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Experimental evidence suggests interaction between the endocrine and immune systems. Functional interactions are likely since immune cells produce hormones and neuropeptides, and endocrine glands can produce a variety of cytokines. In spite of this, the monocytes/macrophages that are normally present in reproductive organs, including the breast; have mainly been studied from an immunological point of view. We propose to expand this horizon with the **novel hypothesis that differentiated local macrophages in breast tissue constitute an *in situ* source of estrogen acting in an autocrine or paracrine manner to regulate breast cell division and differentiation**. The scope of this study is to evaluate immune and non-immune regulatory mechanism on breast cancer cells

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MACROPHAGES, ESTROGEN AND THE MICROENVIRONMENT IN BREAST CANCER.

P.I. F. Naftolin, MD, PhD

Co-Investigator: Gil Mor, MD, Ph.D.

INTRODUCTION

The scope of this study is to evaluate immune and non-immune regulatory mechanism in breast cancer. Experimental evidence suggests interaction between the endocrine and immune systems. Functional interactions are likely since immune cells produce hormones and neuropeptides, and endocrine glands can produce a variety of cytokines. In spite of this, the monocytes/macrophages that are normally present in reproductive organs, including the breast; have mainly been studied from an immunological point of view. We proposed to expand this horizon with the **novel hypothesis that differentiated local macrophages in breast tissue constitute an *in situ* source of estrogen acting in an autocrine or paracrine manner to regulate breast cell division and differentiation**.

BODY

Macrophages, estrogen and the microenvironment of breast cancer

Estrogen is a breast cell promoter and has in many ways been associated with the incidence and cause of breast cancer. Estrogen sources include ovarian and extraglandular sites, including the breast, itself. In addition to the circulating estrogens, the sources that maintain estrogen concentrations in benign and malignant breast tissue remain unclear. Although macrophages may comprise up to 50% of the mass of breast carcinomas, they have never been investigated as possible sources of local estrogen formation. We developed new evidence that breast macrophages constitute an important *in situ* source of estradiol and that the amount produced is sufficient to mediate cellular proliferation via inducing growth factors and cytokines, or by direct action. We utilized immunohistochemistry and RT-PCR to study cell-specific aromatase expression in: (i) 29 breast tissue biopsies, (ii) human monocytes/macrophages and (iii) a myeloid leukemia cell line (THP-1) capable of differentiating into macrophages. Use of a breast cancer cell line (MCF-7) provided biological confirmation of the role of aromatization in cell proliferation. We demonstrated considerable amounts of immunoreactive-aromatase (irARO) in breast tissue macrophages and a positive correlation between the proportion of irARO present in macrophages and lesion severity. Using *in vitro* techniques, we demonstrated that monocytes and THP-1 cells require differentiation into macrophages to

produce aromatase in amounts approaching placental levels. Estrogen production by macrophages in breast tissue appears to be sufficient to stimulate the proliferation of adjacent epithelial cells and to autoregulate cytokine production. The amount of estrogen produced by THP-1 cells stimulated MCF-7 cells to proliferate, an effect blocked by aromatase inhibitors. These findings represent a new dimension of cellular regulation in breast tissue with major biologic implications that are amenable to pharmacological manipulation.

This work has been published in the journal of "Steroid Biochemistry and Molecular Biology" :67: 403-411, 1998 (See Appendixt 1)

The Fas/Fas ligand system: a mechanism for immune evasion in human breast carcinomas

Breast tumors are frequently associated with a predominantly lymphocytic infiltrate, which constitutes a cellular immune response against the tumor. In spite of this massive infiltrate, the immune response appears to be inefficient and the tumor is able to evade it. *We propose that in breast cancer, escape from immunological surveillance results from the induction of apoptosis of Fas[Fas Ligand (Fas L) receptor]- bearing activated lymphocytes by FasL-bearing breast cancer cells. This is because the binding of FasL results in caspase-induced apoptosis of the Fas-bearing cells.*

To test our proposal we studied the expression of FasL by human breast carcinomas and the MCF-7 breast cancer cell line using RT-PCR, immunohistochemistry and Western blot analysis. Moreover, we studied the presence of apoptosis and Fas expression in the lymphocytic population surrounding the tumor. Strong FasL membranous- and cytoplasmic staining was detected in ductal carcinomas and hyperplastic breast tissue, but it was absent from normal womens' breast tissue. No staining was found in normal glands in the non-tumor quadrants, however the normal appearing ducts surrounding the carcinoma (tumor quadrant) showed intense immunoreactivity. Apoptosis was found predominantly among the lymphocytic population, as well as in the blood vessels and fibro-fatty tissue close to the tumor. Further characterization of the apoptotic cells demonstrated that they were CD3-positive.

Our results suggest that breast tumors may elude immunological surveillance by inducing apoptosis of activated lymphocytes via the Fas/FasL system. [We have also demonstrated FasL RNA in other tumor types. This will be the subject of further scientific articles and funding applications]. Up-regulation of FasL expression in hyperplastic and normal breast ducts close to the tumor also suggests a possible role in early neoplastic transformation and proliferation

This work has been published in the journal "Breast Cancer Research and Treatment" :54: 245-253, 1999 (See Appendix 2)

Regulation of Fas Ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen

During neoplastic growth and metastasis, the immune system responds to the tumor by developing both cellular and humoral immune responses. In spite of this active response, tumor cells escape immune-surveillance. We previously showed that FasL expression by breast tumor plays a central role in the induction of apoptosis of infiltrating Fas-immune cells, providing the mechanism for tumor immune privilege. In the present study, we showed that FasL in breast tissue is functionally active, and estrogen and tamoxifen regulate its expression.

We have identified an estrogen-recognizing element (ERE)-like motif in the promotor region of the FasL gene, suggesting direct estrogen effects on FasL expression. This was confirmed by an increase in FasL expression (both RNA and protein) in hormone-sensitive breast cancer cells treated with estradiol. This effect is receptor-mediated since tamoxifen blocked the estrogenic effect. Interestingly, tamoxifen also inhibited FasL expression in estrogen-depleted conditions.

Moreover, while an increase in FasL in breast cancer cells induces apoptosis in Fas-bearing T cells, tamoxifen blocks the induction of apoptosis. These studies provide evidence that tamoxifen by inhibiting FasL expression, by avoiding the immune sanctuary and allowing the killing of cancer cells by activated lymphocytes. This partially explains the protective effect of tamoxifen against breast cancer.

This work has been published in the Journal of Steroid Biochemistry and Molecular Biology, 73 (2000) 185-194 (See Appendix 3)

Macrophage-derived growth factors modulate Fas ligand expression in cultured breast cancer cells.

Fas/Fas ligand (FasL) interactions play a significant role in the immune-privileged status of certain cell populations. Several macrophage-derived cytokines and growth factors appear to modulate this system. When a FasL expressing cell binds a Fas-bearing immune cell, it triggers its death by apoptosis. During this project, we demonstrated that macrophage-conditioned media induced FasL expression by breast cancer cells in a dose-dependent manner. To elucidate which macrophage product was responsible for the up-regulation of FasL, MCF-7 cell cultures were treated with the macrophage products PDGF, TGF-β_1, and ß FGF (see below). The first two, known to be elevated in breast tissue from women with breast cancer, induced a dose-dependent up-regulation of FasL expression, which was inhibited by specific antibodies to PDGF or TGF-β_1. Interestingly, ß FGF (which is not elevated in breast cancer) did not induce any response. These results suggest that the pro-inflammatory nature of the breast microenvironment induces the FasL expression by glandular epithelial cells, and signals Fas-mediated cell death of activated immune cells. This could be a mechanism for cancer cells to escape immune surveillance, grow and metastasize.

The present work is in preparation for publication. We summarize the results obtained so far:

Results:

a. Effect of macrophage-conditioned media(CM) on FasL mRNA expression

To evaluate the effect of activated macrophage secreted soluble products on FasL expression, normal mouse breast epithelial cells HC-11 cells, which normally do not express FasL, were incubated with CM obtained from differentiated macrophages/THP-1 cells. FasL expression was analyzed after a 24-hour incubation using RT-PCR analysis. As shown in figure 1, CM induced a marked increase in FasL expression detected at both the mRNA and protein level.

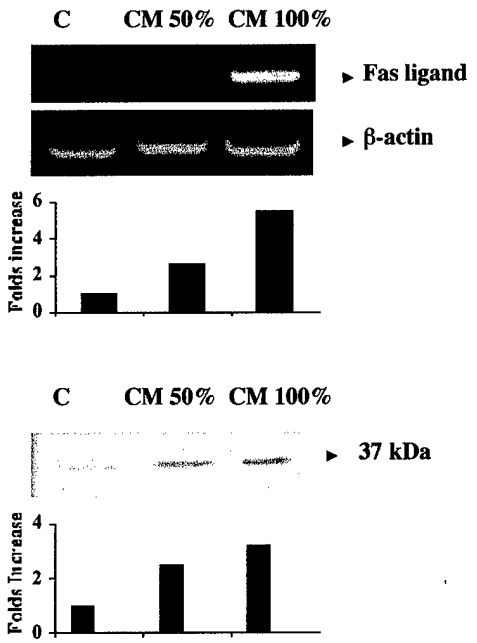

Fig 1 Effect of CM on normal epithelial breast cells. **1A**. RT-PCR. **1B** Western Blot analysis

b. PDGF, TGF-β_1, and βFGF modulation of FasL mRNA expression in beast cells

To characterize the macrophage-secreted soluble growth factors which could mediate the observed effect of CM on FasL expression, HC-11 cultures were treated with PDGF, TGF-β_1, and βFGF and the expression of FasL was analyzed both, at the RNA and protein levels.

- Time-course experiments:

MCF-7 cells were treated with PDGF (10 ng/ml), TGF-β_1 (1 ng/ml) and βFGF (10

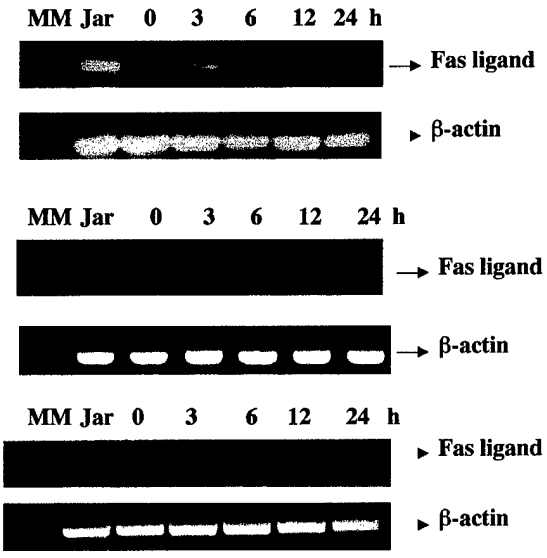

Fig. 2 Effect of cytokines on normal epithelial breast cells. **2A**. RT-PCR of cells treated with PDGF. **2B** RT-PCR of cells treated with TGF-β_1. **2C** RT-PCR of cells treated with bFGF

ng/ml), and incubated for time intervals of 0, 3, 6, 12, and 24 hours, after which the experiment was terminated. Treatment with PDGF or TGF-β_1 induced an early increase in the transcription of FasL observed at 3 hours, and which gradually decreased by 6 and 12 hours. Interestingly, no up-regulation of FasL expression was observed with βFGF, (Fig. 2).

c. Effect of CM, PDGF, and TGFβ_1 on FasL expression at the protein level

The FasL protein expression was examined by Western blot analysis of whole cell lysates. As demonstrated in figure 3, FasL was present in cell lysates obtained from HC-11 cultures treated for 24 hours with either CM, PDGF or TGF- β_1, confirming the previous mRNA findings.

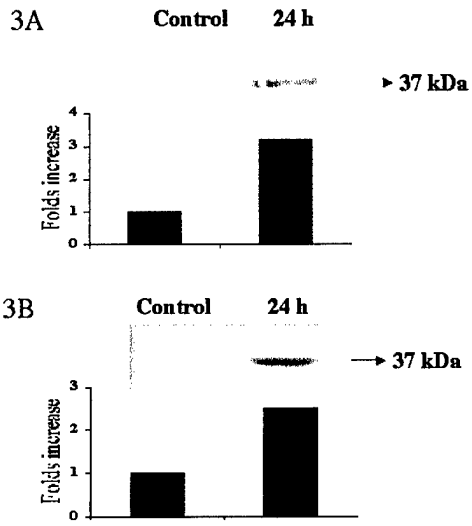

Fig 3. Effect of cytokines on normal epithelial breast cells. **3A**. Western Blot analysis of cells treated with PDGF. **3B** Western Blot analysis of cells treated with TGF-β_1

EXPRESSION OF FAS AND FAS LIGAND DURING PREGNANCY, LACTATION AND INVOLUTION, AND ITS POTENTIAL ROLE DURING MAMMARY GLAND REMODELING

Mammary involution is associated with degeneration of the alveolar structure and programmed cell death (PCD) of mammary epithelial cells. In this study we evaluated the expression of Fas and FasL in the mammary gland tissue and its possible role in the induction of apoptosis of mammary cells.

FasL-positive cells were observed in normal mammary epithelium from pregnant and lactating mice, but not in non-pregnant/virgin mammary tissue. Fas expression was observed in epithelial and stromal cells in non-pregnant mice but was absent during pregnancy. At day one after weaning high levels of both Fas and FasL proteins and caspase 3 were observed and coincided with the appearance of apoptotic cells in ducts and glands. During the same period, no apoptotic cells were found in the Fas-deficient (MRL/*lpr*) and FasL-deficient (C3H/*gld*) mice. Increase in Fas and FasL protein was demonstrated in human (MCF10A) and mouse (HC11) mammary epithelial cells after incubation in hormone-deprived media, before apoptosis was detected.

These results suggest that Fas-FasL interactions play an important role in the normal remodeling of mammary tissue. Furthermore, this autocrine induction of apoptosis may prevent accumulation of cells with mutations and subsequent neoplastic development. Failure of the Fas/FasL signal could contribute to tumor development.

This work has been published in the Journal of Clinical Investigation 106 (2000) 1209-1220 (See Appendix 4)

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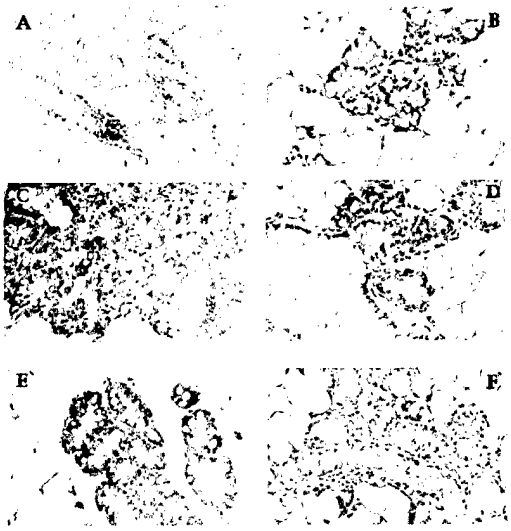
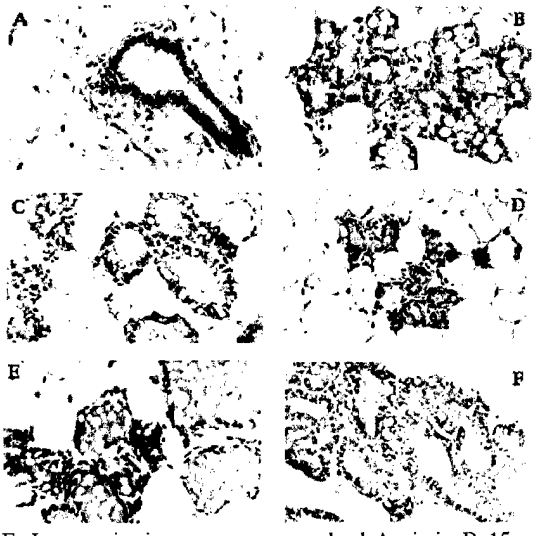
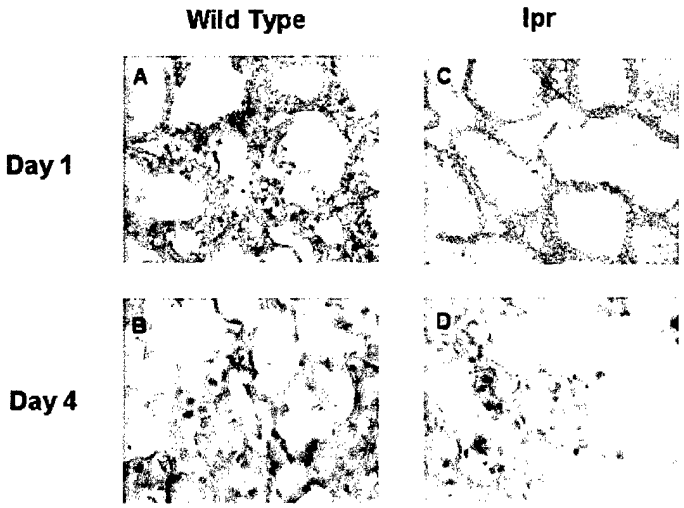


Fas expression in mouse mammary gland. A: virgin; B: 15 days of pregnancy; C: lactation; Involution: D: day 1; E: day 3, F: day 5



FasL expression in mouse mammary gland. A: virgin; B: 15 days of pregnancy; C: lactation; Involution: D: day 1; E: day 3, F: day 5



Localization of apoptosis in mouse mammary tissue during involution

METHYL-TESTOSTERONE INHIBITS AROMATASE ACTIVITY IN JAR CHORIOCARCINOMA CELLS AND MACROPHAGE-LIKE THP-1 CELLS IN CULTURE

17α-methyl-testosterone is a synthetic androgen with affinity for the androgen receptor. Methyl-testosterone is used widely as a component of hormone replacement therapy. Previous reports have indicated that contrary to testosterone, methyl-testosterone is not aromatized. However, methyl-testosterone still could affect local estrogen formation by regulating aromatase expression or by inhibiting aromatase action. Both possibilities have important clinical implications.

To evaluate the effect of methyl-testosterone on the expression and activity of aromatase, we tested the choriocarcinoma Jar cell line, a cell line that express high levels of P450 aromatase and macrophage-like THP-1 cells, which express aromatase only after undergoing differentiation.

We found that in both cell lines methyl-testosterone inhibits aromatase activity in a dose-related manner. The curve of inhibition parallels that of letrozole and gives 86% inhibition at 10^{-6} methyl-testosterone, determined by the tritium release assay. Methyl-testosterone does not have detectable effects on aromatase RNA and protein expression by JAR cells. Undifferentiated THP-1 cells had no aromatase activity and showed no effect of methyl-testosterone, but differentiated THP-1 (macrophage-like) cells had a similar inhibition of aromatase activity by methyl-testosterone to that seen in JAR cells. The Lineweaver-Burke plot shows methyl-testosterone to be a non-completive aromatase inhibitor.

