because there is preferential use of the proximally located P450arom promoters I.3 and II by the tumor, instead of the much more distally located fetal liver-type promoter I.4. (*J Clin Endocrinol Metab* **83:** 1797–1800, 1998)

MILD GYNECOMASTIA at puberty is a common but self-limiting condition, which ordinarily disappears by the end of pubertal development. On the other hand, the development of severe gynecomastia in prepubertal and adolescent boys and young men prompts the physician to immediately rule out an estrogen-secreting tumor. Although testicular Sertoli cell tumors and the syndrome of excessive peripheral aromatization are some of the best studied causes of gynecomastia of prepubertal onset, other testicular and adrenal tumors have been reported to secrete sufficient quantities of estrogen to give rise to rapid development of severe gynecomastia at any age (1, 2). It should be pointed out here that gynecomastia in young boys may also be caused by inadvertent application of exogenous estrogens such as ointments that contain diethylstilbestrol (3). Several cases of gynecomastia also have been reported in association with the presence of a liver tumor (4–6). In none of these reports, however, was aromatase expression in the tumor tissue studied. This is the first report in which aromatase expression in a liver carcinoma was demonstrated, which gave rise to extremely high plasma levels of estradiol-17β, gynecomastia, and hypogonadotropic hypogonadism. In the steroidogenic pathway, aromatase is the enzyme that converts C_{19} steroids to estrogens in a number of human cells, such as the placental syncytiotrophoblast, ovarian granulosa cells, and adipose and skin fibroblasts (7). Aromatase expression also is detected at very high levels in the fetal liver (the second highest, only next to placenta, among fetal tissues) (8). Aromatase expression in liver, however, disappears in the postnatal period. Aromatase expression in human tissues is regulated by use of several alternative promoters, giving rise to transcripts with promoter-specific untranslated 5′-ends but with an identical coding region (7). For example, whereas the distally located promoter I.1 is exclusively used in the placenta, giving rise to transcripts containing exon I.1; promoters I.3 and I.4 are responsible for aromatase expression in the adipose tissue in a hormone-dependent fashion, giving rise to transcripts containing exons I.3 and I.4 (7). In the ovary and testis, on the other hand, only the proximally located promoter II is used (7, 9). Aromatase expression in the fetal liver

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is under the control of promoter I.4 (8, 10). We have demonstrated that promoters I.3 and II are preferentially used for inappropriate aromatase expression in a number of human tumors, such as endometrial carcinoma (11), uterine leiomyomas (12), adipose fibroblasts surrounding breast cancer (13), testicular Sertoli cell tumors, and feminizing adrenal carcinoma (2, 9). In the present report, we demonstrated the use of promoters I.3 and II in this fibrolamellar liver carcinoma.

Subject and Methods

This 17½-yr-old boy was evaluated for development of severe gynecomastia. He was born after a normal pregnancy with normal birth weight and height. He had no major illnesses or surgeries during childhood or early adolescence. He developed mild gynecomastia at age 12 yr. Gynecomastia never regressed but did not progress for 4 yr. From age 16½ yr, breast size rapidly increased to reach a severe degree of gynecomastia. He had pain in his right flank for the past 6–9 months and mentioned recent wt loss of 5 pounds. Aside from gynecomastia, he had a normal pubertal development and had erections and nighttime ejaculations. His family history is negative for gynecomastia.

On physical examination, his height was 171.2 cm (just under the 25th percentile) and his wt was 50.6 kg (<5th percentile). He had bilateral severe gynecomastia with mammary tissue, on the right side, of 6 × 6 × 6-cm size and, on the left side, of 8 × 8 × 7-cm size. He had a fair amount of axillary hair and apocrine secretory activity. His liver was enlarged (vertical span of 12 cm) with an ill-defined lower margin. Tanner stage 4 pubic hair development was noted. His phallus was 13 cm stretched; and his testes, which measured 4.5 × 2.3 × 1.9 and 5.1 × 2.8 × 2 cm, were soft in consistency without any evidence of palpable masses. His serum estradiol-17β level was extremely high, whereas gonadotropins and testosterone were low. Hormone levels are reported in Table 1. The rest of the blood work was essentially normal, except for mildly elevated liver enzymes. Bone age was consistent with chronological age. Ultrasonic examination of the testes and adrenals indicated no abnormalities. Abdominal ultrasound revealed a large mass, with a mean diameter of 15 cm, in the right liver lobe and an adjacent 3.6-cm mass in the quadrate lobe. An magnetic resonance imaging scan of the abdomen confirmed these findings. A chest x-ray was strongly suggestive of left pulmonary metastases.

The hepatic tumor in the right lobe, the quadrate lobe, and a portion of the left lobe was removed surgically. The specimen weighed 1590 g. Metastatic tumors were also resected from the left lung. Pathological diagnosis was fibrolamellar hepatocellular carcinoma. After tumor resection, hormone levels were restored to essentially normal for an adult man (Table 1). Subsequently, he was given chemotherapy and underwent bilateral mastectomies within 6 months. Within 1 yr, a recurrence was noted in the liver, and the patient underwent a second tumor resection.

Tissue acquisition

All tissue samples were obtained at the time of surgery and were immediately frozen in liquid nitrogen until RNA isolation, or processed and paraffin embedded for immunohistochemistry. Total RNA was isolated using the guanidinium thiocyanate-cesium chloride method, as previously described (14). For the following studies, we obtained written informed consent, which was approved by the Institutional Review Committee of the University of North Carolina at Chapel Hill.

TABLE 1. Serum hormone levels of boy with hepatic cancer and gynecomastia

	Before removal of tumor	After removal of tumor
Estradiol-17β	312 pg/mL	<50 pg/mL
Estrone	1200 pg/mL	74 pg/mL
Testosterone	1.53 ng/mL	6.85 ng/mL
Androstenedione	0.39 ng/mL	
FSH	1.3 mIU/mL	6.3 mIU/mL
LH	2.8 mIU/mL	3.7 mIU/mL
β-HCG	<5 mIU/mL	

Immunohistochemistry

The anti-aromatase P450 (anti-P450arom) antibody used in this study was a rabbit IgG fraction of an antiserum raised against the enzyme purified from human placenta (15). The immunohistochemical procedures were performed, as previously described, on 2.5-μm-thick sections mounted on poly-L-lysine-coated slides using the biotin-streptavidin amplified technique with a Histone immunostaining kit (Nichirei, Tokyo, Japan) (16). Briefly, the staining procedure was performed as follows: 1) routine deparaffinization; 2) inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in methyl alcohol for 30 min at 23 C; 3) blocking with 1% goat serum for 45 min at 23 C; 4) incubation with the primary antibody at 4 C for 18 h; 5) incubation with biotinylated goat antirabbit antibody for 30 min at 23 C; 6) incubation with peroxidase-conjugated streptavidin for 30 min at 23 C; 7) colorimetric reaction with a solution containing 0.05% Tris-HCl (pH 7.6), 0.66 mol/L 3,3′-diaminobenzidine, and 2 mol/L H_2O_2; and 8) counterstaining with 1% methyl green.

Northern blot analysis and quantitative RT-PCR

Northern blot analysis was performed as described previously (9). Total RNA samples from all indicated tissues and cells were size-fractionated by electrophoresis on formaldehyde-agarose (1%) gel and transferred to a nylon membrane by capillary elution. Hybridization was conducted for 16 h at 42 C using a human complementary DNA probe radiolabeled with [^{32}P]-deoxycytidine triphosphate.

Exon-specific competitive RT-PCR was performed according to a recently standardized method (17). For total P450arom transcript levels, coding region was amplified using specific oligonucleotides, as previously described (17). We also used sense oligonucleotides specific for untranslated first exons to amplify promoter-specific transcripts. The antisense primer was designed from a sequence in the coding exon III. To check the integrity and comparative quantity of RNA used in amplification of *CYP19* gene transcripts, transcripts of glyceraldehyde-3-phosphate dehydrogenase (a housekeeping gene) were amplified by the RT-PCR method, as described previously (17). A trace amount of [^{32}P]-deoxycytidine triphosphate was added to each of the PCR samples. The reaction products were analyzed on 4% nondenaturing polyacrylamide gels. Gels were either autoradiographed with an x-ray film or scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantitatively analyzed using ImageQuant software. PCR amplified products obtained were of expected sizes.

Results and Discussion

Regulation of inappropriate aromatase expression in a liver cancer tissue, which gave rise to the development of severe gynecomastia, was studied. To date, only two reports have been published describing a link between hepatocellular carcinoma and gynecomastia. In the first case (a 38-yr-old man), serum estrogen levels did not change after the removal of the tumor (4), and in the second case (a 15-yr-old boy), the patient died before the tumor could be resected (5). In neither case was aromatase expression in the tumor studied. In the present study, we hypothesized that the massive amounts of estrone and estradiol-17β were secreted directly by the liver cancer. This pertains because levels of estrogens, testosterone, and gonadotropins promptly returned to normal after removal of the carcinoma (Table 1). Low serum testosterone before surgery was a result of low LH that was suppressed by extremely high levels of circulating estradiol-17β, giving rise to hypogonadotropic hypogonadism.

The next set of questions pertains to the sources of testosterone and androstenedione, the substrates for aromatase in this boy's liver cancer. Were these C_{19} steroids synthesized

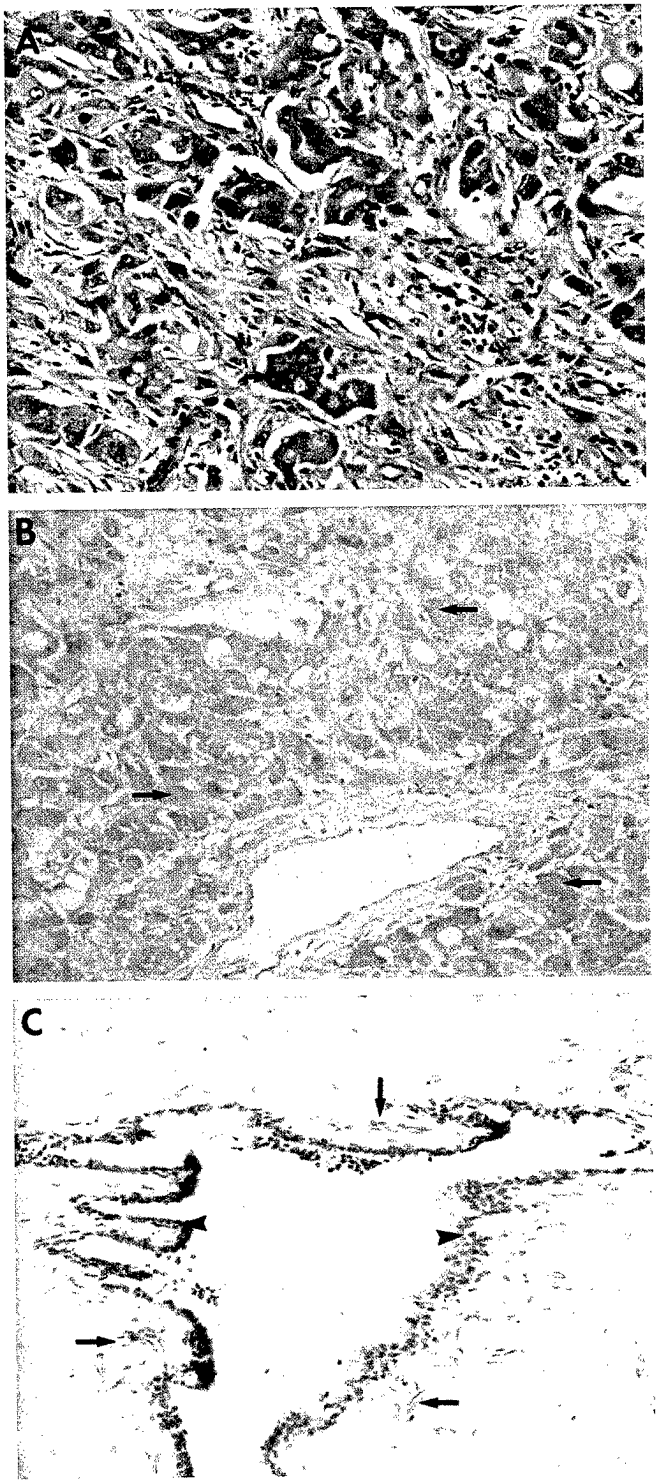

FIG. 1. Immunohistochemical detection of P450arom (*brown stain*) in the histological sections of the fibrolamellar hepatocellular carcinoma. A, Hematoxylin and eosin staining of the liver tumor show large polygonal cells with abundant cytoplasm and sharply demarcated borders (*arrows*). These neoplastic cells are separated into sheets and nests by relatively acellular collagen-rich stroma containing fibroblasts (*arrowheads*). B, Abundant immunoreactive aromatase (*light brown stain*) is detected primarily in the cytoplasm of neoplastic cells of the hepatocellular carcinoma (*arrows*). C, No immunoreactive aromatase is detected in glandular epithelial cells (*arrowheads*) or stromal fibroblasts (*arrows*) of the breast tissue of the patient.

within the liver tumor, or did the rates of testosterone and androstenedione production, as calculated from their plasma levels and metabolic clearance rates, account for the estrogen secretion by the tumor? Both of these mechanisms might have provided the C_{19} precursors for aromatase activity in the tumor. We, however, do not have an answer, because variables such as the metabolic clearance rates of these C_{19} steroids and estrogens and the rates of aromatization by the liver tumor and hepatic blood flow were not determined.