Our results show for the first time that methyl-testosterone acts as a non-competitive aromatase inhibitor. These findings are relevant for understanding the effects of methyl-

testosterone as a component of hormone replacement therapy. Methyl-testosterone may, as a functional androgen and inhibitor of endogenous estrogen production, also offer special possibilities for the prevention/treatment of hormone-sensitive cancers.

This work has been submitted for publication in the journal of "Steroid Biochemistry and Molecular Biology" :2000 (See Appendix 5)

KEY RESEARCH ACCOMPLISHMENTS

We have shown, for the first time, the presence of an immune regulatory mechanism, the Fas/Fas Ligand system, in human breast tissue. This system responds to factors produced by macrophages and is sensitive to regulation by sex hormones, mainly estrogen. We have demonstrated a number of the control loops involved in the function of the immune regulatory systems in human and murine mammary gland.

RELEVANCE OF THE STUDY:

- Our results suggest that breast tumors may elude immunological surveillance by regulating via the Fas/FasL system, and thereby apoptosis of activated lymphocytes.
- Macrophages present at the tumor site increase, through cytokines and estrogen, the expression of FasL in the tumor, and further contribute to the "immune escape" of the tumor.
- We have described the regulation of FasL expression by cancer cells by estrogen. Moreover, these studies provide evidence that tamoxifen inhibits FasL expression, allowing the killing of cancer cells by activated lymphocytes. This partially explains the protective effect of tamoxifen against breast cancer.

• We have shown a role for the Fas/FasL system during mammary gland involution and in tissue remodeling. Understanding the mechanisms of tissue remodeling will help us to understand the protective effect of pregnancy and lactation against breast cancer.

REPORTABLE OUTCOMES

Manuscripts:

- Mor, G., Yue,W., Santen, R.J., Gutierrez, L., Eliza, M., Bernstein, L., Harada, N., Wang, J., Lysiak, J., Diano, S., Naftolin, F. (1998). Macrophages, estrogen and the microenviron- ment of breast cancer. Journal of Steroid Biochemistry and Molecular Biology: 67: 403-411
- Garcia-Velascol, J., Arici, A., Naftolin, F., Zreick, T., **Mor, G.** (1999) Macrophage-derived growth factors regulate Fas Ligand expression in endometrial stromal cells. A role in endometriosis. Molecular Human Reproduction. 5:642-6
- Diano, S.; Horvath, T.; Mor, G.; Register, T.; Adams, M.; Harada, N.; Naftolin, F. (1998) Aromatase and estrogen receptor immunoreactivity in the coronary arteries of monkeys and humans. Menopause: 6 (1) 21-28
- Gutierrez, L., Eliza, M., Niven-Fairchild, T., Naftolin, F., Mor, G. (1999). Fas/Fas-Ligand system induced apoptosis in human breast carcinoma: A mechanism for immune evasion. Breast Cancer Research and Treatment. 54 (3):245-253
- Mor, G., Kohen, F., Brown, S., Nilsen, J., Brown, W., Naftolin, F. (2000). Regulation of Fas Ligand expression in breast cancer cells by estrogen: Functional differences between estradiol and tamoxifen. Journal of Steroid Biochemistry and Molecular Biology:: 73: 185-194

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Personal

PI: Frederick Naftolin MD, D.Phil.

Co-Investigator: Gil Mor MD, Ph.D.

Degrees Obtained:

- Mariel Eliza (Yale class of 1999), presented her thesis for the Medical degree based on this work and received Honors.
- Rebecca Rosen (Yale class of 1999) presented her thesis for the Bachelor degree based on this work.
- Wendi Brown (Yale class of 1999) presented her thesis for the Medical degree based on this work and received Honors.
- Amanda Muñoz (Yale class of 2000) presented her thesis for the Bachelor degree based on this work.
- Rich Redlinger (Yale class of 2000) presented her thesis for the Bachelor degree based on this work.

CONCLUSION

The findings summarized in this report represents a new dimension of cellular regulation in breast tissue with major biological implications, amenable to pharmacological manipulation. We have shown not only that immune cells are a source of sex hormones, cytokines and growth factors affecting normal mammary cells and breast cancer cells'

growth and proliferation. We have also provided evidence that tumor cells employ immune regulatory mechanism to "escape" from immune surveillance.

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Appendixes

We provide five Appendices.

PERGAMON

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Macrophages, Estrogen and the Microenvironment of Breast Cancer

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Estrogen is a major mitogenic stimulus to established breast cancer. Estrogen sources include ovarian, extraglandular sites and breast tissue. Which source primarily maintains benign and breast cancer tissue estrogen concentrations remains unclear. While macrophages may comprise up to 50% of the mass of breast carcinomas, previous studies neglected to study them as possible sources of estrogen. We present evidence that breast macrophages constitute an *in situ* source of estradiol and that the amount produced is sufficient to mediate cellular proliferation. We utilized immunohistochemistry and RT-PCR to study cell-specific aromatase expression in (i) 29 breast biopsies, (ii) human monocytes/macrophages and (iii) a myeloid cell line (THP-1) capable of differentiating into macrophages. Use of a breast cancer cell line (MCF-7) provided biologic confirmation of the role of aromatization in cell proliferation. We demonstrated considerable amounts of immunoreactive-aromatase (irARO) in breast tissue macrophages and a positive correlation between the proportion of irARO present in macrophages and lesion severity. Using *in vitro* techniques, we demonstrated that monocytes and THP-1 cells require differentiation into macrophages to produce aromatase in amounts approaching placental levels. The amount of estrogen produced by THP-1 cells stimulated MCF-7 cells to proliferate, an effect blocked by aromatase inhibitors. Estrogen production by macrophages in breast tissue appears sufficient to stimulate the proliferation of adjacent epithelial cells and to autoregulate cytokine production. These findings represent a new dimension of cellular regulation in breast tissue with major biologic implications, amenable to pharmacological manipulation. © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Complex interactions between epithelial, stromal, endothelial and lymphoid cell components influence breast tissue proliferation. These dynamic cell–cell interactions require a well-developed array of intercellular communication signals. So far, most attention has been directed to the stromal and epithelial components of the breast and their products, whereas the potential role(s) and relative importance of migratory white blood cell types have received relatively limited attention.

Macrophages are a major component of the normal breast's stroma and comprise a substantial cellular component of the cell mass of breast carcinomas [1, 2]. Although they constitute a stable presence in normal breast tissue, a marked influx of macrophages from the circulation characterizes breast disease. For example, macrophages are found in large numbers during the first phase of tumor growth [3]. A salient feature of these cells is their ability to communicate with each other via a complex network of extracellular signals, including many cytokines and their soluble receptors. These macrophage factors are thought to be produced largely within the tumor and to act locally in an autocrine and/or paracrine manner [4]. Thus, contrary to their expected

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cytotoxicity towards tumor cells, tumor associated macrophages (TAM) may promote tumor growth through their secretion of growth factors [2].

Despite the low estrogen plasma levels characteristic of menopause, breast tumors excised from postmenopausal women contain estradiol concentrations similar to those from premenopausal patients [5]. Increased uptake from the circulating plasma estradiol produced in extraglandular sites via aromatase provides one explanation for the maintenance of high tissue estradiol levels [6]. *In situ* estrogen synthesis from plasma androgens via local estrogen synthetase aromatase (ARO) provides a more plausible explanation. Previous data from others and us have demonstrated aromatase in both epithelial and stromal breast cells. The activity in isolated and cultured stromal cells from breast has been shown to respond to known enhancers of aromatase with up to a 30,000 fold stimulation of aromatase expression [7]. Furthermore, recent evidence indicates that breast tumors produce factors able to enhance the activities of the enzymes involved in estrogen synthesis, thereby promoting an (estrogenic) environment favorable for tumor growth [8]. Many of these factors are macrophage products [9–11] and their expression has been shown to be regulated by estrogen [12, 13]. Thus, macrophages could be a major source of tissue estrogen, for example, during the climacteric.

Our prior studies and those of others neglected to examine the macrophage cells in breast tissue [14, 15]. Consequently, in the present study we investigated the relationship between TAM and local estradiol production in the breast using a variety of immunohistochemical, biochemical, molecular and biologic methods. We found that these cells may not only be the target for estrogen action, but may constitute a local source of estrogen to furnish both autocrine and paracrine regulation of the breast microenvironment.

MATERIALS AND METHODS

Clinical material-human breast tissue specimens

Human breast tissue from biopsies of normal breast ($N = 8$), ductal hyperplasia ($N = 9$) and *in situ* breast carcinoma ($N = 12$) were obtained from the Department of Pathology, Yale University School of Medicine. A signed written consent was obtained from each patient and the use of tissues was approved by the Yale University Human Investigation Committee.

Cells and chemicals

THP-1 cell line was purchased from the American Type Culture Collection (Rockville, MD). MCF-7 cells were kindly provided by Dr Bruggemeier (Ohio State University, Columbus, OH). RPMI 1640 medium and FBS were purchased from Life Technologies (Grand Island, NY). DMEM and penicillin–streptomycin solution were obtained from Gibco BRL (Gaithersburg, MD). Testosterone and estradiol were purchased from Steraloids (Wilton, NH). [1-^{3}H] Androstenedione (24.5 Ci/mmol) and [^{3}H] thymidine (20 Ci/mmol) were purchased from NEN Dupont (Boston, MA). Letrozole and ICI 182,780 were gifts from Novartis and ICI Pharmaceuticals, respectively. All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO).

Immunocytochemistry

The peroxidase–antiperoxidase immunocytochemical method described by Naftolin *et al.* was used [14]. This technique employ the polyclonal antibody of Dr Harada directed against highly purified aromatase [16].

Double staining of breast cancer tissue sections for ARO and CD68 was carried out to determine the identity of ARO+ cells morphologically resembling macrophages. For this, slides were incubated with CD68 and ARO as described above, but using SAP and DAB substrate for color development of CD68 (brown color) and Alkaline Phosphatase substrate Blue Kit (Vector) for ARO.

Induction of differentiation of THP-1 cells

THP-1 cells were induced into macrophage-like phenotype by incubation with PMA at a concentration of 6 ng/ml.

Aromatase (tritiated water release) assay

Aromatase activity was measured using the tritiated water release assay as previously described by Lipton [17]. Aromatase activity was expressed as fmol of estrone produced/mg of protein/h. Cell monolayers or pellets were lysed with 0.1 N sodium hydroxide to determine their protein content. The protein concentration of the homogenate was measured as described by Lowry *et al.* [18].

Preparation of conditioned media

THP-1 cells were seeded in 6-well plates at the density of 1×10^6 cells/well in phenol red-free RPMI-1640 with 5% DCC-FBS. The culture media was supplemented with PMA (6 ng/ml) or PMA plus testosterone (10^{-8} M) with or without the aromatase inhibitor, letrozole (10^{-7} M). The cells were cultured at 37°C in a CO_2 incubator for 24 h. The media was then removed from the plates, centrifuged and stored at 4°C for <48 h. These media were designated as THP-1-conditioned media (CM). Nonconditioned media consisted of fresh phenol red-free RPMI-1640 with 5% DCC-FBS. Supplementation of the nonconditioned media was carried out as for the THP-1-CM.

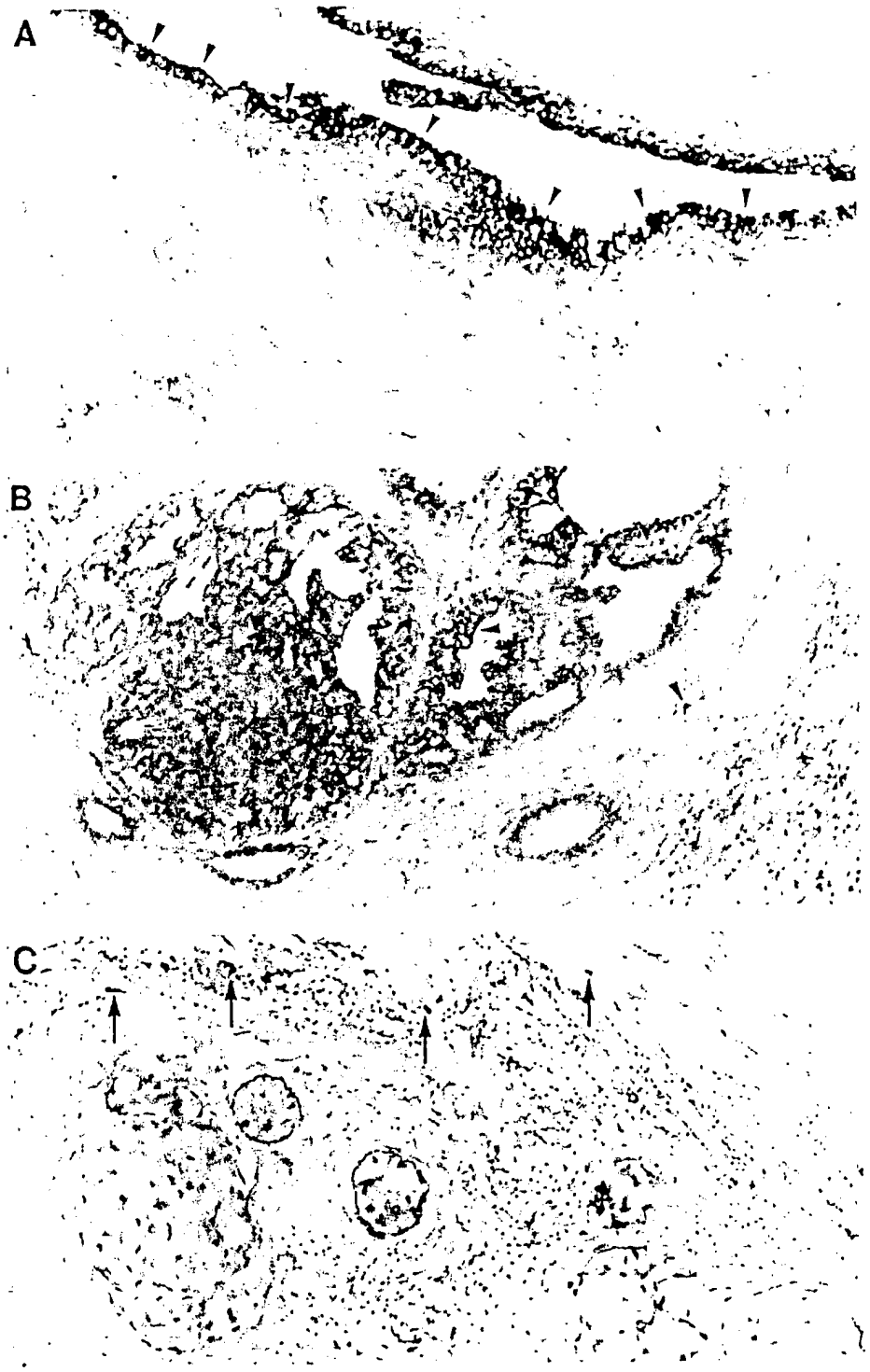

Fig. 1. Aromatase immunocytochemistry. (A) Section of fibrocystic breast tissue with normal ductal epithelium stained for irARO. Staining is localized to the breast ductal epithelium. irARO positive cells are absent from the stroma and fibrous tissue. Magnification, ×400. (B) Breast tissue section showing atypical ductal hyperplasia stained for irARO. Staining is mainly localized to the breast ductal epithelium; note, however, the presence of several irARO positive cells in the stroma. Magnification, ×400. (C) Section of an intraductal *in situ* breast carcinoma stained for irARO. Notice increased staining of the nonepithelial compartment of the lesion. Close inspection of the morphological characteristics of these irARO positive cells suggests they are macrophages (arrows, see Fig. 2). Magnification, ×400.

[^{3}H] Thymidine incorporation proliferation assay

MCF-7 cells were seeded in 12-well plates at a density of 0.15 million cells/well in regular DMEM and incubated for 18 h with the THP-1 CM or non-conditioned media, followed by a 2 h incubation with [^{3}H] thymidine (1 μCi/well). DNA extraction was carried out using the phenol–chloroform procedure. Two aliquots were made from the DNA solution: one for spectrophotometric measurement and the other for scintillation counting. Incorporated [^{3}H] thymidine was expressed as dpm/μg of DNA.

Human peripheral monocyte isolation/cell separation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood (taken at random throughout the cycle) from healthy females (without exogenous hormone treatment) and male volunteer donors between the ages of 24–32. PBMC were separated by fractionation on Ficoll Lymphocyte Separation Media (specific gravity 1.077). The interface (containing lymphocytes and monocytes) was removed and washed in an equal volume of PBS by centrifugation for 10 min at 1500 rpm. The pellet was then resuspended in 80 ul of phosphate buffered saline (PBS) with 5 mM EDTA and 0.5% BSA per 10^7 cells. A pure monocytic population was obtained as previously described [19] using the MiniMACS separation system from Miltenvi Biotec Inc. [20]. The positive fraction, containing the monocytes, was isolated for further studies.

RT-PCR and hybridization for ARO mRNA

Total RNA was prepared from frozen normal human placentas, breast tissue and from 2×10^6 monocytes and macrophages, using TRIZOL reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Macrophages were differentiated in culture for 48–72 h prior to RNA extraction. RT-PCR was performed using the RT-PCR kit from Pharmacia BioTech (Piscataway, NJ) according to the manufacturer's protocol. cDNA synthesis was carried out with 0.2 μg of pd(N)$_6$ and 10 μg of total RNA. The primers used for amplification of ARO mRNA have been recently described [21]. The PCR product was then separated by electrophoresis and transferred to blotting membrane by capillary elution. Southern hybridization was performed with the ECL 3′-oligolabelling and detections system (Amersham, U.K.) according to the instructions of the manufacturer.

RESULTS

Immunohistochemical localization of ARO in normal and malignant breast tissue

We initially compared the degree of breast aromatase immunostaining in biopsies from normal breast, ductal hyperplasia with accompanying fibrocystic disease and ductal *in situ* breast carcinoma. Localization of ARO in these breast tissues involved an affinity-purified antiserum antibody specific against ARO [16]. irARO was detected in stromal cells and tumor epithelial cells, as previously described [14]. The breast ductal epithelium exhibited variable staining for irARO; the intensity of irARO staining appeared to be inversely related to the degree of dysplasia, but this was not substantiated by formal morphometric analysis [Fig. 1(a)–(c)]. Conversely, staining of the nonepithelial compartment (stroma and fibrous tissue) was more intense in the more advanced lesions. irARO was also localized to the local inflammatory cells, more specifically to cells morphologically resembling macrophages [arrows, Fig. 1(c)]. To establish that these cells were macrophages, tissues were doubled-stained with a monoclonal antibody recognizing the tissue macrophage-specific antigen, CD68 and an antibody recognizing ARO. CD68 and ARO-positive cells were localized to the local inflammatory cells around and within the tumor [Fig. 2(a) and (b)]. Colocalization of ARO and CD68 immunoreactivity identified the cells expressing irARO as macrophages [Fig. 2(c)].

irARO, ARO and estrogen production by PMA-differentiated THP-1 myeloid leukemic cells and their effect on the proliferation of MCF-7 breast cancer cells

To study irARO expression in THP-1 myeloid leukemia cells and to show whether there is a biological effect of THP-1 cell-formed estrogen on nearby cells,

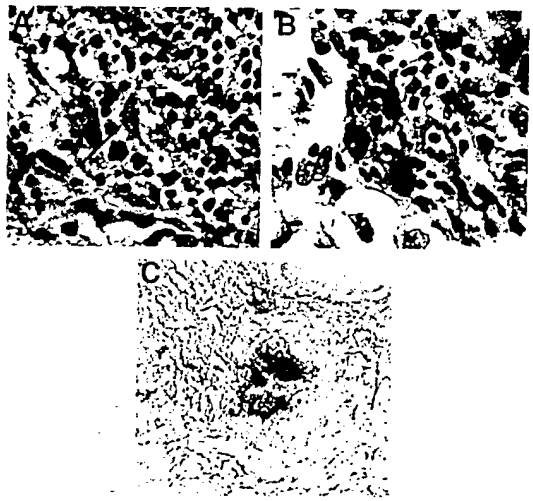

Fig. 2. Staining of breast cancer tissue sections for ARO and CD68. Immunostaining was carried out to establish the identity of ARO+ cells morphologically resembling macrophages, as shown in Fig. 1(B) and (C). For this, serial slides were incubated with anti-ARO (A) and anti-CD68 antibodies (B). Magnification, ×400. (C) A high magnification view of TAMs showing colocalization of irARO and CD68 immunoreactivity. Magnification, ×1000.

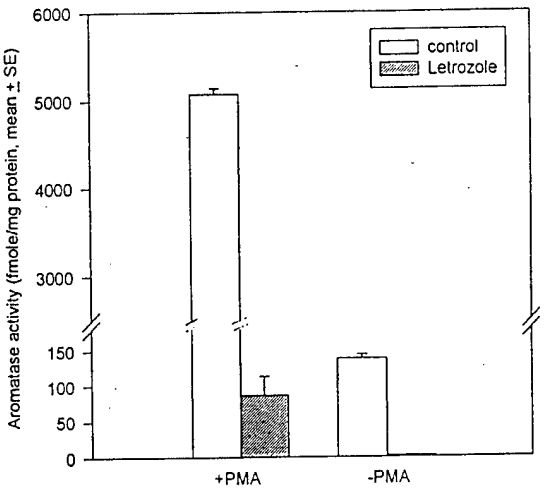

Fig. 3. Aromatase activity in THP-1 cells. ARO activity, as measured by the [^{3}H] water release method, is expressed as the amount of estrone produced by THP-1 cells cultured with or without PMA (6 ng/ml) for 24 h. PMA treatment resulted in a 30-fold increase in ARO activity in relation to basal ARO activity in the nontreated cells ($p < 0.001$). Both basal and PMA-induced ARO activity were suppressed by the ARO inhibitor letrozole (10^{-7} M).

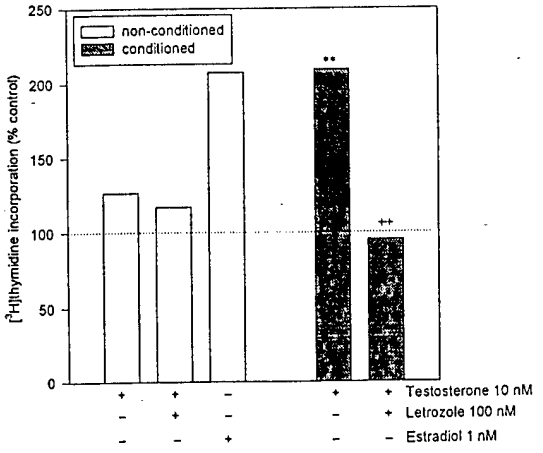

Fig. 4. Effect of THP-1 CM on MCF-7 cell proliferation. MCF-7 cells were treated with THP-1 CM or non-CM for 20 h. [^{3}H] thymidine incorporation into DNA is expressed as a percent of the levels of incorporation by MCF-7 cells in untreated cultures. Culture of MCF-7 in the presence of THP-1 CM resulted in a doubling of [^{3}H] thymidine incorporation. A similar proliferative effect was observed when MCF-7 cells were incubated in regular medium supplemented with 10^{-9} M of estradiol (E_2), as a control for the E_2 group. The corresponding medium containing PMA (6 ng/ml) was used as control for the other treatment groups. All experiments were repeated at least three times. The standard errors for all means were <10% of the mean. **$p < 0.01$ compared with PMA control; ++ $p < 0.01$ compared with PMA + T. (Student's t-test).

THP-1 cells were induced to differentiate in culture, using PMA. Using the ^3H_2O release method, undifferentiated THP-1 cultures incubated in the presence of ^{3}H testosterone (10^{-8} M) were found to produce 0.14 pmol estrone/mg of protein/h, while differentiated cells produced 5.1 pmol estrone/mg of protein/h (Fig. 3). Addition of the aromatase inhibitor, letrozole (10^{-7} M), during the incubation period significantly inhibited both basal and poststimulation aromatase activity levels (Fig. 3). Using the thymidine incorporation assay, THP-1 CM from the differentiated cell cultures was found to stimulate the *in vitro* growth (as evidenced by increased levels of thymidine incorporation) of MCF-7 breast cancer cells. Our ability to block THP-1 CM-induced MCF-7 cell growth by adding the aromatase inhibitor letrozol, confirms that the effect on proliferation was in fact due to estrogen (Fig. 4).

Immunohistochemical localization of ARO in peripheral human monocytes (PHM)

We studied whether expression of ARO depends on differentiation and whether this phenomenon is tissue-specific. To establish an *in vitro* model, we began by testing the ability of cultured PHM to express ARO. PHM were isolated using anti-CD-14-coated magnetic beads and cultured. In culture, PHM differentiated from typical monocytes [Fig. 5(a)] to the macrophage phenotype [Fig. 5(b)]. While irARO was absent in PHM at the beginning of culture [Fig. 5(c)], PHM cultured for >48 h were positive for irARO [Fig. 5(d)]. Similar results were obtained for the THP-1 cells [Fig. 5(e) and (f)].

Expression of ARO mRNA

To evaluate the expression of ARO mRNA, total RNA from human placentas, monocytes and *in vitro* differentiated macrophages was tested using RT-PCR. As illustrated in Fig. 6, ARO mRNA was identified in normal placenta (Fig. 6, lane 1). A similar size product (272 bp) was detected in the differentiated macrophages and THP-1 cells (Fig. 6, lanes 2 and 5). ARO mRNA was absent from the monocytes, lymphocytes and undifferentiated THP-1 cells (Fig. 6, lanes 3, 4 and 6).

DISCUSSION

Despite low estrogen plasma levels after menopause, breast tumors excised from postmenopausal women contain estradiol concentrations similar to those from premenopausal patients [22]; a possible explanation is *in situ* estrogen synthesis from plasma androgens via local ARO [23]. The present study further substantiates previous work establishing aromatase expression in breast tissue and suggests that a significant portion of the tissue estrogen may arise from TAMs. Therefore the study achieves its

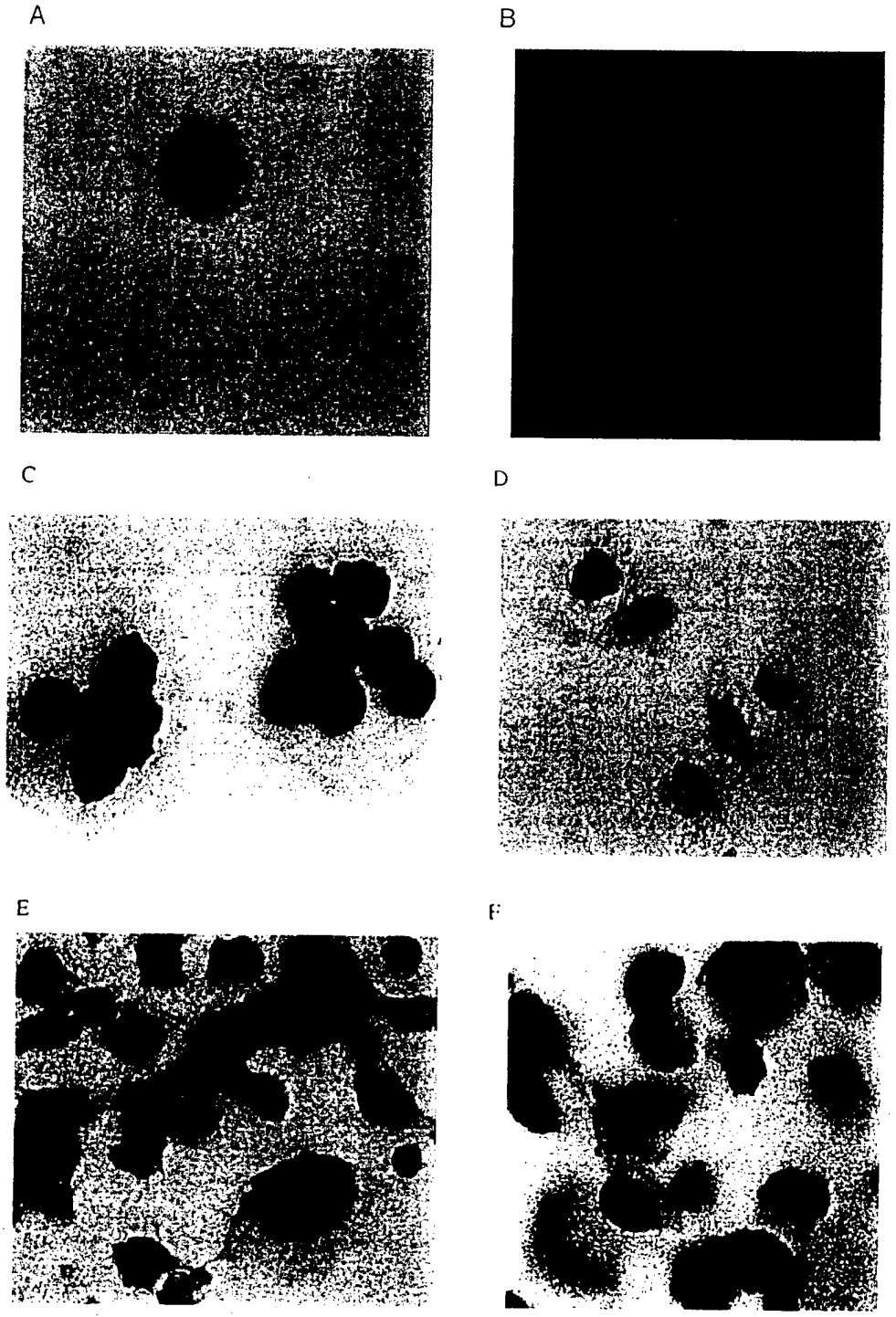

Fig. 5. Light micrograph of monocytes/macrophages. Note the differences in morphology between the two cell lines. (A) Freshly isolated monocytes are round with a horseshoe-shaped nucleus and a nuclear-cytoplasmic ratio of approximately one. (B) In contrast, after 48 h in culture, the macrophage-like morphology with pseudopods and a nuclear-cytoplasmic ratio <1 are evident. Magnification, ×1000. (C)–(D) Human monocytes stained for irARO immediately or after >48 h in culture. While no irARO was detected in freshly isolated monocytes (C), positive irARO was found in cells cultured >48 h. whose morphology resembles that of tissue macrophages (D). Magnification, ×1000. (E) PMA/differentiated THP-1 cells stained with anti-aromatase antibody show strong cytoplasmic irARO. (F) No staining is detected when the first antibody is omitted.

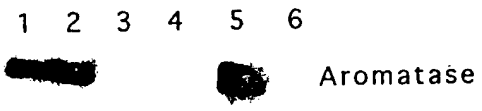

Fig. 6. ARO mRNA expression. To evaluate the expression of ARO mRNA, total RNA from human placentas, monocytes and *in vitro* differentiated macrophages was tested using RT-PCR. Hybridizable amplification products of ARO mRNA was identified in normal placenta (lane 1). A similar size product (272 bp) was detected in the differentiated macrophages and THP-1 cells (lanes 2 and 5). ARO mRNA was absent from peripheral monocytes, lymphocytes and undifferentiated THP-1 cells (lanes 3, 4 and 6).

objective of identifying the source of local estrogen formation in breast tissues and breast cancer. It is beyond the scope of the present study to quantify the actual amount of estrogen made in specific cell types in tissue sections which were obtained for the specific purpose of identification. Similarly, further studies will be required to assess the growth kinetics of the involved cells. This study also uncovers a new level of interaction between the immune and endocrine systems, which may be applicable to other organs throughout the body: that is, estrogen production by immune cells [24, 25].

In addition to the previously described presence of irARO in breast cells, detailed examination of breast tumors stained for ARO showed positive immunoreactivity among the leukocytic population, more specifically irARO was localized to cells morphologically resembling macrophages. To establish that these cells were indeed macrophages, sections were stained with a monoclonal antibody recognizing CD68, a macrophage-specific antigen. Immunoreactive cells were localized to the leukocytes around and within the tumor; thus, positive staining for CD68 identified the cells expressing ARO as macrophages.

Although their function within the tumor is not clear, up to 80% of the leukocytes in some breast carcinomas are macrophages [26, 27]. Tissue macrophages are recruited from peripheral monocytes, which are known to express estrogen receptors (ER) [19]. Upon entering the tissues, monocytes differentiate into macrophages capable of performing tissue-specific functions. Under the effect of local factors they may lose their ability to produce some proteins while becoming able to express the ones required for their new role [28]. Our *in vitro* studies suggest that the aromatase complex is one such product, since freshly isolated monocytes are irARO-negative, while cultured monocytes which have acquired the tissue macrophage phenotype become irARO-positive. Since ARO is not constitutively expressed by circulating monocytes, it must be induced, e.g. by differentiation. Whether this irARO expression is specific to the macrophages in breast tissue and the factors inducing it is presently under investigation.

Our *in vitro* studies using the THP-1 cells (a human premyeloid leukemia cell line that can be induced to differentiate by vitamin D_3 or PMA) support this theory of aromatase expression following differentiation of monocytes into tissue macrophages. Undifferentiated THP-1 cells were found to be negative for irARO, while PMA stimulated cells did not only acquire the morphology of tissue macrophages, but showed both strong cytoplasmic irARO and CD68 immunoreactivity.

Jakob *et al.* [29] have previously reported the expression of aromatase mRNA and enhancement of aromatization of testosterone in vitamin D_3 and PMA-treated THP-1 cells. In the present study, we compared and measured aromatase activity in PMA-treated THP-1 cells using the tritiated water release assay. This differentiation resulted in a dramatic increase in aromatase activity (more than 30-fold) compared to basal activity levels. In addition, a 98–99% decrease in both basal and poststimulation aromatase activity levels was achieved by culturing the THP-1 cells in the presence of the aromatase inhibitor, letrozole.

The biological significance of aromatase expression in PMA-differentiated THP-1 cells was examined by studying the effect of THP-1 CM on cultured MCF-7 cells. Incubation of MCF-7 cells for 20 h in testosterone (10^{-8} M) containing THP-1 CM resulted in a 2-fold increase in [^{3}H] thymidine incorporation. In the presence of regular media containing an identical concentration of testosterone no stimulation or changes in [^{3}H] thymidine incorporation by MCF-7 were observed. Furthermore, the stimulatory effect of testosterone (10^{-8} M) containing THP-1 CM on MCF-7 cells was abolished in the presence of the ARO inhibitor letrozole during the incubation period. The degree of stimulation, as reflected by the amount of [^{3}H] thymidine incorporation by MCF-7 cells incubated with THP-1 CM, was similar to that induced by the addition of estradiol (10^{-9} M) to the cultures. Likewise, the amount of estradiol synthesized by 1×10^6 THP-1 cells during a 24 h period was similar to previously reported estradiol concentrations produced by vitamin D_3 or PMA stimulated THP-1 cells [29]. These studies clearly demonstrate that growth stimulation of MCF-7 cells cultured in THP-1 CM is mediated by estrogen resulting from the aromatization of testosterone by THP-1 cells.

The presence of irARO in tissue macrophages indicates that these cells may be a major source of *in situ* estrogen formation in both normal and diseased breast tissues. These *in vitro* studies also suggest that macrophage-derived estrogen could contribute to the development and progression of breast tumors. Thus, *in situ* production of estrogen by the macrophages may constitute a new level of cellular regulation in the normal and malignant breast tissue. As mobile

regulatory cells, macrophages are free to reach regions of the breast where they could cooperate with factors that induce neoplasia or other pathologic changes. This characteristic mobility is concordant with reports of increased tissue estrogen levels in the breast quadrants where the tumor is located [30].

Although much is known about the general role of estrogen in the breast, the biological role of locally formed estrogen is not completely understood. Based on our studies with THP-1 CM and MCF-7 cells, we believe that estrogen production by macrophages in the breast may affect adjacent cells and autoregulate cytokine production from the macrophages, themselves (Fig. 7). This is an area of great importance, owing to the pleiotropic properties of many cytokines, such as IL-1, IL-6 and TNF-a. These cytokines have been shown to be involved in the pathogenesis of most age-related diseases, i.e. atherosclerosis, fibrosis, osteoporosis and others [31–34]. Recent studies have elucidated the role of cytokines such as IL-6, TNFα, IL-1 and IGF II in the regulation of estrogen synthesis [8]. We believe that the regulatory effect of these cytokines may not only be restricted to the breast cells, but to the macrophages as well, in what could potentially constitute a regulatory loop between the different cell components of the breast microenvironment. In addition, chemokine-attracting factors, such as MCP-1, have been shown to attract monocytes into a variety of tissues, including the breast [35, 36]. This is a self-regulating system [37], which implies a feedback loop between TAMs and local estrogen synthesis. Of course, the presence of this estrogen regulated influx of 'inflammatory cells' does not obviate the presence of a more traditional immune response.