To study aromatase expression in the liver cancer tissue, we used immunohistochemistry, northern blot analysis, and exon-specific RT-PCR techniques. Upon hematoxylin and eosin staining of the liver tumor, we observed large polygonal cells with abundant cytoplasm and sharply demarcated borders (Fig. 1A). These sheets of neoplastic cells were separated by collagen-rich stroma containing fibroblasts (fibrolamellar variant). Using a polyclonal antibody, abundant immunoreactive aromatase expression was detected in the cytoplasm of large neoplastic cells of the hepatocellular carcinoma (Fig. 1B). Immunoreactive aromatase was also detected in the stromal fibroblasts. Higher staining intensity in overwhelming numbers of the neoplastic hepatocellular cancer cells, however, suggested that aromatase expression in these cells accounted for the largest portion of estrogen production in the tumor tissue. No immunoreactive aromatase was detected in the breast tissue of this patient, indicating that peripheral aromatization in the adipose tissue did not significantly contribute to estrogen excess in this boy (Fig. 1C). Northern blot analysis (Fig. 2) showed readily detectable P450arom mRNA in total RNA samples from the liver tumor and fetal liver, whereas no transcripts were detected in total RNA from the adjacent liver, recurrent tumor, or normal liver tissues (obtained from persons who died in traffic accidents). Placenta and adipose stromal cells, treated with dibutyryl AMP, were used as positive controls for northern analysis. Similar results were obtained using RT-PCR as a more sensitive technique, and we were also able to observe aromatase expression, to a lesser degree, in the recurrent tumor (Fig. 3).

To characterize the regulation of aromatase expression in the hepatocellular carcinoma, it was essential to determine the alternative promoter(s) responsible for gene transcription

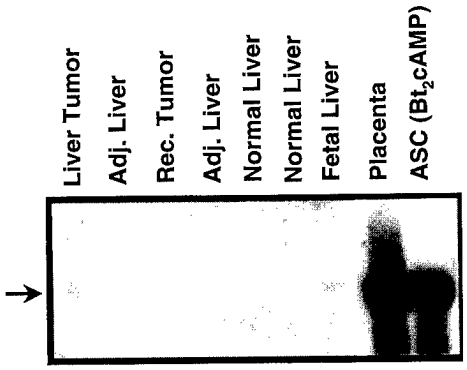

FIG. 2. Northern blot analysis showing expression of P450arom. An equal amount of total RNA (20 μg) from each tissue was used. Adj. Liver, Normal liver tissue adjacent to the tumor; Rec. tumor, recurrent tumor; ASC (Bt_2cAMP), adipose stromal cells treated with dibutyryl cAMP; Normal liver, disease-free control liver samples from two adults who died in traffic accidents.

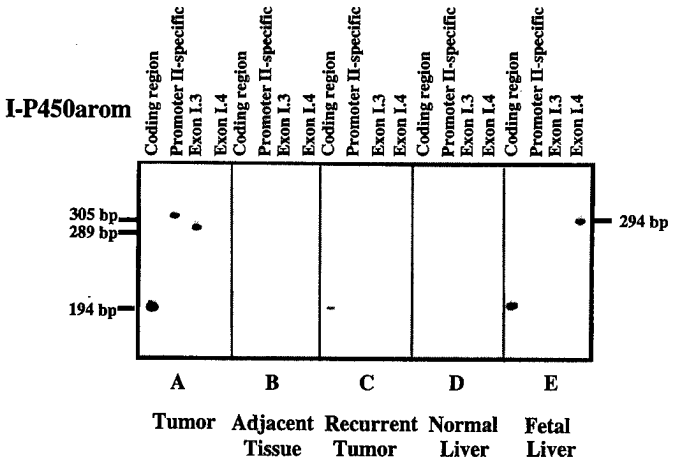

FIG. 3. I, RT-PCR of promoter-specific P450arom transcripts in total RNA from liver tumor (A), adjacent normal liver tissue (B), recurrent tumor (C), normal liver from a disease-free adult (D), and fetal liver (E). Coding region, Coding exons II and III that are common to all P450arom transcripts (size: 194 bp); Promoter II-specific, promoter II-specific transcripts (size: 305 bp); Exon I.3, promoter I.3-specific transcripts (size: 289 bp); Exon I.4, promoter I.4-specific transcripts (size: 294 bp). PCR-amplified products were of expected size (17). II, Amplification of the ubiquitous marker, glyceraldehyde-3-phosphate dehydrogenase, from total RNA of above mentioned tissues.

in this tissue. Therefore, we used an exon-specific RT-PCR technique to determine the promoter-specific 5′-ends of P450arom transcripts in the liver tumor (17). We observed that promoters I.3 and II were used in the liver cancer, whereas promoter I.4-specific sequences were found in the fetal liver tissue, as expected (Fig. 3). This was somewhat surprising, because regression to use the fetal-type promoter I.4 might be expected in hepatic cells after neoplastic transformation. Instead, the cAMP-inducible promoters, I.3 and II, were used in this liver cancer tissue. This is similar to our recent findings in breast cancer, where promoter switching takes place from promoter I.4 in the disease-free breast fat to promoters I.3 and II in the tumor-bearing breast adipose tissue (13). Interestingly, in an adrenal cancer too, promoter II was used for aromatase expression, as was the case also in Sertoli cell tumors (2, 9). High expression of aromatase transcripts and protein in these tumors and the use of promoters II and I.3 indicate some common mechanism of regulation of P450arom gene expression in various endocrine tumors. Because in nonneoplastic tissues, use of promoter II is dependent on steroidogenic factor 1 and cAMP response element binding protein, it will be of interest to investigate the role of these factors in aromatase expression in these neoplastic tissues, including liver cancer (18).

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Chapter 5

Tissue-Specific Expression of the *CYP19* (Aromatase) Gene

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INTRODUCTION

Aromatase (Thompson, et al., 1974; Mendelson et al., 1985; Nakajin et al., 1986; Kellis and Vickery, 1987) (P450arom, the product of the *CYP19* gene (Nelson et al., 1993)) is the enzyme which catalyses the biosynthesis of estrogens. *CYP19* is a member of the P450 superfamily of genes which currently contains over 300 members in some 36 gene families (Nelson et al., 1993). Estrogen biosynthesis appears to occur throughout the entire vertebrate phylum including mammals, birds, reptiles, amphibians, teleost and elasmobranch fish, and *Agnatha* (hagfish and lampreys) (Callard et al., 1900, 1980; Callard, 1981). It has also been described in the protochordate *Amphioxus* (Callard et al., 1984). To our knowledge, estrogen biosynthesis has not been reported in non-chordate animal phyla. This is in spite of the fact that the CYP19 family appears to be an ancient lineage of P450 gene products, diverging as much as 10^9 years ago (Nelson et al., 1993). In most vertebrate species that have been examined, aromatase expression occurs in the gonads and in the brain. This is true of the fish and avian species that have been examined as well as most mammals (such as rodents). In many species, estrogen biosynthesis in the brain has been implicated in sex-related behavior such as mating responses, and frequently a marked sexually dimorphic difference has been demonstrated. This is true, for example, in avian species in which the song of the male is important in courtship behavior (Hutchinson, 1991). In the case of humans and a number of higher primates, there is a more extensive tissue distribution of estrogen biosynthesis, since this also occurs in the placenta of the developing fetus as well as in the adipose tissue of the adult. The ability of the placenta to synthesize estrogen is also the property of a number of ungulate species such as cows, pigs, and horses. However, at least in cattle, there is no evidence of estrogen biosynthetic capacity in adipose, whereas in rodent species such as rat and mice, as well as in rabbits, neither adipose nor placenta has any ability to synthesize estrogens.

The physiological significance of estrogen biosynthesis in the placenta and adipose of humans is unclear at this time. The C_{18} steroid produced in each tissue site of biosynthesis is quite tissue-specific. For example, the human ovary synthesizes primarily estradiol, whereas the placenta synthesizes estriol and adipose synthesizes estrone. This appears to reflect primarily the nature of the C_{19} steroid presented to the estrogen-synthesizing enzyme in each tissue site. Thus, in the case of adi-

pose tissue, the principal source of substrate is circulating androstenedione produced by the adrenal cortex. In the case of the placenta, the major precursor is 16α-hydroxydehydroisoandrosterone sulfate derived as a consequence of the combined activities of the fetal adrenal and liver. Although the human placenta produces very large quantities of C_{18} steroids, particularly estriol, its physiological importance is unclear. This pertains because in pregnancies characterized by placental sulfatase deficiency, the placenta is essentially deprived of C_{19} substrate and hence synthesizes, relatively speaking, minute quantities of estrogen, yet such pregnancies are quite uncomplicated (France and Liggins, 1969). At most, parturition is delayed by several days. Similarly, at this time no physiological significance has been attributed to estrogen biosynthesis by human adipose; however, the latter has been implicated in a number of pathophysiological conditions. Estrogen biosynthesis by adipose tissue not only increases as a function of body weight but as a function of age (Hemsell et al., 1974; Edman and MacDonald, 1976) and has been correlated directly with the incidence of endometrial cancer as well as with postmenopausal breast cancer. Furthermore, evidence is accumulating to suggest that the estrogen implicated in the development of breast cancer is that which is produced locally within the adipose tissue of the breast itself (Miller and O'Neill, 1987). On the other hand, estrogen biosynthesis in adipose tissue may have beneficial consequences since osteoporosis is more common in small, thin women than in large, obese women. While this may be, in part, the consequence of the bones of the latter being subject to load-bearing exercise, nonetheless it seems likely that the increased production of estrogens by the adipose of obese women is a significant factor.

THE *CYP19* GENE

Some years ago we and others cloned and characterized the *CYP19* gene which encodes human P450arom (Means et al., 1989; Harada et al., 1990; Toda et al., 1990) (Figure 1). The coding region spans nine exons beginning with exon II (Figure1). Sequencing of rapid amplification of cDNA ends (RACE)-generated cDNA clones derived from P450arom transcripts present in the various tissue sites of expression revealed that the 5'-termini of these transcripts differ from one another in a tissue-specific fashion upstream of a common site in the 5'-untranslated region

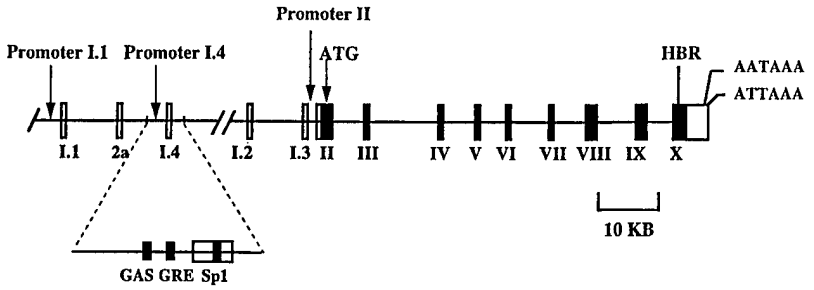

Figure 1. Schematic representation of the human *CYP19* gene. The closed bars represent translated sequences. The septum in the open bar in exon II represents the 3'-acceptor splice junction for the untranslated exons. The sequence immediately to the left of the septum is that present in mature transcripts whose expression is driven by promoter II. The five untranslated exons, I.1, 2a, I.2, I.3, and I.4, are indicated in their approximate locations. Also shown are promoters I.1 and II and putative promoters I.2, I.3, and I.4. The heme-binding region (HBR) is indicated in exon X, as are two alternative polyadenylation signals that give rise to the two species of P450arom transcript of 3.4 and 2.9 kb. The genomic region shown spans a distance of at least 75 kb; however, as the gap between exons I.4 and I.2 has never been bridged, the true size is unknown.

(Means et al., 1991; Kilgore et al., 1992; Jenkins et al., 1993; Toda and Shizuta, 1993). Using these sequences as probes to screen genomic libraries, it was found that these 5'-termini correspond to untranslated exons which are spliced into the P450arom transcripts in a tissue-specific fashion, due to the use of tissue-specific promoters. Placental transcripts contain at their 5'-ends untranslated exon I.1 which is located at least 40 kb upstream from the start of translation in exon II (Mahendroo et al., 1991; Means et al., 1991). This is because placental expression is driven from a powerful distal placental promoter, I.1, upstream of untranslated exon I.1. On the other hand, transcripts in the ovary contain sequence at their 5'-ends which is immediately upstream of the start of translation. This is because expression of the gene in the ovary utilizes a proximal promoter, promoter II (Jenkins et al., 1993). By contrast, transcripts in adipose tissue contain yet another distal untranslated exon, I.4, which is located in the gene 20 kb downstream from exon I.1 (Mahendroo et al., 1993). A number of other untranslated exons have been characterized by ourselves and others (Harada et al., 1993; Toda et al., 1994), including

one specific for brain (Honda et al., 1994). Splicing of these untranslated exons to form the mature transcripts occurs at a common 3'-splice junction which is upstream of the start of translation. This means that although transcripts in different tissues have different 5'-termini, the protein encoded by these transcripts is always the same, regardless of the tissue-site of expression, thus there is only one human P450arom enzyme encoded by a single-copy gene.