The concept of local estrogen and its role in the breast microenvironment has wide implications, for example in the confusing area of the role of estrogen replacement therapy (ERT) and the pathogenesis of breast cancer. So far, the possible role of exogenous estrogen in breast carcinogenesis has been addressed without regards to local estrogen production and secretion. The effect of local estrogen formation could be powerful without overtly affecting circulatory estrogen, conversely, the amount of circulating estrogen levels would have to achieve extremely high levels in order to compete with the effects of local estrogen synthesis. The results of this study lead us to believe that the determination of the source, amount and role of *in situ* estrogen formation in breast disease could resolve apparent inconsistencies in theories that only consider the circulating estrogen levels to explain the

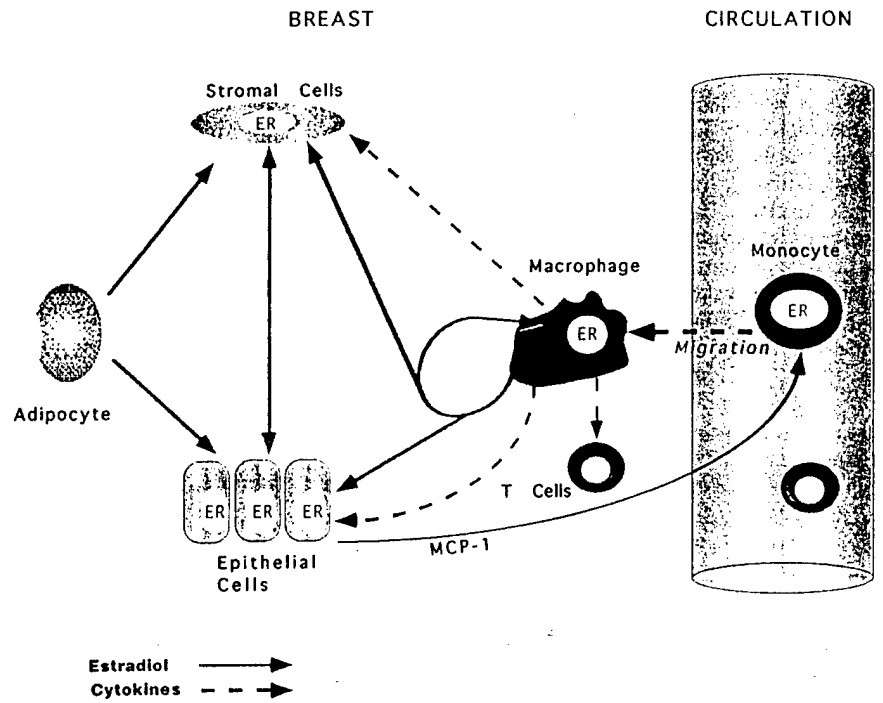

Fig. 7. Sources and effects of local estrogen formation in breast tissue. The life cycle of the peripheral monocytes includes entry into the tissues, to become macrophages. In the breast there is also a nonimmune interaction between the macrophages and nearby cells which includes autocrine and paracrine effects of macrophage-derived estrogen. There is also an inverse relationship between the secretion of chemokines (e.g. MCP-1) and local estradiol, which closes this regulatory loop. ER, estrogen receptors; MCP-1, monocyte chemotactic protein-1.

role of physiologic ERT in breast cancer pathogenesis [38]. Similarly, understanding how and how much estrogen-producing macrophages contribute to the microenvironment of the breast could help explain the success of antihormone treatments and open new therapeutic possibilities for breast cancer.

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Report

The Fas/Fas-ligand system: a mechanism for immune evasion in human breast carcinomas

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Key words: apoptosis, breast carcinoma, immunology, tolerance/suppression, tumor immunology

Summary

Breast tumors are frequently associated with a predominantly lymphocytic infiltrate, which constitutes an immune response against the tumor. In spite of this massive infiltrate, the immune response appears to be inefficient and the tumor is able to evade it. We propose that in breast cancer, tumor escape from immunological surveillance results from the induction of apoptosis of Fas-bearing activated lymphocytes by FasL-bearing breast cancer cells.

To test this proposal we studied the expression of FasL by human breast carcinomas and the MCF-7 breast cancer cell line by RT-PCR, immunohistochemistry, and Western Blot. Moreover, we describe the presence of apoptosis and Fas expression in the lymphocytic population surrounding the tumor. Strong membranous and cytoplasmic staining was detected in ductal carcinomas and hyperplastic breast tissue, but it was absent from normal breast tissue. No staining was found in normal glands in the non-tumor quadrants; however, the normal appearing ducts surrounding the carcinoma (tumor quadrant) showed intense immunoreactivity. Apoptosis was found predominantly among the lymphocytic population, as well as in the blood vessels and fibro-fatty tissue close to the tumor. Further characterization of apoptotic cells demonstrated that they were CD3+ cells.

Our results suggest the breast tumors may elude immunological surveillance by inducing, via the Fas/FasL system, the apoptosis of activated lymphocytes. Recent data have demonstrated FasL RNA in other tumor types. Upregulation of FasL expression in hyperplastic and normal breast ducts close to the tumor also suggests a possible role in early neoplastic transformation and proliferation.

Abbreviations: Con A: concanavalin A; FasL: Fas ligand; RT-PCR: reverse transcription-polymerase chain reaction.

Introduction

An important question in tumor immunology is why neoplasms expressing tumor-specific antigens are not eliminated as 'foreign' by the immune system. This process of evasion, also called 'tumor escape', has been suggested to result from the inability of the immune system to react to the tumor, because of either non-recognition of tumor antigens or non-reactivity secondary to insufficient co-stimulation, anergy, tolerance, or immunosuppression [1, 2].

Local immune-suppression due to factors derived from the tumor is a feature of many cancer types. In 1993, for example, O'Mahony et al. described the non-necrotic cell death (apoptosis) of activated lymphocytes associated with esophageal squamous cell carcinoma [3]. Further studies have identified tumor-derived immunomodulatory molecules, such as cytokines, amino sugars, and gangliosides [2, 4], as responsible for such a state which may result in tumor evasion.

Recent reports showing the expression of FasL in Sertoli cells of the testis and ocular tissues [5, 6], and more recently in the placental trophoblast [7], have provided new insights into the concepts of tolerance and immune-privilege. When grafted under the

kidney capsule, Sertoli cells expressing FasL were protected for longer periods against rejection. In the eye, constitutive FasL expression was shown to control the proliferation of Fas+ lymphoid cells entering this organ. Recently, we described the expression of FasL in the human placenta and its neoplastic form, the choriocarcinoma, and the presence of apoptotic cells within the surrounding inflammatory infiltrate [8, 9]. Moreover, FasL has been found to be expressed in several tumor cells [10, 11], suggesting that this system may constitute one of the mechanisms by which tumor cells successfully escape immune surveillance.

The Apo-1/CD95 (Fas) and CD95-ligand (FasL) are cell surface molecules that induce the programmed cell death or apoptosis of lymphoid cells and play an important role in maintaining an appropriate immune response [12, 13]. Following antigen-induced activation, T cells express Fas and can then be triggered to undergo apoptosis in response to FasL expressed by other activated T cells. This system is involved in the deletion of autoreactive lymphocytes and elimination of excess lymphocytes during and after an immune response to foreign antigens. Both activated T and B cells express high levels of Fas and are highly susceptible to apoptosis upon binding of Fas by its ligand [14].

Breast cancer is the most common and dreaded malignancy affecting women. It has an unpredictable course and a sustained risk of metastasis that spans 20 years or more after diagnosis and initial treatment. Unfortunately, the incidence of breast cancer is increasing, and it is estimated that one in nine women will develop the disease during her lifetime. Much is known about the hormonal and genetic aspects of the disease; however, very little is known about why the tumor, in spite of the presence of a massive infiltrate of leukocytes, is not rejected but rather continues growing and even metastasizes.

In the present report, we studied the expression of FasL by human breast carcinomas and the MCF-7 breast cancer cell line. We also studied the presence of apoptosis and Fas expression in the lymphocytic population surrounding the tumor. Our results suggest that breast tumors may elude immunological surveillance by inducing, via the Fas/FasL system, the apoptosis of the activated lymphocytes that would have otherwise mediated tumor rejection.

Materials and methods

Clinical material – human breast tissue specimens

Human breast tissue from mammoplasty surgery (n = 12), ductal invasive breast carcinoma (n = 18), and normal breast tissue from breast reductions (n = 7) were obtained from the Department of Pathology, Yale University School of Medicine. A signed written consent was obtained from each patient and the use of tissues was approved by the Yale University Human Investigation Committee. Following surgical excision, breast tissue specimens were frozen in liquid nitrogen for RNA extraction or fixed in 10% buffered formalin and paraffin-embedded for immunohistochemistry. Tissue blocks were stored at room temperature until 5-μm sections were cut.

Cells and chemicals

The human T cell line Jurkat and the breast carcinoma cell line MCF-7 were purchased from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium and FBS were purchased from Life Technologies (Grand Island, NY). All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture conditions

Jurkat cells were maintained in RPMI-1640 nutrient medium supplemented with 10% FBS, penicillin (100 unit/ml) and streptomycin (100 μg/ml), pH 7.15, and incubated at 37°C in a 5% CO_2 atmosphere. MCF-7 cells were cultured in phenol red DMEM supplemented with 5% FBS.

Immunohistochemistry for FasL, Fas, CD45, and CD3

Detection of FasL and Fas expression was performed using a rabbit polyclonal IgG containing anti-human FasL or Fas, respectively (Q-20 and N-20, Santa Cruz Biotechnology, Santa Cruz, CA). Detection of CD3 and CD45 positive cells was performed using mouse anti-human monoclonal antibodies (DAKO, San Diego, CA). Deparaffinized and rehydrated 5-μm thick sections were blocked for endogenous peroxidase activity with 0.3% H_2O_2 in methanol, washed in PBS, and pre-incubated with 10% normal goat or horse serum in PBS-5% BSA for 30 min. One microgram per milliliter of FasL and Fas antibodies were applied. For CD3 and CD45 detection, two

drops of the already diluted anti- CD3 antibody and CD45 respectively were applied. Slides were then incubated for 1 h at room temperature in a humidified chamber. After washing, sections were incubated for 1 h with a biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA) for Fas and FasL or a biotinylated horse anti-mouse for CD45. Streptavidin-peroxidase incubation for 30 min and color development with diaminobenzidine or amino-ethyl-carbazole (AEC) were then carried out. Following incubation with the first antibody, slides for CD3 detection were immersed directly in diaminobenzidine.

In situ 3′ end labeling of DNA for cell death detection

The presence in breast tissue sections of single strand DNA breaks indicating apoptosis was assessed using the TUNEL technique (*In situ* Cell Death Detection Kit, Fluorescent, Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Briefly, following deparaffinization and re-hydration of previously stained sections with anti-CD3, slides were incubated with proteinase K (20 μg/ml in 10 mM Tris/HCl, pH 7.4) for 30 min at 37°C. Samples were then treated with terminal deoxynucleotidyl transferase enzyme and fluorescein-labeled nucleotides for 60 min at 37°C in the dark. After washing with PBS, the slides were cover-slipped and analyzed under the fluorescence microscope. For Fas/apoptosis co-localization, slides already stained for Fas were treated as described above. An alkaline phosphatase-antifluorescein antibody was added and a nitro-blue-tetrazolium alkaline phophatase kit (Vector) was used for color development.

RT-PCR for FasL mRNA

Total RNA was prepared from frozen normal breast, breast carcinomas, and 2×10^6 Jurkat cells using TRIZOL reagent (GIBCO BRL, Grand Island, NY), according to the manufacturer's instructions. Prior to RNA extraction, Jurkat cells were stimulated in culture for 24 h with 5 μg of ConA. RT-PCR was performed using the RT-PCR kit from Pharmacia Bio Tech (Piscataway, NJ), according to the manufacturer's protocol. cDNA synthesis was carried out with 0.2 μg of $pd(N)_6$ and 5 μg of total RNA. The primers used for amplification of FasL have been recently described [15] and have the following sequence; upstream: 5′-ATAGGATCCATGTTTCTGCTCCTTCCACCTACAGAAGGA-3; downstream: 5′-ATAGAATTCTGACCAAGAGAGAGCTCAGATACGTTGAC-3. Each PCR cycle consisted of: a denaturation step at 94°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min for 10 cycles, followed by 35 cycles modified by a cumulative 5 s increase of extension time per cycle. The PCR products were analyzed on a TAE % agarose gel with ethidium-bromide.

Cloning and sequencing of FasL RT-PCR products

The RT-PCR products were cloned into PCR-Script Amp SK(+) plasmid vector (Stratagene, La Jolla, California) according to the manufacturer's instructions. Briefly, cDNA amplified with Pfu polymerase was purified by ethanol precipitation. Blunt-ended ligation was done at room temperature for 1 h. The ligation product was added to Epicurian Coli XL1-Blue MRF' Kan supercompetent cells for transformation and the cells were grown on LB plates with 50 μg/ml ampicillin, x-gal, and IPTG. Positive colonies were identified according to the method described by Sandhu et al. [16]. The cloned FasL RT-PCR product was sequenced at Yale's Keck DNA Sequencing facility using a fluorescent dideoxy chain termination system (Applied Biosystems, Inc.).

Western Blot analysis

Proteins were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed after blocking the membranes with 5% powdered milk. The primary antibodies (FasL monoclonal antibody, clone 33 (Transduction Laboratories, Lexington, KY), and FasL polyclonal antibody N-20 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1000 dilution. The secondary antibody (peroxidase-labeled horse anti-mouse, or goat anti-rabbit, Vector, Burlingame, CA), were developed with TMB Peroxidase substrate kit (Vector, Burlingame, CA).

Results

Immunohistochemical localization of FasL in malignant and normal human breast tissue

Paraffin sections from normal breast tissue ($n = 7$) and breast tumors ($n = 18$) were studied for FasL expression using a specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). In each case, intense immunoreactivity of tumor cells was detected,

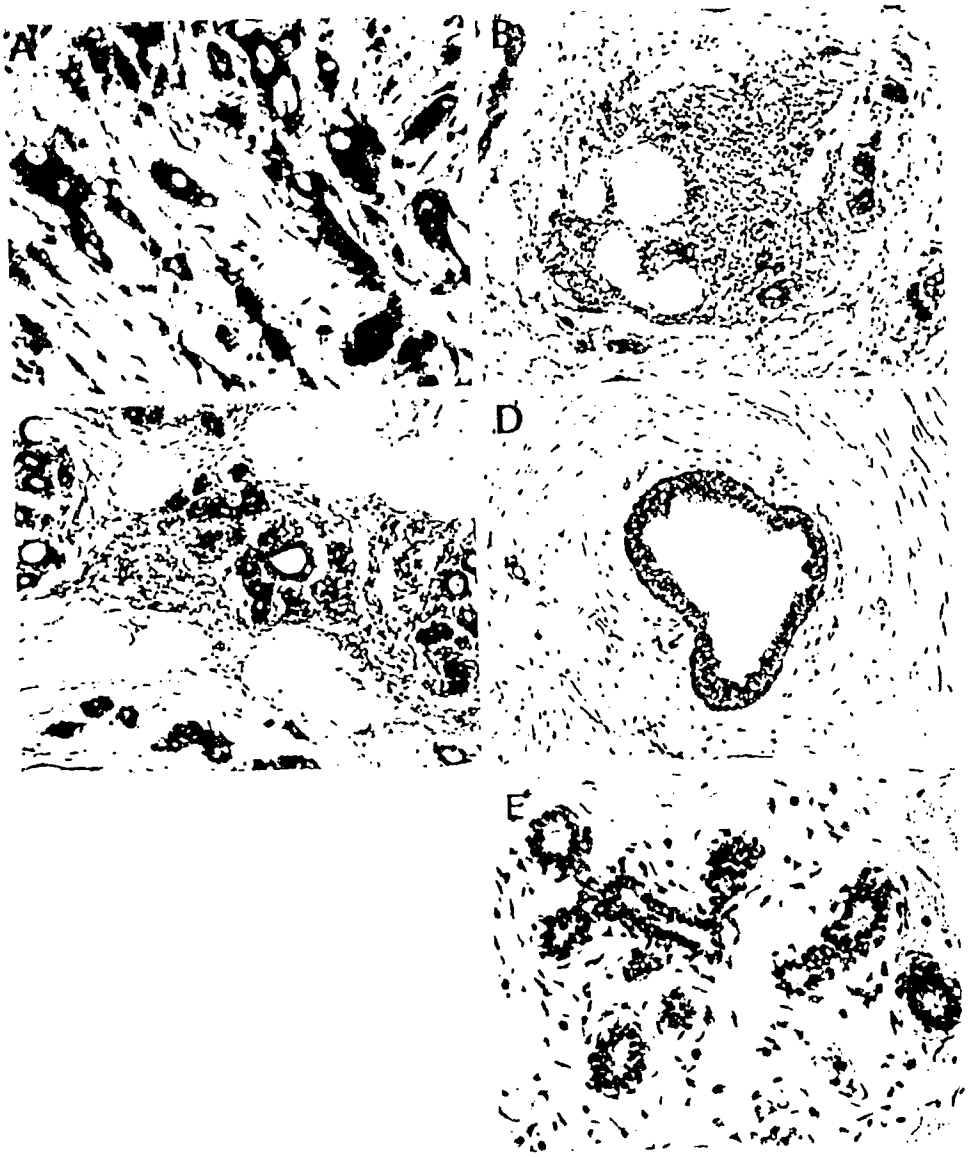

Figure 1. Immunohistochemical study of FasL expression in breast tissue: Paraffin sections of breast tumor were stained with anti-human FasL antibody. A. Invasive ductal carcinoma, showing strong cytoplasmic immunoreactivity, as well as few fibroblasts and macrophages within the stroma (magnification: 400×). B. FasL-positive tumor cells surrounded by an intense lymphocytic infiltrate. C. Apparently normal breast glands surrounding the tumor (T) exhibit positive immunoreactivity for FasL. Note the intense lymphocytic infiltrate. The ductal carcinoma as well shows strong FasL immunoreactivity (magnification: 400×). D. Paraffin section from hyperplastic duct obtained from non-tumor breast tissue: positive cells are found predominantly in the myoepithelium of the glands. The stroma is FasL-negative with the exception of some immunoreactivity present in blood vessels and fibroblasts near the ducts (magnification: 400×). E. Normal breast tissue obtained from a different quadrant of the tumor: No staining is evident.

including both cytoplasmatic and surface distribution. The staining was stronger in cells at the periphery of the tumor, mainly in areas of tumor invasion (Figure 1A). A high number of FasL-positive tumor cells surrounded by an intense lymphocytic infiltrate was also noted (Figure 1B). The lymphocytic cell population was predominantly FasL-negative, but FasL-positive lymphocytes were found in the area of the tumor (Figure 3C). Interestingly, FasL-positive immunoreactivity was found in normal appearing ducts and hyperplastic breast located in the same quadrant of the tumor, and was always surrounded by a strong inflammatory infiltrate (Figure 1C). In contrast, no immunoreactivity was found in the normal ducts in

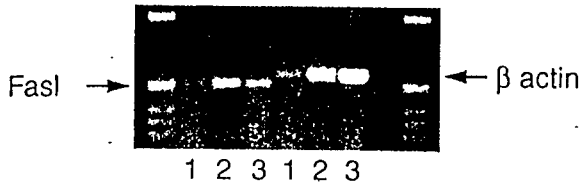

Figure 2. Expression of FasL mRNA in human breast tissue. FasL and β-actin mRNA were detected by RT-PCR in: the breast cancer cell line MCF-7 (lane 1), breast tumor (lane 2), and hypertrophic breast tissue (mammoplasty) (lane 3). The PCR product is consistent with the known FasL sequence.

quadrants located farther away from the tumor (Figure 1E). FasL expression in the hyperplastic breast tissue samples obtained after mammoplastic surgery showed a different pattern of immunoreactivity: positive cells were found in the basal epithelial layer of the glands while the luminal epithelial layer was predominantly negative (Figure 1D).

Expression of FasL mRNA in the MCF-7 breast carcinoma cell line, non-tumor breast tissue, and breast carcinoma

To confirm the results obtained from the immunohistochemical studies, we evaluated the expression of FasL mRNA in human breast tissue and the MCF-7 human breast cancer cell line. Total RNA was extracted and tested using RT-PCR. As illustrated in Figure 2, FasL RNA was identified in MCF-7 cells (Figure 2, lane 1), tissue from breast carcinomas (Figure 2, lane 2), and tissue from mammoplastic surgery (Figure 2, lane 3). The FasL RT-PCR products were cloned and their sequence determined. The sequence of the 521-bp product matched that previously published for the human FasL [17].

In situ cell death detection – terminal deoxy (d)-UTP nick end-labeling (TUNEL)

To determine the possible role of FasL expression by tumor cells in the induction of apoptosis of lymphocytes, we studied the presence of apoptosis in breast carcinomas. Apoptotic cells were detected by staining of DNA fragments with the TUNEL technique. TUNEL-stained nuclei were mainly found among the inflammatory infiltrate directly contacting the tumor (Figure 3A). No apoptosis, or very scattered apoptotic nuclei, was detected within the tumor. The lymphocytic infiltrate surrounding normal glands near the tumor displayed numerous apoptotic cells (Figure 3D). Further characterization of these cells with anti-CD45 and anti-CD3 antibodies identified these apoptotic cells as leukocytes and T cells respectively (Figures 3B and 3E). We were also able to detect apoptotic cells surrounding the blood vessels and in the fibro-adipose tissue (data not shown). On close inspection, apoptotic cells were detected inside the lumen, around the periphery of the blood vessels, and migrating through the walls of the vessels.

Co-localization of Fas and apoptosis

The induction of apoptosis by the FasL is mediated by the Fas receptor present in the target cell. The staining of breast tumor sections with an anti-Fas antibody (Santa Cruz, Santa Cruz, CA) revealed numerous Fas-positive cells. These Fas-immunoreactive cells were localized mainly to the inflammatory infiltrate around the tumor, as well as near normal appearing glands, vessels, and fibro-fatty tissue. In all the cases studied, the cells showed membranous staining (Figures 3C and 3F). The tumor component was Fas-negative, with only scattered immunoreactive intratumor leukocytes. Double staining for Fas and TUNEL revealed that these Fas-positive cells showed apoptotic features (Figure 3C; inset).

Western blot analysis

The specificity of the antibodies used for immunocytochemistry was evaluated using Western Blot analysis. Whole cell lysates from MCF-7 cell cultures and breast tissues, from which we had previously obtained mRNA, were electrophoresed in a polyacrylamide gel as described in 'Material and methods'. As seen in Figure 4, Clone N-20 and clone 33 recognize the same 37-kDa protein in a dose-dependent manner.

Discussion

Breast tumors are frequently associated with a predominantly lymphocytic infiltrate that constitutes an immune response against the tumor. Despite this massive infiltrate, the immune response appears to be inefficient and tumor cells evade it and metastasize.

Blockade of the responsiveness of T cells to tumor-specific antigens had major implications for tumor immunology. Apoptosis is one way by which the immune system generates tolerance towards antigens, and the Fas/FasL system is one mediator of apoptosis. In this study, we demonstrate FasL expression

Figure 3. *In situ* detection of apoptotic cells in breast tissue. Apoptotic cells were detected by staining of DNA fragments with the TUNEL assay. A. Apoptotic cells are present in the vicinity of breast tumor. TUNEL negative areas correspond to tumor sheets (T) (magnification: 600×). B. CD45 staining of breast tumor sections showing the distribution of the leukocytic infiltrate (I) and its relation to the tumor mass (T) (magnification: 1000×). D and E. Co-localization of apoptotic cells and CD3 antigen. Paraffin sections from the breast tissue were stained first with the anti-CD3 antibody and then with the TUNEL assay. Fluorescent microscopy reveals apoptotic cells in contact with a normal duct and within the fibroconnective tissue (white arrows) (D). Staining of the same section for CD3 (interface) demonstrates that the apoptotic cells are immunopositive (black arrows) (E). C and F. Fas expression is found in the leukocyte infiltrate (I) as well as in the fibroadipose tissue (F). C (Inset). Co-localization by double staining for Fas (pink cell membrane) and TUNEL (blue nucleus; immunohistochemistry with avidin-biotin-immunoperoxidase and alkaline phosphatase for TUNEL detection)(magnification: 1000×).

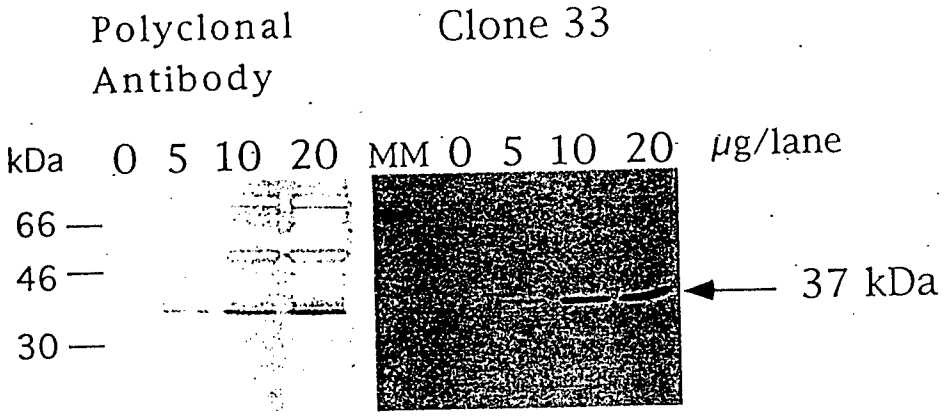

Figure 4. Immunoblot analysis of FasL protein in human breast cancer cell line. Serial dilutions: 20, 10, and 5 µg of protein samples derived from MCF-7 cell cultures were subjected to Western blotting with a polyclonal antibody N-20 and a monoclonal antibody clone 33. Both antibodies recognize the same 37-kDa band corresponding to the FasL, in a concentration-dependent manner.

by breast tumor cells and describe their spatial relationship to Fas-bearing apoptotic lymphocytes in the proximity of the tumor.

Previously proposed mechanisms for tumor evasion of immune surveillance include: (a) aberrant regulation of antigen processing and presentation [18]; (b) release of cytotoxic substances [3], or immunemodulatory amino sugars or gangliosides [1]; and (c) changes in the immunologic profile of the cell surface [19]. Contrary to the above described mechanisms, the Fas/FasL induced apoptosis of activated lymphocytes represents an 'on-the-spot' active, cell–cell mediated, and specific immunosuppressive process which defeats the immune system by locally inducing immune tolerance. The role of FasL in local immuneregulation was demonstrated by Ferguson and Griffith [20]. These investigators showed that injection of a virus into the anterior chamber of the eye in normal mice results in an inflammatory infiltrate which undergoes apoptosis while inflammatory cells in the B6 *gld* (FasL deficient) mice do not die. These results, together with the finding of FasL expression in the eye, suggest that it is the FasL in ocular tissues that controls inflammation.

In our study, we found that breast carcinoma tissues express FasL, as determined by RT-PCR (Figure 2). To confirm that the presence of FasL mRNA in the whole breast tissue is not a product of leukocytic contamination, we tested its expression in the cloned breast carcinoma cell line MCF-7. A similar PCR product to the one found in breast cancer was detected in MCF-7 cells (Figure 2). At the protein level, two different antibodies recognized the same 37-kDa protein corresponding to the FasL in whole lysates obtained from MCF-7 cells (Figure 4).

In breast cancer sections, FasL was localized to the cytoplasm and membrane of tumor cells and to the normal appearing glandular epithelium in the tumor quadrant. Strong immunoreactivity was also detected in the 'normal appearing' and hypertrophic glands near the tumor, but no staining was found in the tissue located farther away from the tumor quadrant. This data raises the question about these apparently 'normal' cells: are they already biochemically abnormally? Is the presence of FasL an early signal or marker of malignant transformation? Alternatively, could this unexplained expression of FasL be the result of paracrine stimulation from nearby cancer cells or other cells such as macrophages [21]?

We also found apoptotic cells among the leukocytic infiltrate localized near the tumor and glands, as well as in the blood vessels and fibro-fatty tissue close to the tumor. These apoptotic cells are CD3 immunopositive and therefore constitute apoptotic T cells. Previous findings have reported a progressive decrease in T lymphocytes consistent with the spread of the cancer [22]. Our findings of apoptotic CD3− and Fas-positive cells in the proximity of FasL positive tumor cells may explain the mechanism behind the reported progressive decline in the number of T cells. Apoptotic cells were not detected in the normal breast tissue or among the intratumor lymphocyte population. The absence of apoptotic CD3+ lymphocytes inside the tumor could be related to the dual role of these immune cells as reported by several investigators. There is now evidence that cytokines produced by cancer infiltrat-

ing leukocytes can either down-regulate or stimulate cancer cell growth [23]. This dilemma has not been solved and the role of these intratumor lymphocytes is under investigation. We hypothesize that the apoptotic T cells could correspond to activated lymphocytes expressing Fas, [24] while the intra-tumor CD3+ cells may represent a T cell population at a different stage of activation [25].

We found two different patterns of apoptotic cell distribution in the leukocyte population: (a) a large number of apoptotic cells expressing Fas were detected among the lymphocytic infiltrate in direct contact with the tumor, as well as in the apparently normal breast surrounding the tumor; and (b) a second group of apoptotic leukocytes was also observed in the fibro-fatty tissue and blood vessels. This last group of apoptotic leukocytes could represent a population susceptible to secreted (paracrine) FasL or cytokines produced by the tumor [26].

The finding of FasL expression in 'normal' breast tissue adjacent to the tumor, as opposed to the non-tumor quadrants, is important in understanding normal and tumor microenvironment, as well as tumor progression. We hypothesize that the immunological changes detected in the tumor are also present in the surrounding, apparently normal tissue as a result of the interaction or crosstalk between the two tissues. Tumors have been shown to secrete growth factors and other products and these may affect the surrounding normal tissue. Thus, an early step in the progression towards neoplastic transformation may include the expression and upregulation of factors such as FasL. This could be of value as a 'tumor marker' of the earliest nature. In any case, FasL expression results in neoplastic cells becoming able to modify the immune response directed against them by affecting the tumor microenvironment in a way that favors the successful escape of the tumor from immune surveillance.

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Regulation of Fas ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen

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Abstract

During neoplastic growth and metastasis, the immune system responds to the tumor by developing both cellular and humoral immune responses. In spite of this active response, tumor cells escape immune surveillance. We previously showed that FasL expression by breast tumor plays a central role in the induction of apoptosis of infiltrating Fas-immune cells providing the mechanism for tumor immune privilege. In the present study, we showed that FasL in breast tissue is functionally active, and estrogen and tamoxifen regulate its expression. We identified an estrogen recognizing element like-motif in the promoter region of the FasL gene, suggesting direct estrogen effects on FasL expression. This was confirmed by an increase in FasL expression in both RNA and protein levels in hormone sensitive breast cancer cells treated with estradiol. This effect is receptor mediated since tamoxifen blocked the estrogenic effect. Interestingly, tamoxifen also inhibited FasL expression in estrogen-depleted conditions. Moreover, an increase in FasL in breast cancer cells induces apoptosis in Fas bearing T cells and, tamoxifen blocks the induction of apoptosis. These studies provide evidence that tamoxifen inhibits FasL expression, allowing the killing of cancer cells by activated lymphocytes. This partially explains the protective effect of tamoxifen against breast cancer.

Keywords: FasL; Estrogen; Tamoxifen; Breast cancer

1. Introduction

Tumor growth and metastatic spread are not random mechanical phenomena. They are regulated by the interaction between breast cells, stroma, immune cells and surrounding tissue. This interaction is mediated in part by steroid hormones, growth factors and various cytokines that influence the behavior and phenotypic expression of breast cells [1].

Estrogen and progesterone have major roles in the normal physiology of the breast by regulating cell proliferation [2–4]. In addition, estrogens have been shown to influence the breast by regulating production of locally acting hormones, growth factors and cytokines [5]. The latter represent a system of signals that organize and coordinate cellular proliferation, migration and the interaction with other cell types such as the immune system [6]. At basal physiological levels, these factors provide a homeostatic environment, but at elevated levels, the balance shifts toward rapid cell division and transformation that can lead to neoplastic proliferation. In addition, growth factors and hormones acting through paracrine and autocrine mechanisms play important roles in other aspects of neoplastic transformation [7,8]. This report addresses these local actions in breast cancer and how they affect the 'escape' of transforming or neoplastic cells from immune surveillance. The 'immune escape' which contributes to successful tumor growth and metastasis may be due to the inability of the immune system to react normally to reject the tumor. This could be a consequence of non-recognition or non-reactivity of tumor antigens, in-

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duced by anergy, tolerance or immunosuppression [9–11]. We and others have shown that the escape of tumor cells from immune surveillance is an active process and is mediated by the Fas–FasL system [12–14].

The Fas–Fas ligand (Fas–FasL) system is a primary mechanism for the induction of apoptosis in cells and tissues [15]. The Fas–FasL interaction is the mechanism for peripheral clonal deletion and control of T-cell expansion during immune responses and for killing by cytotoxic T-cells [16]. Fas, also called APO-1 or CD95, is a type I membrane protein of 45 kDa that belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family [15]. FasL, a type II membrane protein of 37 kDa, also belongs to the TNF superfamily [15,17]. Fas is normally expressed in various tissues such as thymus, liver, heart and kidney [18], and its expression by T and B cells is enhanced after lymphocyte activation. In contrast, FasL expression was reported originally to be restricted to activated T cells [15,17].

More recently, FasL expression has been reported in non-immune cells, mainly in cells from immune-privileged tissues, suggesting that the Fas–FasL system may play an important role in the mechanism underlying immune privileged status [19]. These data suggest that FasL expression by tumor cells may contribute to creating an immune privileged site and immunosuppression. Thus, FasL expression has been detected in stromal cells of the retina, Sertoli cells in the testis [20,21] and in the human placenta, mediating trophoblast invasion/proliferation [12,22]. This appears to be true for cancer as well. FasL has been shown to be expressed in melanomas [23], myeloma [24], colon cancer [25], choriocarcinoma [12] and breast cancer [26]. Furthermore, it has been shown that cancer cells induce apoptosis in Fas-sensitive, but not in Fas-insensitive lymphoma cells [27]. Despite all this information, the factors regulating FasL expression on cancer cells, including those in breast cancer, have not been elucidated.

Currently the main indication for the use of anti-estrogens in mammary carcinoma arises from the observation that estrogen is a mitogen in breast cancer and approximately one-third of patients will respond to endocrine therapy. The main therapeutic anti-estrogen that has been used is tamoxifen. Since estrogen is known to regulate many aspects of the immune response, including the production and secretion of cytokines [28,29], and the Fas–FasL system could be an important mechanism for the anti tumor effect of tamoxifen, we tested the hypothesis that, estrogen regulates FasL expression in breast tissue and that tamoxifen acting as an anti-estrogen down regulates FasL, preventing tumor escape from immune surveillance. We used an in vitro system to show by reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot analysis and RNase protection assay that the expression of FasL in hormone sensitive breast cancer cells is regulated by estrogen. Furthermore, we show that FasL present in the cancer cells is active since it induces apoptosis in Fas positive T cells but not in Fas resistant immune cells. In demonstrating that tamoxifen inhibits FasL expression, we found that this occurred independent of the presence or absence of estrogen.

2. Material and methods

2.1. Chemicals

Dulbeccos Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Life Technologies (Grand Island, NY). 17-β estradiol and tamoxifen were purchased from Sigma (St. Louis, MO). The tamoxifen was proven pure by high performance liquid chromatography (HPLC).

2.2. Cell culture

The human breast cancer cell line MCF-7 and the ductal breast carcinoma cell line T47D, were purchased from ATCC (Rockville, MD), and cultured in DMEM media containing antibiotics-antimycotics (1% vol/vol) and fetal bovine serum (10% vol/vol) at 37°C in a humidified chamber (5% CO_2 in air). Cells were passed by standard methods of trypsinization, plated in six-well dishes and allowed to replicate to 80% confluence. Afterward cell cultures were treated in 'estrogen-depleted conditions' consisting of serum-free and phenol red-free DMEM-F12 media for 24 h before the treatment with sex hormones/anti hormones was initiated.

2.3. Preparation of total RNA and protein samples for Western blot analysis

Total RNA and protein were prepared from MCF-7 and T47D cells using TRIzol® reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. The TRIzol® method allowed us to extract RNA and protein from the same cells. This is an advantage since we were able to study the same samples at both the mRNA and protein level.