EXPRESSION OF AROMATASE IN HUMAN OVARY

As indicated previously, aromatase P450 is expressed in the pre-ovulatory follicles and corpora lutea of ovulatory women by means of a promoter proximal to the start of translation (PII) (Means et al., 1991; Jenkins et al., 1993). Aromatase expression in the granulosa cells of the ovary is primarily under the control of the gonadotropin follicle-stimulating hormone (FSH), whose action is mediated by cAMP. To understand how this transcription is controlled by cAMP, we constructed chimeric constructs containing deletion mutations of the proximal promoter 5'-flanking DNA fused to the rabbit β-globin reporter gene (Michael et al., 1994). Assay of reporter gene transcription in transfected bovine granulosa and luteal cells revealed that basal and cAMP-stimulated transcription was lost upon deletion from -278 to -100 bp, indicating the presence of a functional response element in this region; however, no classical cAMP-responsive element was found. Mutation of a CAAGGTCA motif located at -130 bp revealed that this element is crucial for basal and cAMP-stimulated reporter gene transcription. When a single copy of this element was placed upstream of a heterologous promoter, it could act as a weak cAMP-response element (CRE). Electrophoretic mobility shift assay in the presence of specific antibodies and u/v-cross-linking established that Ad4BP/SF-1 binds to this hexameric element. Steroidogenic factor-1 (SF-1) is an orphan member of the steroid hormone receptor gene superfamily which has been shown to be a critical developmental factor for the gonads as well as the adrenals (Lala et al., 1992; Luo et al., 1994). However deletion mutation analysis revealed the presence of another sequence upstream of the SF-1 site which was also critical for cAMP-responsiveness. This sequence, at -211/-202 bp, is TGCACGTCA, identical to a canonical CRE save for the extra C. A similar element is present in the rat gene (Fitzpatrick and Richards, 1994). In the case of the human, electrophoretic gel mobility shift and antibody super-shift analysis revealed that a number of factors bind to this site (Mi-

chael and Simpson, 1996). Moreover, u/v-cross-linking analysis revealed that the major protein binding to this site appeared to be about 24 kDa in size, much smaller than CRE binding protein (CREB) or, indeed, most other transcription factors. Thus, it is apparent that much remains to be elucidated regarding the mechanism whereby FSH and cAMP regulate aromatase expression in the ovary.

EXPRESSION OF AROMATASE IN HUMAN PLACENTA

As indicated previously, placental expression of aromatase in the human is driven from a powerful distal placental promoter I.1 upstream of untranslated exon I.1 which is located at least 40 kb upstream from the start of translation in exon II (Means et al., 1991). At this time, the true distance is unknown since the genomic clones containing exon I.1 on the one hand and exon II on the other have never been overlapped. Employing various deletion mutations of the upstream flanking region of exon I.1, we and others have examined several putative regulatory sequences within this region and the proteins which interact with these sequences to regulate expression of aromatase in choriocarcinoma cells. Thus Toda and colleagues (1992) have identified a binding site for C/EBP-β which is located between -2141 and -2115 bp relative to the start of transcription in exon I.1. They further identified an element located between -238 and -200 bp which appears to synergize with the C/EBP-β element upstream. Yamada *et al,* (1995) identified two elements within -300 bp upstream of exon I.1 which recognize the same trans-acting factor that binds to the trophoblast-specific element previously located in the enhancer region of the human glycoprotein hormone α-subunit gene.

We have identified an imperfect palindromic sequence 5'-AGGTCATGCCCC-3' located at -183 to -172 bp which is responsible for stimulation of aromatase expression by retinoic acids (Sun et al., 1996). This does not function as a binding site for SF-1 since SF-1 is not expressed in placenta or in the cells, however, it does appear to bind a heterodimer comprising retinoid X receptor α (RXRα) and vitamin D receptor (VDR). It was reported recently that levels of RXR and retinoic acid receptor (RAR receptor expression increased during the process of cytotrophoblast differentiation into syncytiotrophoblasts in the placenta (Stephanon et al., 1994). This is coincident with the increase in aromatase expression. These results suggests that retinoids may indeed play an important role in developmental regulation of aromatase gene expression in the placenta.

EXPRESSION OF AROMATASE IN ADIPOSE TISSUE

We have found that aromatase expression does not occur in adipocytes but rather in the stromal cells which surround the adipocytes, and which may themselves be preadipocytes (Price et al., 1992a). These stromal cells grow in culture as fibroblasts. Consequently we have employed these cells in primary culture as a model system to study the regulation of estrogen biosynthesis in adipose tissue (Ackerman et al., 1981). When serum is present in the culture medium, expression is stimulated by glucocorticoids including dexamethasone (Simpson et al., 1981). Under these conditions P450arom transcripts contain primarily untranslated exon I.4 at their 5'-end (Mahendroo et al., 1993; Zhao et al., 1995a). We subsequently have characterized the region of the *CYP19* gene upstream of exon I.4 (Figure 2) and have found it to contain a TATA-less promoter, as well as an upstream glucocorticoid response element (GRE) and an Sp1 sequence within the untranslated exon, both of which are required for expression of reporter gene constructs in the presence of serum and glucocorticoids (Zhao et al., 1995a). Additionally, we found this region to contain an interferon-γ activating sequence (GAS) element. Such sequences are known to bind transcription factors of the signal transducer and activator of transcription (STAT) (Schindler et al., 1992; Darnell, et al., 1994; Zhong et al., 1994).

We have studied aromatase expression in samples of adipose tissue obtained from women of various ages, using competitive reverse transcription-polymerase chain reaction (RT-PCR) with an internal standard, and have found a marked increase in the specific content of P450arom transcripts in adipose tissue with increasing age (Bulun and Simpson, 1994), thus providing a molecular basis for the previous observation that the fractional conversion of circulating androstenedione to estrone increases with age (Hemsell et al., 1974; Edman and MacDonald, 1976). Furthermore, there are marked regional variations in aromatase expression, with highest values being found in adipose from buttocks and thighs as compared with abdomen and breast (Killinger et al., 1987; Agarwal et al., 1997).

We also used this RT-PCR technique to examine regional variations in aromatase expression in breast adipose tissue and have found that the highest expression occurs in adipose tissue proximal to a tumor, as compared to that distal to a tumor (Bulun et al., 1993a; Agarwal et al., 1996). This is in agreement with previous observations regarding the regional distribution of aromatase activity within breast adipose (O'Neill et al., 1988; Reed et al., 1993), as well as an immunocytochemical study (Sasano et al., 1994). These results

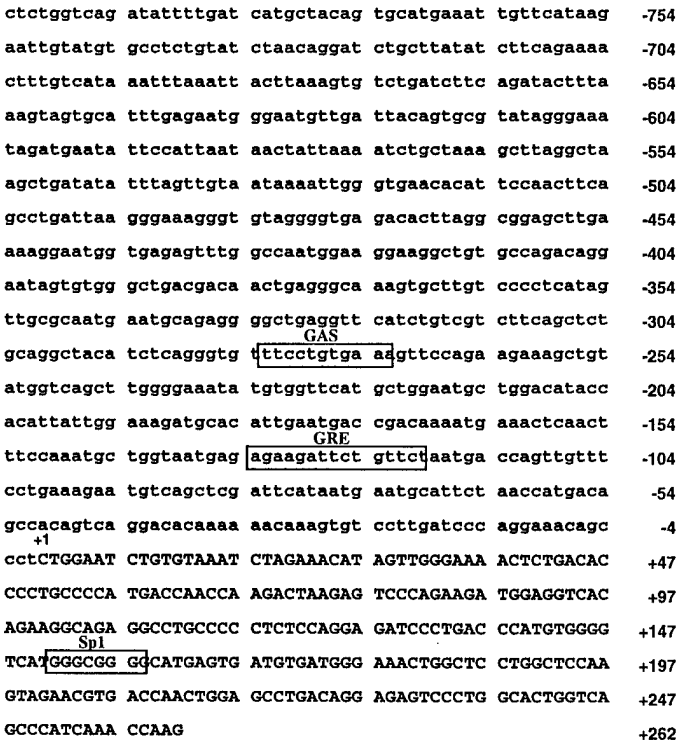

Figure 2. Sequence of the *CYP19* gene upstream of and including exon I.4. The start of transcription, determined by primer extension and S1 nuclease analysis, is indicated as +1. Consensus binding sites, namely GRE, GAS and Sp1, are shown in boxes. The nucleotides comprising exon I.4 are shown in capital letters.

suggest there is crosstalk between a breast tumor and the surrounding adipose cells in terms of the ability of the latter to synthesize estrogens, and that factors produced by developing breast tumors may set up local gradients of estrogen biosynthesis in the surrounding fat via paracrine and autocrine mechanisms(Simpson et al., 1994; Agarwal et al., 1996).

CYTOKINES WHICH STIMULATE AROMATASE EXPRESSION IN ADIPOSE TISSUE

Recently we observed for the first time that the effect of serum to stimulate aromatase expression in human adipose stromal cells (in the pres-

ence of glucocorticoids) can be mimicked by specific factors, namely members of the class I cytokine family, which includes interleukin-11 (IL-11), IL-6, oncostatin-M (OSM), and leukemia inhibitory factor (LIF) (Narazaki et al., 1994; Stahl et al., 1994). Members of this cytokine family employ a receptor system involving two different janus tyrosine kinase (JAK)-associated components, gp 130 and LIFRβ or a related β-component (Stahl and Yancopoulos, 1993). However, the IL-6 receptor complex includes a component whose cytoplasmic domain is apparently not involved in signaling (Stahl and Yancopoulos, 1993), and which can exist in a soluble form (Kishimoto et al., 1992). Recently an α-subunit of the IL-11 receptor complex has been cloned (Hilton et al., 1994), although this does not apparently exist in a soluble form. The concentration-dependence of the stimulation of aromatase by IL-6, IL-11, LIF, and OSM is indicative of high affinity receptor binding.

Addition of class I cytokines to adipose stromal cells resulted in a rapid phosphorylation of JAK1 (Zhao et al., 1995b). By contrast, JAK3 was not phosphorylated under these conditions to any significant extent, whereas JAK2 was phosphorylated to an equal extent both in the presence or absence of IL-11. As indicated by blotting with an anti-phosphotyrosine antibody and by inhibition in the presence of herbimycin A, this phosphorylation occurred on tyrosine residues present in the JAK1. Both gp130 and LIFRβ can associate with and activate at least three members of the JAK family, JAK1, JAK2, and TYK2, but utilize different combinations of these in different cells (Stahl et al., 1994), however, it is apparent that JAK1 is the kinase of choice in human adipose stromal cells.

This activation of JAK1 results in the rapid phosphorylation of STAT3 on tyrosine residues, but this was not the case for STAT1. Recently it has been shown that STAT3 is the substrate of choice for the IL-6/LIF/OSM cytokine receptor family, and that the specificity of STAT phosphorylation is based not upon which JAK is activated (Boulton et al., 1994; Stahl et al., 1994; Zhong et al., 1994), but rather is determined by specific tyrosine-based motifs in the receptor components, namely gp130 and LIFRβ, shared by these cytokines (Stahl et al., 1995). Finally, gel shift analysis indicated that STAT3 can interact with the GAS element present in the promoter I.4 region of the P450arom gene upon addition of IL-11 to these cells. This interaction in turn results in activation of expression, as indicated by transfection experiments employing chimeric constructs in which the region -330/+170 bp of the I.4 promoter region was fused upstream of the CAT reporter gene. The results

indicate that both deletion of the GAS sequence, as well as mutagenesis of this sequence, resulted in complete loss of IL-11- and serum-stimulated expression in the presence of glucocorticoids.

Activation of this pathway of expression by these cytokines is absolutely dependent on the presence of glucocorticoids. This action of glucocorticoids is mediated by the GRE element downstream of the GAS element (Zhao et al., 1995a). Additionally, the Sp1-like element present within untranslated exon I.4 also is required, at least for expression of the -330/+170bp construct (Zhao et al., 1995a). These sequences, while present within a 400 bp region of the gene, are not contiguous and the nature of the interaction between STAT3, the glucocorticoid receptor and Sp1 to regulate expression of the P450arom gene via the distal promoter I.4 remains to be determined. Our present understanding of the regulation of expression of aromatase in adipose tissue is summarized in Figure 3.

Recently, we have found that tumor necrosis factor α (TNFα) also stimulates aromatase expression in adipose stromal cells in the presence of dexamethasone. This action of TNFα is mimicked by ceramide, indicative that sphingomyelinase activity is involved in the TNFα response. This action of TNFα appears to involve promoter I.4, specifically an AP1 site upstream of the GAS element which binds a c-jun/fos heterodimer upon activation by TNFα (Zhao et al., 1996b).