2.4. RT-PCR analysis

RT-PCR was performed using the RT-PCR kit from Pharmacia BioTech (Piscataway, NJ) according to the manufacturer's directions. cDNA synthesis was performed with $pd(N)_6$ 0.2 μg and 5 μg total RNA. The primers used for amplification of FasL have previously been described [27] and have the following sequence: upstream, 5'-ATAGGATCCATGTTTCTGCTCTTC-

CACCTACAGAAGGA-3″; downstream, 5′-ATAGAATTCTGACCAAGAGAGAGCTCAGATACGTTGAC-3″. Each PCR cycle consisted of denaturation at 95°C, 30 s; annealing at 52°C, 30 s; and elongation at 72°C, 1 min, for a total of 35 cycles. The PCR products were analyzed in TBE 1.2% agarose gel with ethidium bromide.

The FasL signal was measured by a densitometer and standardized against the actin signal using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA). The linearity of the system was determined using serial dilutions of cDNA and the regression of dilution factor on amplified cDNA was linear ($y = 2881.125x - 785.75$) and the correlation coefficient was $r = 0.994$ (Fig. 1A).

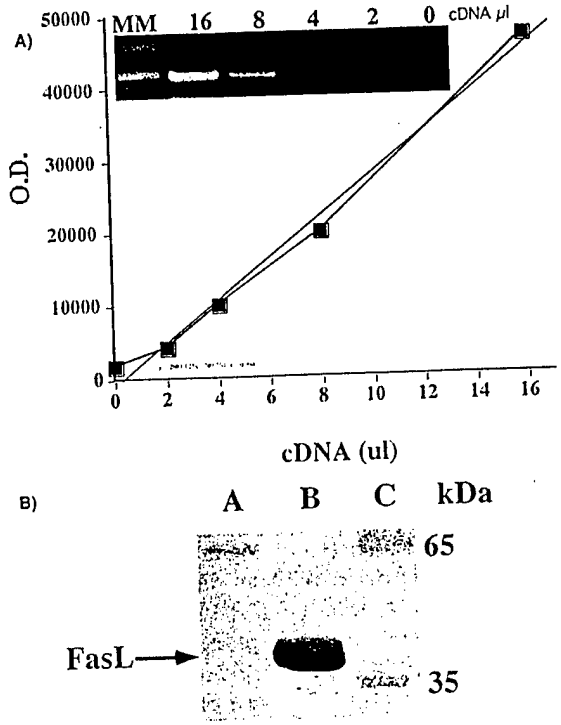

Fig. 1. A: RT-PCR linearity. The linearity of the PCR system was determined using serial dilutions of cDNA. The FasL signal (inset) obtained after RT-PCR was densitometered and standardized against the beta-actin signal using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA). The insert is a representative gel of the RT-PCR. Values are arbitrary units provided by the computer software according to a gray scale. B: Specificity of the monoclonal antibody for FasL, clone 33. Western blot analysis of Jurkat cell lysates was probed with the monoclonal antibody for FasL (clone 33, Transduction Technologies). Clone 33 recognize a 37-kDA protein in PMA (10 ng/ml) stimulated Jurkat cells (lane B) but not in the unstimulated Jurkat cells (Lane A). C = molecular markers.

2.5. Western blot analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immuno-blotting was performed after blocking non-specific binding by the membranes with 5%-powdered milk. The blots were incubated first with the primary antibody (FasL monoclonal antibody, clone 33, Transduction Laboratories, Lexington, KY at 1:1000 dilution) for 1 h. After washing, the membranes were incubated with the second antibody, peroxidase labeled horse anti-mouse gamma globulin (Vector, Burlingame, CA) for another hour. Finally, the blots were developed with TMB Peroxidase substrate kit (Vector, Burlingame, CA). The specificity of clone 33 for FasL was previously demonstrated [30]. Here we further confirmed its specificity by using Jurkat cells stimulated with concavalin A (Fig. 1B). Jurkat cells were previously shown to produce FasL upon concavalin A or anti-CD3 stimulation [31].

2.6. RNase protection assay (RPA)

The synthesis and labeling of the templates was done using the RiboQuant system (PharMingen, San Diego, CA) according to the manufacturer's instructions. In brief the Multiprobe, hAPO-3, which contains templates for FasL, Fas and the housekeeping genes L-32 and GAPDH was labeled with [$-^{32}$P]UTP using T7 RNA polymerase. Ten micrograms of total RNA was hybridize for 16 h at 56°C. mRNA probe hybrids were treated with RNase + Proteinase K and extracted with phenol-chloroform. Protected hybrids were resolved on a 5% acrylamide/bis gel, dried under vacuum and exposed to Kodak film for 24–72 h at −70°C. Densitometry was performed using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA).

2.7. Co-culture DNA fragmentation assay (the JAM test)

Target Jurkat cell (Fas-positive) death resulting from the co-culture with effector MCF-7 tumor cells (FasL-positive) was quantified by measurement of target cell DNA fragmentation using the JAM assay [32]. Adherent MCF-7 breast cancer cells were seeded into the wells of a flat 96-well microtiter plate at a cell number appropriate to give the required E/T ratios. Target Jurkat cells' DNA was labeled by prior incubation with 10 Ci/ml of [^{3}H]TdR at 37°C for 24 h. Labeled Jurkat cells were washed and added to the seeded effector cells in a final volume of 200 µl/well. After co-culture at 37°C for 8–24 h, the cells were removed from the wells and filtrated onto glass fiber filters using an automatic

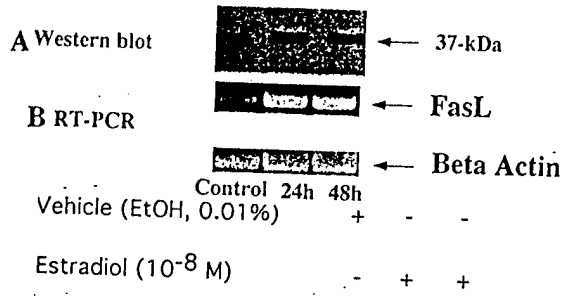

Fig. 2. Effect of estrogen on FasL expression. MCF-7 cells grown in phenol free DMEM and serum free conditions for 24 h were treated with 17-β-estradiol (10^{-8} M) for 24 and 48 h. Control cells received 0.01% Ethanol in the same phenol and serum free media. A: Western blot analysis. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels, transferred to nitrocellulose membranes, and blotted with FasL monoclonal antibody (clone 33). The secondary antibody (peroxidase labeled horse anti-mouse) was developed with TMB Peroxidase substrate kit. The FasL signal was standardized to the amount of protein loaded by staining the membrane with Ponceau Red and analyzed with a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA). The figure is representative of three independent experiments. B: RT-PCR for FasL was performed with total RNA extracted from the same in vitro monolayer cultures of MCF-7 cells as described for the Western blot. β-Actin housekeeping gene was amplified to verify that the same amount of cDNA was loaded in each lane. Each experiment was performed at least three times.

96-well filtration unit. The cells were then lysed with hypotonic buffer and their DNA was washed through the filter by four washes with D/D water. The radioactivity of intact chromosomal DNA retained on each filter was measured by liquid scintillation counting. Specific cell killing was calculated using the following equation according to Matzinger [32]:

$$\% \text{ Specific Killing} = (S - E/S) * 100$$

where E (experimental) is cpm of retained (complete) Jurkat cell DNA in the presence of MCF7 effector cells and S (spontaneous) is cpm of retained DNA in the absence of effector cells.

3. Results

3.1. Effect of estrogen on FasL expression

Previously, we showed that breast tumor cells express FasL and induce apoptosis of immune cells. This allows the tumor cells to escape from immune surveillance [26]. To examine the role of estrogen in the immune-surveillance mechanism, we used the human breast cancer cell lines MCF-7 and T47D, and monitored FasL mRNA and protein expression by RT-PCR, RPA and Western blot analysis. Breast cancer cells were treated with 17-β-estradiol (10^{-8} M) for 24 and 48 h and the relative levels of FasL mRNA were quantified. Fig. 2B shows a representative gel for analysis of PCR products. We found that the treatment of MCF-7 or T47D cells with estradiol resulted in an increase in FasL mRNA levels at 24 and 48 h (Fig. 2B). A similar effect was found at the protein level: Western blot analysis tested with a monoclonal (clone 33) or a polyclonal antibody (clone N-20) for FasL showed an increase in the expression of FasL following treatment with estradiol (Fig. 2A).

3.2. Estrogen modulation of FasL expression in human breast cancer cells

To further characterize the observed effect of estrogen on FasL expression, MCF-7 cells were treated with 17-β-estradiol (10^{-8} M) for time intervals of 0, 3, 6, 18 and 24 h after which the experiment was terminated. Effects of estrogen treatment on FasL mRNA expression were detected by RPA and RT-PCR. This effect presented a biphasic pattern, showing a strong band as early as 3 h of incubation and diminishes thereafter. A secondary increase in the FasL band is seen at 24 h (Fig. 3). Quantitative densitometry of the gels shows maximal FasL mRNA levels after 3 h of estrogen stimulation, followed by a 40% decrease from 6 to 12 h, with a definite but smaller increase at 24 h. At the protein level, increase of FasL expression was present only after 24 h of incubation with estradiol, remaining high up to 48 h (Fig. 2A and Fig. 3). Similar results were found with T47D cells (data not shown).

3.3. Effect of tamoxifen on estrogen-stimulated FasL expression

To determine if the increase of FasL expression was indeed estrogen receptor-mediated, we examined the effect of tamoxifen on estradiol-treated MCF-7 and T47D cells. As expected, and as shown in Fig. 4, the addition of tamoxifen (10^{-7} M) inhibited the stimulatory effect of estrogen, both at the protein (Fig. 4A) and at the mRNA level (Fig. 4B). This supports the idea that estrogen's increase of FasL expression is a receptor-mediated action. However, when both cancer cells were treated with tamoxifen alone we found inhibition of FasL mRNA and protein expression, suggesting an estrogen-independent effect of tamoxifen (Fig. 4 lane 3).

3.4. Inhibitory effect of tamoxifen on FasL expression

We further characterized the inhibitory effect of tamoxifen using RT-PCR and Western blot analysis. MCF-7 cells were incubated with increasing concentrations of tamoxifen for 24 h. RNA was analyzed by RT-PCR. As shown in Fig. 5, treatment with tamoxifen inhibited FasL mRNA expression in a dose-dependent

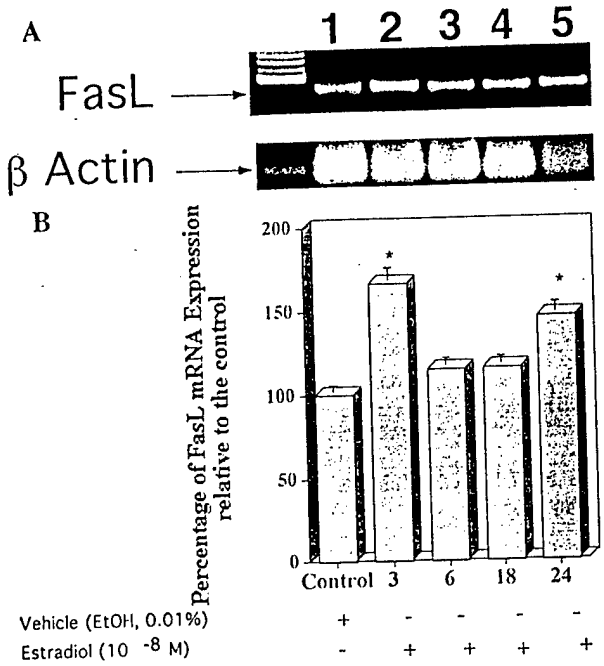

Fig. 3. Effect of estrogen on FasL mRNA expression, time response. MCF-7 cells were treated with 17-β-estradiol (10^{-8} M) for 3, 6, 18 and 24 h. RPA assay and RT-PCR were performed using 5 μg total RNA. The intensity of the products was calculated using a digital imaging and analysis system as described in Section 2. A: Representative PCR gel stained with ethidium bromide. Each experiment was repeated at least three times. B: Quantification of the FasL signal. Estrogen increases FasL mRNA expression after 3 h (lane 2), followed by a decline (lanes 3 and 4) and increases again after 24 h (line 5). $^{*}P < 0.01$ control vs. treated cells. The intensity of the products is given in the y-axis as the percentage expression relative to the control. Beta actin expression was used as internal control for each individual sample. Error bars represent standard error of the mean (S.E.M.). Statistical significance was determined by ANOVA.

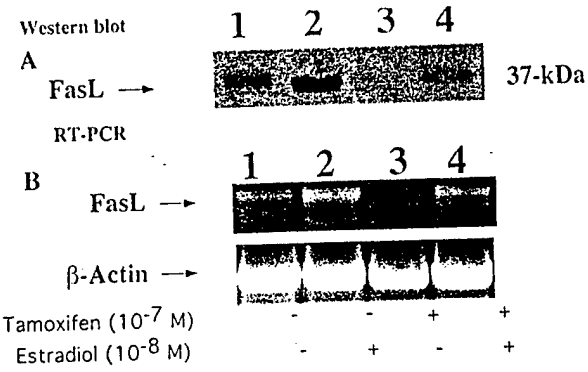

Fig. 4. Effect of estrogen and tamoxifen on FasL mRNA expression. MCF 7 or T47D cells were treated with estradiol (10^{-8} M), tamoxifen (10^{-7} M) or estrogen plus tamoxifen. Twenty micrograms of protein from each sample was subjected to 10% SDS-PAGE and transferred to nitrocellulose. Immuno-blot analysis was performed as described in Section 2. A: Representative Western blot for T47D cells. As shown in Fig. 2A for MCF-7 cells, estrogen also increases the expression of FasL in T47D cells. The administration of tamoxifen to the culture blocked the stimulatory effect of estrogen (Lane 4) and inhibited FasL expression when administrated in estrogen depleted conditions (Lane 3). B: Representative RT-PCR of T47D cell treated with estrogen and tamoxifen. Five micrograms of total RNA from the same samples described in A were reverse transcribed followed by PCR with specific primers for FasL and beta actin. The products were separated by electrophoresis in a 1.5-% agarose gel and stained with Ethidium bromide. The same effect of estrogen and tamoxifen described at the protein level was also found at the mRNA level.

manner (Fig. 5A). Quantification of the signal with a desitometer, shows significant decrease, 53% ($P > 0.001$; n: 5) and 44% ($P > 0.001$; n: 5) at concentrations of tamoxifen of 10^{-6} and 10^{-8} M, respectively. Similarly, using Western blots we found a decrease at the protein level (Fig. 4 and data not shown). When we treated cells with tamoxifen for different periods of time, and tested for FasL mRNA expression, we found a 20% decrease in the signal as early as 3 h of incubation reaching a peak of inhibition at 12 and 24 h (Fig. 6).

3.5. RPA for FasL mRNA

We further tested the direct effect of tamoxifen on FasL mRNA expression by RPA using a series of apoptosis gene templates, each of distinct length and each representing a sequence in a distinct mRNA species. The advantages of the multiple probe-RPA approach are its sensitivity and its capacity to simultaneously quantify several mRNA species in a

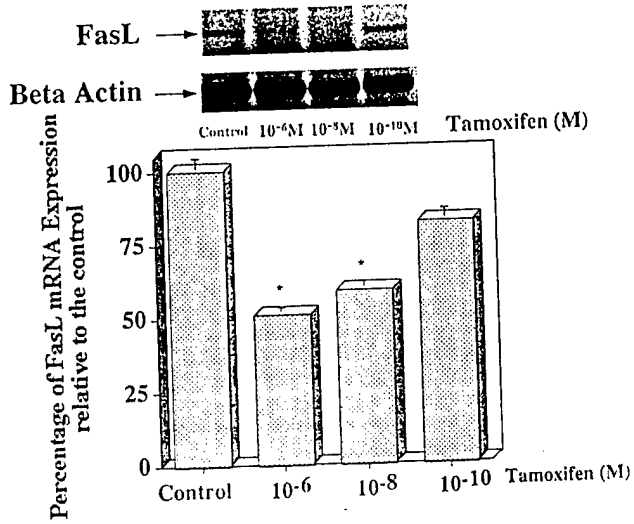

Fig. 5. Effect of tamoxifen on FasL mRNA expression. MCF-7 cells were treated with tamoxifen at concentrations of 10^{-6}, 10^{-8}, and 10^{-10} M for 24 h. Total RNA was analyzed for FasL expression by RT-PCR as described before. A: Representative gel analysis from MCF-7 cells treated with tamoxifen at different doses. B: The intensity of the signal was analyzed using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA) and standardized to the beta-actin signal. The y-axis represents the relative percentage mRNA expression relative to the control. Error bars represent standard error of the mean (S.E.M.). Statistical significance was determined by ANOVA. $^{*}P > 0.001$.

single sample of total RNA. Thus, MCF-7 cells were treated with different concentrations of tamoxifen for 24 h. Total RNA was isolated and analyzed by RPA. As shown in Fig. 7, tamoxifen inhibited FasL mRNA in a dose-dependent manner, but had no effect on Fas associated death domain (FADD) and death receptor 3 (DR3) mRNA expression. It is worth to note that tamoxifen at the concentration of 10^{-8} M had some inhibitory effect on Fas mRNA expression by MCF-7 cells, however, this effect was not consistent and was not statistical significant.

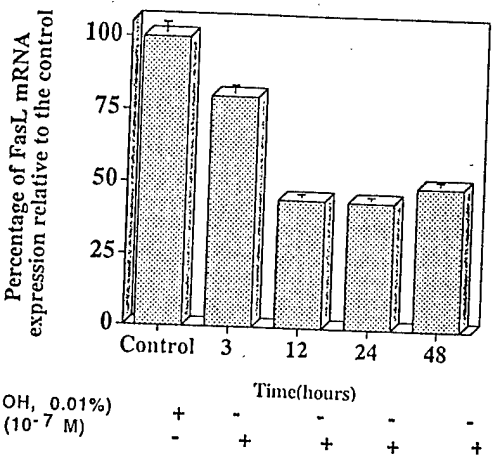

Fig. 6. Time-response effect of Tamoxifen on FasL mRNA expression in human breast cancer cell lines. The breast cancer cell lines MCF-7 and T47D cells were treated for 3, 12, 24 and 48 h with tamoxifen at a concentration of 10^{-7} M. Total RNA was extracted and RT-PCR was performed to study FasL expression. The intensity of the signal was analyzed using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA) and standardized to the beta-actin signal. The *y*-axis represents the relative percentage mRNA expression relative to the control. Error bars represent standard error of the mean (S.E.M.). Statistical significance was determined by ANOVA. $^{*}P > 0.001$. Each experiment was repeated at least three times for each cell line.