MESENCHYMAL - EPITHELIAL INTERACTIONS IN REGULATION OF AROMATASE EXPRESSION IN ADIPOSE TISSUE

As indicated previously, adipose tissue is the major site of estrogen biosynthesis in elderly women and men. The fact that this expression is confined to the stromal cells rather than the adipocytes themselves is consistent with the known actions of IL-6, IL-11, and TNFα to inhibit the differentiation of 3T3 L1 fibroblasts into adipocytes (Keller et al., 1993), thus aromatase is a marker for the undifferentiated fibroblast state. As indicated previously, aromatase expression in adipose increases dramatically with age (Keller et al., 1993; Bulun and Simpson, 1994). There is also a marked regional distribution with expression being greatest in buttock and thigh regions as compared to abdomen and breast (Price et al., 1992b; Bulun and Simpson, 1994). However, within the breast there is also a marked regional variation with expression being highest at sites

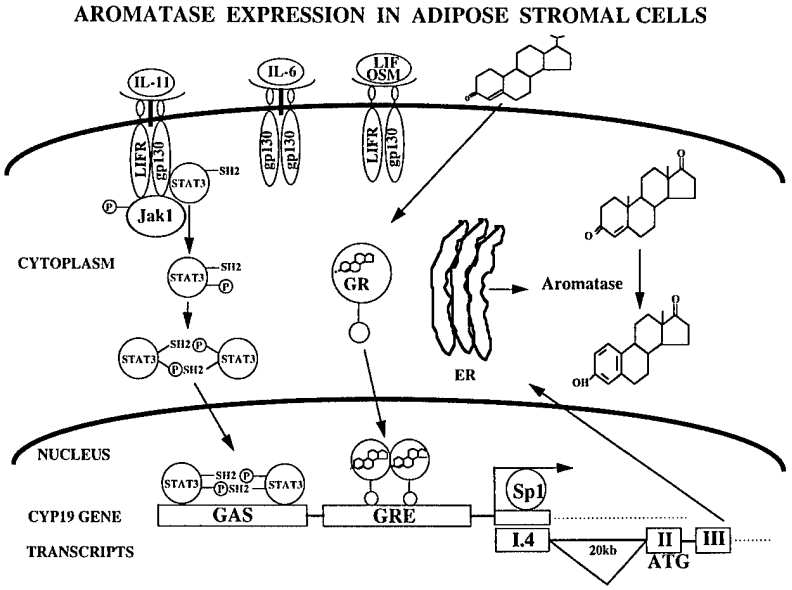

Figure 3. Schematic of second-messenger signaling pathways whereby Group I cytokines stimulate aromatase gene expression in human adipose stromal cells. JAK1 may be bound to the common receptor subunit gp 130 and activated following ligand binding and receptor dimerization, as a consequence of phosphorylation on tyrosine residues. STAT3 is recruited to binding sites on gp130 and is phosphorylated on tyrosine residues by JAK1. These phosphotyrosine residues are recognized by SH2-homology domains on STAT3, resulting in dimerization followed by translocation to the nucleus and binding to the GAS element of promoter I.4 of the aromatase gene. Following binding of glucocorticoid receptors to the GRE and Sp1 to its site on untranslated exon I.4, activation of transcription of the aromatase gene from promoter I.4 is initiated. Splicing of the initial transcript results in formation of mature mRNA which translocates to the ribosomes and is translated to give rise to aromatase protein.

proximal to a tumor as compared to those distal to a tumor (Bulun et al., 1993a; Agarwal et al., 1996).

Recently we developed a competitive RT-PCR technique to measure the levels of the various P450arom transcripts in adipose tissue. We found that in breast, abdomen, buttocks, and thighs of healthy subjects, I.4-containing transcripts predominated, with I.3- and II-specific transcripts in much lower abundance. Based on these findings we suggest that aro-

matase expression in adipose tissue may be under tonic control by circulating glucocorticoids and that regional and age-dependent variations may be the consequence of paracrine and autocrine secretion of stimulatory cytokines such as IL-6 and TNFα, the levels of which have been shown to increase with age (Wei et al., 1992; Daynes et al., 1993).

To our surprise, however, we found that the increase in aromatase expression in a tumor-containing breast was not due to an increase in I.4-specific transcripts, but rather of transcripts specific for promoters II and I.3 (Agarwal et al., 1996). Similar results have been obtained by Harada and colleagues (Harada et al., 1993). Since expression from these promoters is regulated by cAMP, these results strongly suggest that breast tumors secrete a factor(s) which stimulates aromatase expression in the surrounding stroma by increasing adenylate cyclase. Our recent evidence suggests that this factor is prostaglandin E_2 (PGE_2) (Zhao et al., 1996a). PGE_2 is a powerful stimulator of aromatase expression via both the protein kinase A (PKA) and protein kinase C (PKC) pathways. Moreover PGE_2 is known to be produced by breast tumor fibroblasts and epithelium, as well as by macrophages recruited to the tumor site (Schrey and Patel, 1995).

Such local paracrine mechanisms could be important in the stimulation of breast cancer growth by estrogens. Commonly, breast tumors produce a desmoplastic reaction whereby there is local proliferation of stromal cells surrounding the tumor, strongly indicative of the production of growth factors by the tumor. These proliferating stromal cells express aromatase, as indicated by immunocytochemistry (Sasano et al., 1994). It is possible then to propose a positive feedback loop whereby adipose stromal cells surrounding a developing tumor produce estrogens which stimulate the tumor to produce prostanoids, growth factors and cytokines (Dickson and Lippman, 1987). Some of these act to stimulate the further growth and development of the tumor in a paracrine and autocrine fashion. Additionally, these or other factors act to stimulate proliferation of the surrounding stromal cells and expression of aromatase within these cells. Thus a positive feedback loop is established by paracrine and autocrine mechanisms which lead to the continuing growth and development of the tumor.

AROMATASE EXPRESSION IN OTHER TISSUES

Stimulation of aromatase expression by serum in the presence of glucocorticoids is not confined to cells present in adipose tissue, but also has

been reported in skin fibroblasts (Berkovitz et al., 1989) and in hepatocytes derived from fetal liver (Lanoux et al., 1985). In each of these cell types the P450arom transcripts contain exon I.4 as their 5'-terminus (Harada, 1992; Toda et al., 1994); however, the factors which mimic the action of serum to stimulate aromatase expression in these cell types have as yet to be elucidated. Clones from intestine also predominantly contained exon I.4, as did a few from brain. Harada et al., (1993) also reported that a sequence identical to the one we call I.4 was present in transcripts from fetal liver and brain. Sequence-specific Northern and PCR analysis of fetal testes and ovary, and Sertoli cell tumors obtained from patients with Peutz-Jegher Syndrome, a condition characterized by the presence of estrogen-producing bilateral multifocal sex cord tumors, revealed mainly promoter II-specific sequence, similar to the situation in adult ovary (Bulun et al., 1993b). Interestingly, in an estrogen-secreting hepatocellular carcinoma, aromatase expression was driven by promoter II in contrast to the situation in fetal liver, or in adult liver where expression is undetectable (Bulun, unpublished observations). No exon I.1-specific sequences (the predominant sequence in placenta) were present in any clones isolated from these libraries.