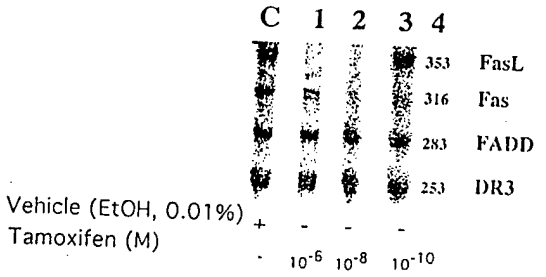

Fig. 7. RNase Protection assay (RPA). In order to confirm the specificity of the tamoxifen effect on FasL expression, similar samples as described in Fig. 5 were analyzed by RPA. MCF-7 and T47D cell cultures were treated with tamoxifen at concentration of 10^{-6} M (Lane 1), 10^{-8} M (Lane 2) and 10^{-10} M (Lane 3) for 24 h. The figure shows a representative RPA assay performed using 10 μg total RNA of MCF-7 cells. Note the inhibitory effect of tamoxifen on FasL mRNA expression at concentrations of 10^{-6} (Lane 1) and 10^{-8} (lane 2) but not on Fas, FDDA and DR3 mRNA. Lane 4 indicates the size of the protected product. Control (C) = MCF-7 cells without any treatment.

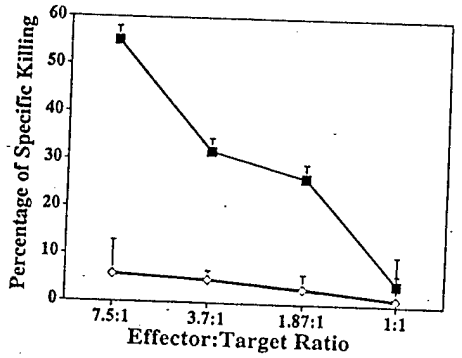

Fig. 8. MCF-7-induction of apoptosis in Fas-bearing cells. [^{3}H]Thymidine labeled target cells were co-cultured with MCF-7 cells at different E:T ratios for 24 h. Induction of apoptosis was quantified using the JAM assay. Co-culture of untreated MCF-7 cells. Co-culture of MCF-7 cells-tamoxifen-treated (10^{-7} M) and Jurkat cells for 24 h.

3.5.1. Fas-positive Jurkat cells are killed by MCF-7 cells

In order to ascertain whether the FasL present on the breast cancer cells is functional we established a co-culture system in which MCF-7 cells attached to 96-well plates were incubated with labeled Fas-positive Jurkat cells. As shown in Fig. 8, 55% of Jurkat cells underwent apoptosis after 24 h of incubation with MCF-7 cells at a ratio of 7.5:1. Cell killing was proportionate to the effector:target ratio (E:T) and statistically significant killing (20%) occurred even when there was only a ~2:1 MCF-7/Jurkat cell ratio. No apoptosis was induced in Fas-resistant Ramos cells (data not shown).

3.5.2. Tamoxifen treatment protects Jurkat cells from being killed by breast cancer cells

To test our hypothesis that tamoxifen inhibits FasL expression, and therefore blocks the protective effect of FasL in tumor cells, we treated MCF-7 cells with tamoxifen (1×10^{-7} M for 24 h) prior and during co-culturing them with labeled (Fas-positive) Jurkat cells. The JAM assay showed that treatment with tamoxifen protected Jurkat cells from killing by the MCF-7 cells (Fig. 8).

3.5.3. Tamoxifen protective effect is ER dependent

To confirm that the effect of tamoxifen on FasL expression is ER-dependent we carried out similar experiments as with MCF-7 cells but instead used the Jar choriocarcinoma cell line. Jar cells express FasL but are negative for estrogen receptors. Under these circumstances, tamoxifen did not have any effect on FasL expression and did not inhibit the induction of apoptosis in Jurkat cells (Fig. 9).

3.6. Presence of an estrogen responsive elements-like motif (ERE) in the promoter region of the FasL gene

The molecular basis for selective transcriptional activation by estrogen is the result of the estrogen-receptor complex interacting with specific nucleotide sequences termed estrogen responsive elements (ERE). To determine if the effect of estrogen on FasL expression could be mediated by the classical ER-ERE pathway, we looked for the presence of ERE at the FasL gene using a computerized gene homology program from the National Institutes of Health. Motifs resembling the consensus ERE were found only at the promoter region of the FasL gene located at nucleotides 543–552 (Table 1). The FasL ERE consist of two palindromic arms separated by 3-bp. One of the arms GGTCA has perfect homology to the canonical ERE while the second arm has two mismatches (bold). A second pathway for transcriptional regulation by the estrogen receptors αand β is through the AP-1 enhancer element. Accordingly, a complete AP-1 sequence TTAGTCAG, was identified at nucleotides 234–241 of the FasL promoter region.

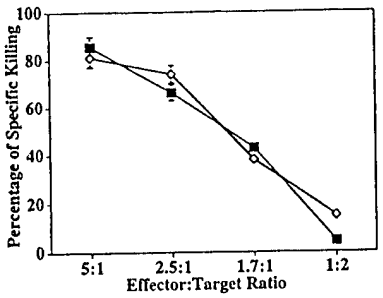

Fig. 9. Induction of apoptosis in Jurkat cells by FasL expressing Jar cells. [^{3}H]Thymidine labeled Jurkat target cells were co-cultured with choriocarcinoma cell line, Jar cells at different E:T ratios for 24 h. Induction of apoptosis was quantified using the JAM assay. No effect on Fas-positive induced apoptosis was found after pre-treatment of Jar cells with tamoxifen (10^{-7} M). Co-culture of untreated Jar cells and Jurkat cells. Co-culture of Jar cells treated with tamoxifen (10^{-7} M) and Jurkat cells.

Table 1
Sequences matching the Xenopus vitellogenin A2 gene ERE and the human FasL promoter region gene by using a sequence analysis program[a]

Vitellogenin	5′ AGGTCANNNTGACC 3′
Human FasL	5′ AGGTCAGGGTAAAT 3′

[a] The sequence in the FasL gene consisted of two pentamers (underlined) with close homology to the canonical ERE. In both genes the two pentamer sequences are separated by three nucleotides. Mismatches are in bold.

4. Discussion

Numerous in vivo and in vitro studies have shown the induction of lymphocyte apoptosis by FasL-bearing tumor cells. These implicate the Fas/FasL system as a mechanism by which tumors escape immune surveillance [19,33]. In the present study, using two human breast tumor cell lines we demonstrated the regulation of FasL expression by estrogen and tamoxifen.

Until recently, T cells were thought to be the major source of active FasL molecules and its role was mainly related to the process of acquisition of peripheral self-tolerance [34]. Further studies revealed that in addition, cells of the testis, retina and trophoblast also express FasL resulting in the establishment of classical immune privileges sites [12,20,22,30,35]. More recently, T cell-derived neoplastic cells [27], ovarian carcinoma cells [36], neuroblastoma cells [37], choriocarcinoma [12] and breast tumor cells [26] have also been shown to express FasL.

These observations indicated that FasL-induced suppression of tumor-specific Fas-bearing T cells might also be one of the mechanisms by which neoplastic cells escape from immune surveillance. Recently, we described the presence of FasL in breast tumor cells as well as in apparently normal epithelium of human breast glands that are located in the same quadrant as the tumor; however, FasL was absent in normal tissue far away from the tumor. We therefore proposed that FasL expression might be associated with early changes in the processes of neoplastic transformation [26]. We now have evidence indicating that this may be due to local estrogen formation by migrating macrophage [38].

In the present study we analyzed FasL expression in the breast cancer cell lines MCF-7 and T47D cells treated with estrogen: 17-β-estradiol, at concentration of 1×10^{-8} M induced a three-fold increase in FasL mRNA levels after 24 and 48 h (Fig. 2B). This effect extended to the protein level as shown by Western blot analysis (Fig. 2A). The effect of estrogen on FasL mRNA expression by both cell lines showed a biphasic pattern, with an early increase after 3 h of incubation followed by a decrease after 6 and 12 h and a second moderate increase at 24 h. Such biphasic effect has been reported for other estrogen regulated genes [39] and could be explained by the conformation of the ERE. The hormone-activated estrogen receptor complex binds to specific ERE located in the promoter region of estrogen-regulated genes. Therefore, we looked for the presence of ERE-like motifs (GGTCANNNTGACC) in the human FasL gene using the NIH Entrez computer program. Indeed, an ERE-like motif was found in the promoter region of the FasL gene (nucleotides 543–552) [40], having the characteristic of the ERE, that is a 13-bp palindromic element consisting two 5-bp arms separated by a 3-bp spacer (Table 1). The FasL

ERE has one arm of the palindromic element sequences with perfect nucleotide homology to the described ERE [41] and a second incomplete set (Table 1). The two arms of the palindrome are separated by the exact spacing (3 bp), which is essential for estrogen receptor action [42] (Table 1).

Structural and functional analysis of estrogen regulated genes have shown that most EREs are imperfectly palindromic and that these changes could be related to the affinity to bind the receptor and the efficiency to regulate gene transcription [43–45]. More over, these differences may have an effect on the sensitivity to partial agonist activities, i.e. tamoxifen and raloxifene. In other words, it is not only the promoter context but also the sequence of the binding site itself, which can allow distinction between receptor activated by agonist and that activated by antagonist [44].

The presence of an ERE in the promoter region of the FasL gene suggest that estrogen's effects described in the present study could be mediated by ER-ERE regulation. The functionality of these recognizing elements is being addressed.

In the rat uterus, it has been shown for example that the *fos* gene in the presence of continuous estrogenic stimuli after a first increase in transcription, the gene become refractory to the hormone. During this time, the concentration of transcriptionaly active ER-complexes increases within the first 2–3 h and then decreases, to a level that is approximately 1/4 of the peak level. Comparable phenomenon we can see with FasL expression. The decrease in FasL mRNA after 6 h of estrogen could be as a result of the dissociation of the weakly bound receptor from the FasL-ERE, with the consequent reduction of transcription of the gene. A similar observation was made with the human pS2 gene ERE, also imperfectly palindrome are less sensitive to the receptor in transcription experiments [43]. Our in vitro studies have shown that the FasL ERE, contrary to the canonical consensus palindromic ERE of the *Xenopus laevis* vitellogenin A2 gene requires about ten times more receptor (HEO) to activate transcription (unpublished data).

We then went on to demonstrate that this regulatory effect is ER-mediated by using the estrogen antagonist, tamoxifen which, at concentration of 10^{-7} M was able to block the estrogen-induced increase on FasL expression (Figs. 4 and 5).

Tamoxifen has been used as an anti-estrogen for treatment of hormone-dependent breast tumors and more recently as primary prophylaxis against breast cancer. The modulation of breast cancer proliferation by tamoxifen has been reported to be mediated mainly by its anti-estrogenic activity, which includes the decrease of c-erbB-2 and c-myc RNA levels, cellular production of factors such as TNF α and β, cyclin D and A, and CD36 [46,47].

All of this notwithstanding, we believe that the inhibitory effect of tamoxifen on FasL expression described in this study could also explain prophylactic actions of tamoxifen on the breast. That is, as pointed out above, the early expression of FasL in breast tissue undergoing neoplastic changes provides the growing cancer cell with a defense mechanism against immune surveillance. If FasL expression is inhibited, for example by tamoxifen, the apoptotic signal to immune cells responding to the presence of the tumor is blocked, allowing the immune system to remove the tumor cells. This scenario is supported by our co-culture experiments in which treatment of MCF-7 cells with tamoxifen inhibited the induction of apoptosis of Fas bearing T cells (Fig. 8) but not in Fas insensitive Ramos cells (data not shown). The observation that tamoxifen inhibits almost completely the induction of apoptosis in Fas bearing cells while decreases 30–50% the cell expression of FasL is intriguing and rather difficult to understand. It is possible that tamoxifen could have also effect in the transport of de novo synthesized FasL to the membrane and/or depleting the protein from the cytoplasm. Another explanation could be a direct effect of tamoxifen on a different apoptotic pathway such as p53 or c-myc [48].

The mechanism of action of tamoxifen in FasL expression seems to be mediated by ER but independent of the presence of estrogen. Jar cells, a human choriocarcinoma cell line, express high levels of FasL and induce apoptosis in activated immune cells [12]. However, using similar conditions as with MCF-7 cells, we find no effect of either estrogen or tamoxifen on FasL expression by Jar cells (Fig. 9). Thus since Jar cells are ER negative, we may conclude that the effect of estrogen and tamoxifen on human breast cancer cells is clearly ER mediated.

Of interest, when tamoxifen was added to T47D or MCF-7 cells in estrogen depleted media, FasL expression was almost completely inhibited (Fig. 4, line 3). In repeating this study, we further characterize this effect and found it to be dose- and time-dependent. Thus, as early as 3 h after treatment, tamoxifen decreased FasL mRNA levels by 25%, in relation to the control (Fig. 6).

In explanation, a possible mechanism by which tamoxifen exerted its direct inhibitory effect on the FasL gene could involve the ER/AP-1 pathway. We have found the presence of a perfect AP-1 motif (TTAGTCAG) to be located at the 5'-flanking region of the human FasL gene (nucleotides 234–241). Fos–Jun heterodimers and Jun–Jun homodimers are the principal components of the AP-1 transcription factor family, which interact with genes containing AP-1 specific sequences at the promoter region. Several lines of evidences have suggested that nuclear receptors, such as the glucocorticoid receptor [49,50], retinoic acid recep-

tor [51,52] and ER [53–55], can modify the effect of Fos–Jun complex. Furthermore, it has been shown that most of the modifications by these receptors result in negative effects on the AP-1 enhancer activity [56], possible because interactions with overlapping binding sites or inhibition of the activity through direct protein–protein interaction [57]. More recently, Paech et al. [58] have reported that the tamoxifen-ER complex binds at the AP-1 site and constitutes an alternative ER-regulatory pathway.

In addition, the fact that the FasL ERE is an imperfect palendomic sequence could also explain the inhibitory effect of tamoxifen on FasL expression. As we pointed previously, the physiological distinction between receptor activated by agonist and that activated by antagonist it is not only the promoter context but in the sequence of the binding site itself [44].

In conclusion, it is important to consider that the progression of a tumor is not only dependent on its proliferative rate but also in how it interacts with other cells and systems of the body, specifically the immune system. In recent years a growing number of reports have made evident the relevant role played by immune cells and their products in the regulation of the microenvironment normal and tumor tissues [38]. Alterations in local cell kinetics are followed by activation of several factors, allowing further proliferation, and calling for immune rejection. However, the presence or administration of agents that prevent the expression immune-regulatory factors such as FasL may frustrate this.

This study provides new evidence for the interaction of sex hormones, cancer cells and the immune system. The recognition that these factors influence growth and dissemination of breast cancer will provide new targets for therapeutic and preventive intervention.

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Roles of Fas and Fas ligand during mammary gland remodeling

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Mammary involution is associated with degeneration of the alveolar structure and programmed cell death of mammary epithelial cells. In this study, we evaluated the expression of Fas and Fas ligand (FasL) in the mammary gland tissue and their possible role in the induction of apoptosis of mammary cells. FasL-positive cells were observed in normal mammary epithelium from pregnant and lactating mice, but not in nonpregnant/virgin mouse mammary tissue. Fas expression was observed in epithelial and stromal cells in nonpregnant mice but was absent during pregnancy. At day 1 after weaning, high levels of both Fas and FasL proteins and caspase 3 were observed and coincided with the appearance of apoptotic cells in ducts and glands. During the same period, no apoptotic cells were found in the Fas-deficient (MRL/*lpr*) and FasL-deficient (C3H/*gld*) mice. Increase in Fas and FasL protein was demonstrated in human (MCF10A) and mouse (HC-11) mammary epithelial cells after incubation in hormone-deprived media, before apoptosis was detected. These results suggest that the Fas-FasL interaction plays an important role in the normal remodeling of mammary tissue. Furthermore, this autocrine induction of apoptosis may prevent accumulation of cells with mutations and subsequent neoplastic development. Failure of the Fas/FasL signal could contribute to tumor development.

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Introduction

Breast cancer is a major cause of death in women, and its occurrence has particular relevance to women's health worldwide. The fact that the mammary gland is the source of the most frequent malignancy in the female population and the knowledge that mammary tumorigenesis is influenced by the reproductive history of the individual emphasizes the importance of a better understanding of how pregnancy and lactation influences breast development and differentiation (1–3). Pregnancy, lactation, and the postlactational period entail a remodeling of the mammary gland mediated by programmed cell death (PCD) (apoptosis) necessary for successive lactation cycles. The apoptosis associated with breast involution has been implicated in resistance to tumorigenesis while inappropriate survival of the mammary secretory epithelial cells increases susceptibility to tumor development (4–6). Accordingly, conditions that limit proliferation or cause cell death could reduce the risk for breast cancer in humans (1–3).

Apoptosis is a process regulated by many genes and factors, including the Fas/Fas ligand (FasL) system (7–9). Fas (Apo-1/CD95) is a 45-kDa cell-surface receptor of the TNF/nerve growth factor receptor family whose signal transduction pathway mediates apoptosis of Fas-bearing cells after binding with FasL (10, 11). Although the Fas/FasL system was originally described in the context of lymphocyte-mediated apoptosis, new data have shown that Fas and FasL are widely expressed and function in many tissues outside the immune system. Under these circumstances, the expression of FasL in cells of the testis (12), the anterior chamber of the eye (13), the trophoblast (14–16), and the brain (17) may confer protection against immune responses by locally inducing the death of activated Fas-bearing immune cells that infiltrate into these tissues. As a result, FasL has been proposed to obviate immune suppression by cancer cells, allowing cancer or other abnormal cells to "escape" from immune surveillance by the induction of apoptosis of activated Fas-bearing immune cells (18–21). We reported recently the presence of FasL in hyperplastic ducts and breast cancer cells, but not in normal glandular epithelium, suggesting that the Fas/FasL system might play an important role in the development of breast carcinoma (18). These observations have lead us to study the expression of FasL in normal mammary epithelial cells during pregnancy and involution to evaluate whether FasL expression correlates with the stage of mammary epithelial cell differentiation (18).

Terminal differentiation of the mammary gland normally takes place only during pregnancy and lactation when there is a cycle of lobulo-alveolar and ductal division followed by a period of lactation, after which cells of the secretory mammary epithelium and ducts become committed to apoptotic cell death. Gestational and lactogenic hormones regulate this pathway of differentiation. Changes in the hormonal environment at the end of lactation trigger the apoptotic signal required for "breast remodeling" (22, 23) perhaps also through the Fas-FasL system.