A major finding in the last few years was the discovery of a new exonic sequence expressed in the brain of rat, monkey, and human (Honda et al., 1994; Mouri et al., 1995). This "brain-specific" sequence is the major 5'-terminus of transcripts in rat amygdala, and is also present in transcripts in the hypothalamus-preoptic area (HPOA) (Kato et al., 1996). Regulation of aromatase in brain differs from that in other tissues in that expression appears to be increased by androgens and either suppressed or not affected by cAMP (Lephart et al., 1992). In cultured cells derived from mouse embryonic hypothalamus, aromatase expression is elevated by α1-adrenergic agonists, but not those selective for α2- or β-adrenergic receptors. Substance P, cholecystokinin, neurotensin, and brain natriuretic peptide as well as phorbol esters and Bt_2 cGMP all increased aromatase expression, suggesting a major role of PKC and PKG pathways in this regulation, which is presumably mediated via the brain-specific promoter (Abe-Dohmae et al., 1996).

However, promoter II-specific transcripts have also been detected in amygdala and HPOA regions (Kato et al., 1996) and, as mentioned above, I.4-specific transcripts have also been detected in brain (Toda et al., 1994). So it may be that different promoters are employed in the various brain loci of expression, and that consequently the regulation is quite

different in different brain sites. It should also be noted that transcripts derived from the brain-specific promoter have been detected by RT-PCR in other non-neural cells, namely ovary, placenta, and in a human acute monocytic leukemia cell line (THP-1)(Shozu et al., 1996), although these were present in low abundance in these sites.

PHYLOGENY AND ONTOGENY OF AROMATASE EXPRESSION

Based on comparison of the sequences of the P450arom cDNAs with those of other members of the cytochrome P450 superfamily, it appears that P450arom is only distantly related to other steroidogenic forms of P450 and indeed is one of the most ancient of the cytochrome P450 lineages, apparently evolving more than 1,000 million years ago (Nelson et al., 1993). Certainly aromatase is present throughout the entire vertebrate phylum, but to our knowledge, has not been described in invertebrates. It would be of considerable interest therefore to know if the ancestral gene product is expressed in non-vertebrate phyla, and what reaction it catalyzes.

Inactivation of the SF-1 gene in mice by means of homologous recombination has indicated that this transcription factor is a critical developmental factor for the gonads as well as the adrenals, since these organs fail to develop in such animals (Luo et al., 1994). In the normal embryonic mouse, SF-1 expression is first detected in the genital ridge around embryonic day nine (Ikeda et al., 1994). It subsequently is expressed in the developing adrenal cortex. In genotypic males, testicular development is initiated following a burst of sex-determining region on Y (SRY) expression on embryonic day 11.5, and subsequently the testes express SF-1 in both the Leydig cells and in the Sertoli cells. In females, SF-1 is subsequently expressed in the developing ovary. This developmental expression of SF-1 in the embryonic mouse correlates well with the expression of steroidogenic enzymes as well as of anti-Müllerian hormone in the case of the male. In the mouse embryo, SF-1 is also expressed in the cells which give rise to the hypothalamus around embryonic day 11. As the hypothalamus develops, SF-1 is expressed in the ventral medial region. Subsequent to this, aromatase expression in the hypothalamus is detectable and increases with embryonic development reaching a maximum just prior to the onset of gestation (Ikeda et al., 1995).

An interesting question then arises as to the role of SF-1 in regulating the developmental expression of aromatase in the brain. The brain-specific promoter of the human aromatase gene contains an SF-1-like sequence in the intron immediately downstream from the brain-specific 5'-untranslated exon (Honda et al., 1994b). It remains to be determined if this element plays an important role in the expression of aromatase in this region of the brain. Aromatase is also expressed in other regions of the brain including the amygdala and preoptic nucleus. It is not clear whether the brain-specific promoter is responsible for expression of aromatase in all of these regions of the brain, since promoter II- and I.4-specific transcripts have also been detected in various brain sites (Toda et al., 1994; Kato et al., 1996).

Although little is known about expression of the aromatase gene in lower vertebrates, the sequence of an aromatase gene from the rainbow trout was recently published (Tanaka et al., 1995). In this case, the sequence immediately upstream from the start of transcription contained two sites with close homology to the mammalian SF-1 site, probably indicating that this is the promoter region for aromatase in the gonads of fish. Thus, not surprisingly, this proximal promoter is a primordial promoter of aromatase throughout the entire vertebrate phylum. In lower vertebrates such as fish, aromatase is also expressed to a very high level in the brain (Callard et al., 1978, 1980; Callard, 1981). Recently it has been shown that in the goldfish, two transcripts for aromatase exist, one in ovary and one in brain, that differ significantly throughout the entire coding region, indicative of the presence of two separate genes (Callard and Tchoudakova, 1996). Thus the question of the evolution of the human gene with a single coding region, but with multiple untranslated first exons, becomes a very interesting and complex issue. Another variant on this theme occurs in the pig (Corbin et al., 1995) in which placental transcripts differ from those in ovary at several regions in the coding region. This is consistent with one gene with alternatively-spliced coding exons. Thus the phylogenetic evolution of the aromatase gene will likely turn out to be very complex.

It is clear that in most vertebrates aromatase expression is confined to the brain and the gonads. However as previously indicated, in a number of mammals including primates and some ungulate species such as cow, pig, and horse, expression also occurs in the placenta. In all of these species it appears that a unique placental-specific distal promoter is employed (Hinshelwood et al., 1995). However, there appears to be little

sequence homology between the placental promoter regions of the human and bovine genes, making it difficult to propose that these arose from a common ancestral sequence. Furthermore, the creation of transgenic mice expressing a reporter gene downstream of the human promoter I.1 sequence has shown that expression of the reporter occurs in the mouse placenta, and only at that site (Graves et al., 1996). Since mouse placenta does not express endogenous aromatase activity this means that, nevertheless, the requisite regulatory proteins and transcription factors are present in the mouse placenta.

Since SF-1 is apparently not expressed in placenta this may explain why the proximal promoter of aromatase is not employed in this fetal tissue but rather a distal promoter is used which is regulated by mechanisms totally independent from those involving SF-1. Thus, whereas SF1 may play a critical role in both the phylogenetic and ontogenetic regulation of aromatase expression, the diversity of promoters of aromatase found in a number of mammalian species may reflect, in part, the need for alternative means of regulating aromatase gene expression in tissues where SF-1 is not present or else is not functional.

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