To test the role of the Fas/FasL system in breast remodeling we studied the expression of Fas and FasL in the mammary tissue of normal BALB/c, MRL, C3H, as well as in Fas-deficient (MRL/*lpr*) and FasL-deficient (C3H/*gld*) mice through the different stages of their lactogenic differentiation. We also evaluated the presence of apoptosis during involution in wild-type MRL and C3H mice and compared it with the natural knockout MRL/*lpr* and C3H/*gld* mice. To confirm our in vivo data we also studied caspase activation and apoptosis in normal human and mouse mammary epithelial cells. In this report we describe how Fas and FasL expression is altered during pregnancy, lactation, and postlactational period, and show how these changes are hormonally regulated. We also demonstrated the role of the Fas/FasL system as a mediator of apoptosis of mammary epithelial cells during the first stage of mammary gland involution.

Methods

Cells and culture condition. Cells were cultured in DMEM/F-12 media containing antibiotics and antimycotics (1% vol/vol) and proper serum at 37°C in a humidified chamber (5% CO_2 in air). Cells were passed by standard methods of trypsinization, plated in tissue culture dishes for RNA and protein extraction, and allowed to replicate 80% confluence. The standard media for culture of HC-11 contained dexamethasone (1 μM), insulin (5 μg/ml), prolactin (5 μg/ml), and FBS (10% vol/vol). The media for culture of MCF-10A contained EGF (20 ng/ml), insulin (1 ng/ml), hydrocortisone (500 ng/ml), cholera toxin (100 ng/ml), and horse serum (5% vol/vol). After cell cultures were continued with standard media for 72 hours, starvation media (SM) excluding serum and hormones, and phenol red was initiated for each planned schedule.

DMEM/F-12, FBS, and horse serum were purchased from Life Technologies (Grand Island, New York, USA). HC-11 cells were a gift from Nancy Hynes, and MCF-10A cells were purchased from the American Type Culture Collection (Rockville, Maryland, USA). All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA).

Animal experiment specimens from mice. All studies involving animals were approved by Yale Institutional Review Boards for Animal Care. Cycling female (60–90 days old) BALB/c mice, MRL/MpJ (MRL), and the Fas-deficient MRL/*lpr* (*lpr*) mice came from an in-house colony and were originally obtained from The Jackson Laboratories (Bar Harbor, Maine, USA). The MRL/*lpr* mouse is a natural mutation of the MRL/MpJ mouse, therefore its genetic background is 100% MRL/MpJ (24). C3H/He (C3H) and C3H/*gld/gld* (*gld*) were purchased from The Jackson Laboratories, and their genetic background is 100% C3H/HeJ (n = 10) (25). Animals were housed under controlled temperature (22°C), humidity, and light (14 hours light, 10 hours dark). After deep ether anesthesia and excision, each mammary gland specimen was frozen in liquid nitrogen for RNA and protein extraction or fixed in 4% freshly made paraformaldehyde, then paraffin-embedded for immunohistochemistry. Tissue blocks were stored at room temperature until 5-μm sections were cut and mounted. Mice were sacrificed after the removal of the tissue.

Preparation of total RNA and protein samples. Total RNA and proteins were prepared from frozen mouse mammary gland tissue, frozen human breast tissue, and cultured breast cell lines using TRIzol reagent (Life Technologies Inc., Gaithersburg, Maryland, USA) according to the manufacturer's instructions. We used the TRIzol protocol to extract RNA and protein from the same cells. This method allowed us to study the same samples at both the mRNA and protein level.

RT-PCR. Details of the characterization for the RT-PCR were described previously (26, 27). In short, reverse transcription was performed using the RT-PCR kit from Pharmacia BioTech (Piscataway, New Jersey, USA) according to the manufacturer's directions. The cDNA synthesis was performed with pd$(N)_6$ 0.2 μg and 5 μg total RNA. The primers used for amplification of FasL have been described previously (28) and have the following sequence: for humans, upstream, 5′-ATAGGATCCATGTTTCTGCTCTTCCACCTACAGAAGGA-3′ and downstream, 5′-ATAGAATTCTGACCAAGAGAGAGCTCAGATACGTTGAC 3′; for mice, upstream, 5′-AAGCTTCAGCTCTTCCACCTG-3′ and downstream, 5′ ATGAATTCCTGGTGCCCATG-3′. Each PCR cycle for human FasL consisted of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and elongation at 72°C for 1 minute, for a total of 35 cycles. Each PCR cycle for mice FasL consisted of denaturation at 94°C for 1 minute, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, for a total of 30 cycles. The primer used for amplification of Fas has the following sequence: for humans, upstream, 5′-AAGGAGTACACAGACAAAGCCC-3′ and downstream, 5′-AAGAAGAAGACAAAGCCACCC-3′; for mice, upstream, 5′-GAGAATTGCTGAAGACATGACAATCC-3′ and down stream, 5′-GTAGTTTTCACTCCAGACATTGTCC-3′. Each PCR cycle for human Fas consisted of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 30 seconds, for a total of 30 cycles. Each PCR cycle for mouse Fas consisted of denaturation at 94°C for 1 minute, annealing at 53°C for 30 seconds, and elongation at 72°C for 30 seconds, for a total of 35 cycles. The PCR products were analyzed in Tris-EDTA (TAE) 1–2% agarose gel with ethidium bromide.

The Fas and FasL signals were measured by a densitometer and standardized against the actin signal using a digital imaging and analysis system (AlphaEase; Alpha Innotech Corporation; San Leandro, California, USA). The linearity of the system was determined using serial dilution of cDNA, and the regulation of dilution-factor on amplified cDNA was linear ($y = 2\,881.125x - 785.75$) and the correlation coefficient was $r = 0.994$, as described previously (26, 27).

Western blot analysis. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membrane. Immunoblotting was performed after blocking nonspecific binding by the membranes with 5% powdered milk. The blots were incubated first with the primary Ab for FasL (mAb clone 33, 1:1000 dilution; Transduction Laboratory, Lexington, Kentucky, USA) or primary Ab for Fas (polyclonal Ab M-20 at 1:500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) for 1 hour. After washing, the membranes were incubated with the secondary Ab, peroxidase-labeled horse anti-mouse or goat anti-rabbit gamma globulin (Vector Laboratories, Burlingame, California, USA), for another hour. Finally, the blots were developed with TMB Peroxidase substrate kit (Vector). The specificity of the anti-FasL mAb clone 33 used in this study was evaluated by Western blot analysis, comparing it with two different clones, the polyclonal Ab N-20 from Santa Cruz Biotechnology Inc. and clone NOK-1 from PharMingen (San Diego, California, USA). (See refs. 26, 27 for details.)

Immunohistocytochemistry for Fas and FasL. Detection of Fas and FasL expression was performed using a rabbit polyclonal IgG containing anti-human FasL or mouse monoclonal IgG containing anti-human Fas (N-20 or B-10, respectively; Santa Cruz Biotechnology Inc.). Deparaffinized and rehydrated 5-μm sections were blocked for endogenous peroxidase activity with 0.3% H_2O_2 in methanol, washed in PBS, and preincubated with 10% normal goat or horse serum in PBS/5% BSA for 1 hour to block nonspecific binding of the Ab's. The primary Ab (1:200 dilution for FasL and 1:50 dilution for Fas) in dilution buffer (PBS plus 5% BSA and 0.1% saponin) was applied. Slides were then incubated for 12 hours overnight at 4°C in a humidified chamber. After washing, sections were incubated for 1 hour with a biotinylated horse anti-mouse Ab or biotinylated goat anti-rabbit Ab (Vector Laboratories) for Fas and FasL, respectively. Detection of the secondary Ab was amplified using the ABC system according to the manufacturer's instructions (Santa Cruz Biotechnology Inc.). Color development was done with the 3'3-diaminobenzidine HCL (DAB) substrate (Immunopure metal-enhanced DAB substrate kit; Pierce Chemical Co., Rockford, Illinois, USA), and sections were counterstained with hematoxylin. In the negative control samples, the primary Ab was omitted or preabsorbed with recombinant Fas or FasL, as described previously (17).

DNA fragmentation assay (JAM assay). Cell death of mammary epithelial cells resulting from the elimination of hormones from the culture was quantified by measurement of target-cell DNA fragmentation using the JAM assay (29). Adherent mammary epithelial cells (MCF-10A or HC-11 cells) were seeded into the wells of a flat, microwell, 96-well Falcon plates at a cell number appropriate to give the required confluence. Target-cell DNA was labeled by prior incubation with 10 Ci/ml of [^{3}H]-thymidine at 37°C for 24 hours. Labeled cells were washed and cultured in 200 μl/well of hormone-deprived media. After culture at 37°C for 6–24 hours, the cells were removed from the wells and filtrated onto glass fiber filters using an automatic 96-well filtration unit. The cells were then lysed with hypotonic buffer, and their DNA was washed through the filter by four washes with double-distilled water. The radioactivity of intact chromosomal DNA retained on each filter was measured by liquid-scintillation counting. Specific cell death was calculated using the following equation (29): percentage of specific cell death = $(S - E/S) \times 100$, where E (experimental) is counts per minute of retained (complete) mammary epithelial cell DNA in the hormone-deprived media and S (spontaneous) is counts per minute of retained DNA in hormone-containing media.

In situ localization of apoptotic cells: annexin V. Staining of apoptotic cells was done using the ApoAlert annexin V system (CLONTECH Laboratories, Palo Alto, California, USA), according to the instructions of the manufacturer. In short, cells were cultured in four-well chamber slides. After treatment, cells were incubated for 10 minutes with annexin V-FITC, followed by three washings with PBS, then fixed with 4% paraformaldehyde. For counterstaining, cells were incubated with DAPI (4,6,-diamidino-2-phelylindole). Positive cells were detected by fluorescent microscopy (Carl Zeiss Inc., Jena, Germany).

In situ 3´ end labeling of DNA for cell death detection. The presence in mammary tissue sections of single-strand DNA breaks indicating apoptosis was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (In Situ Cell Death Detection Kit, Fluorescent; Boehringer Mannheim Biochemicals Inc., Indianapolis, Indiana, USA), according to the manufacturer's instructions. Briefly, after deparaffinization and rehydration, slides were incubated with proteinase K (20 mg/ml in 10 mM Tris/HCL, pH 7.4) for 30 minutes at 37°C. Samples were then treated with terminal deoxynucleotidyl transferase enzyme and fluorescein-labeled nucleotides for 60 minutes at 37°C in the dark. In the negative control sections the enzyme was omitted. An alkaline phosphatase–anti-fluorescein Ab was added and a nitro-blue tetrazolium alkaline phosphatase kit (Vector) was used for color development.

Fas-induced apoptosis. The effect of SM on Fas sensitivity was evaluated by incubating mammary epithelial cells with an anti-human Fas mAb (R&D Systems, Minneapolis, Minnesota, USA). One-hundred fifty microliters of 5×10^3 – 1×10^4 cells in DMEM-F12 with 5% horse serum were seeded in 96-well flat-bottomed

microplates (Becton-Dickinson, Franklin Lakes, New Jersey, USA) and incubated for 48 hours. Then the medium was exchanged, and the cells were incubated with various doses of the mouse monoclonal IgG anti-Fas Ab in 200 µl of medium with or without serum. Normal mouse IgG Ab (Santa Cruz Biotechnology Inc.) was used as control. Cells were also treated with anti-FasL (100 ng/ml NOK-1 mAb; PharMingen) or a mouse IgG1 isotype-matched control mAb (100 ng/ml; PharMingen) at the time of plating. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) dye was added into each well 4 hours before the end of the incubation. The supernatant was removed and 100 µl of acidified isopropyl alcohol was added to solubilize the reactive crystals. Absorbency was measured at 540 nm by an automatic microplate reader (Model 550; Bio-Rad Laboratories Inc., Hercules, California, USA). Background consisted of medium only and no cells. Percentage of the absorbency relative to the control was calculated, and the survival graph was drawn. Each assay was performed at least three times in triplicate.

Statistical analysis. Data were analyzed for statistical significance by using Student's *t* test. All experiments were repeated two or three times with similar results.

Results

Fas/FasL mRNA expression in the mouse mammary gland. Terminal differentiation of the mammary gland normally takes place only during pregnancy and lactation. Gestational and lactogenic hormones regulate this pathway of differentiation. Changes in the hormonal environment at the end of lactation trigger the apoptotic signal required for "breast remodeling". We tested the hypothesis that the Fas/FasL system could mediate apoptotic signals during tissue remodeling by studying the expression of Fas and FasL in mice mammary tissue. Studying mice, we analyzed Fas and FasL expression at the mRNA and protein level. RT-PCR was performed on samples (n = 3/group) obtained from nonpregnant virgin mice during pregnancy days 15 and 19, after 10 days of lactation, and days 1, 2, 4, and 12 after weaning. The intensity of the signal was analyzed by densitometry as described in Methods and plotted as a bar in Figure 1. Fas mRNA was clearly detectable in all tissues studied (Figure 1). In contrast, FasL mRNA was absent in nonpregnant, virginal mice (Figure 1, lane 1), began to appear by day 15, and was greatest on day 19 of pregnancy (Figure 1, lanes 2 and 3). During lactation and the first day of weaning, FasL mRNA was also present in high levels (Figure 1, lanes 4 and 5), but decreased dramatically by postlactational days 4 and 12 (Figure 1, lanes 6–8)

Western blot analysis for Fas, FasL, and caspase 3 expression in the mouse mammary gland. To compare the expression of Fas and FasL mRNA with protein expression we performed Western blots on protein preparations from the same samples we employed for mRNA analysis. The intensity of the staining was analyzed by densitometry as described above. As shown in Figure 2, Fas and FasL protein expression differed strikingly from their patterns of mRNA expression. Mammary glands from virgin mice express high protein levels of Fas, but not FasL, protein (Figure 2, lane 1). However, during pregnancy and lactation, Fas protein expression was suppressed (Figure 2, lanes 2–4), while FasL was expressed in high amounts compared with the nonpregnant mouse mammary gland (Figure 2, lanes 2–4).

Removal of the litters, which constitute the primary factor for mammary gland involution, had a profound effect on Fas expression; 24 hours after the removal of the litters, Fas protein level increased dramatically (Figure 2, lane 5). Quantification of the signal indicated a statistically significant increase ($P < 0.001$) of Fas protein levels on days 1, 2, 4, and 8 of involution compared with the protein levels detected during pregnancy (Figure 2, lanes 5, 6, 7, and 8, respectively). FasL protein levels remained high during the first two days of involution and were significantly reduced from days 4 to 12 after weaning ($P < 0.05$) (Figure 2, lanes 5, 6, 7, and 8, respectively). Similarly, the active form of caspase 3 was detected by Western blot analysis during mammary gland remodeling. Thus, high concentration of the active form of caspase 3 was mainly detected during the first 4 days of involution (Figure 3, lanes 3–6), decreasing afterward (Figure 3, lanes 7 and 8). On the other hand, the active form of caspase 3 was not expressed in the mammary tissue from nonpregnant/virgin mice (Figure 3, lane 1).

Localization of FasL and Fas expression in the normal mouse mammary gland. To determine which cells expressed Fas and FasL, we performed immunohistochemistry on

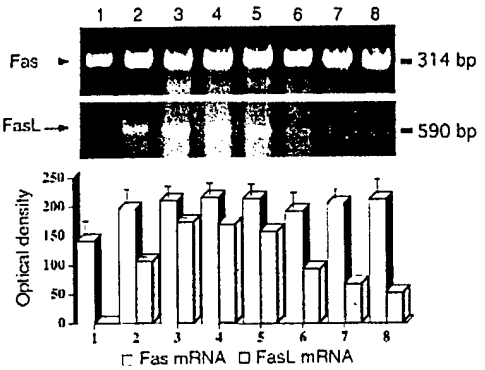

Figure 1
Expression of Fas/FasL mRNA in mouse mammary tissue. RT-PCR for Fas/FasL was performed with total RNA extracted from BALB/c mice during pregnancy, lactation, and involution. FasL mRNA was expressed during pregnancy, lactation, and early involution, but not in breast tissue from virgin mice. Fas mRNA was present in all the tissues evaluated. The intensity of the signal was analyzed using a digital imaging analysis system (AlphaEase). Bars represent the average ± SD from three independent experiments. The figure of the gel is a representative experiment of the three experiments. Lane 1, virgin; lane 2, day 15 of pregnancy; lane 3, day 19 of pregnancy; lane 4, day 10 of lactation; lane 5, day 1 after weaning (AW); lane 6, day 2 AW; lane 7, day 4 AW; lane 8, day 12 AW.

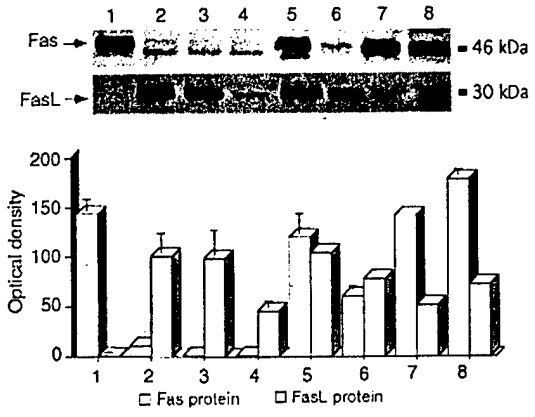

Figure 2
Expression of Fas/FasL protein in mouse mammary tissue. Western blot analysis for Fas and FasL was performed with protein samples obtained from the same samples described in Figure 1. Fas expression is found in breasts from virgin mice but is not expressed during pregnancy and lactation. High levels of Fas protein appear 1 day after weaning. FasL is expressed during pregnancy, lactation, and involution, but not in the virgin mouse. The intensity of the signal was analyzed using a digital imaging analysis system. Bars represent the average ± SD from three independent experiments. The figure of the immunoblot is a representative experiment of the three experiments. Lane 1, virgin; lane 2, day 15 of pregnancy; lane 3, day 19 of pregnancy; lane 4, day 10 of lactation; lane 5, day 1 AW; lane 6, day 2 AW; lane 7, day 4 AW; lane 8, day 12 AW.

paraffin sections of the mammary gland from normal mice during pregnancy, lactation, and weaning. Sections were stained with a polyclonal Ab for FasL (clone N-20; Santa Cruz Biotechnology Inc.) and for Fas using the mAb B-10 (Santa Cruz Biotechnology Inc.). Mammary gland tissues were collected from virgin mice and during different days of pregnancy (as described in Methods), lactation, and involution. Mammary gland samples from virgin mice were immune negative for FasL (Figure 4a), but positive for Fas (Figure 5a). However, at days 15 and 19 of pregnancy abundant FasL-positive immunoreactive cells were found in the glandular epithelium (Figure 4b). The number and intensity of positive immunoreactivity for FasL increased significantly as pregnancy progressed (from day 5 to day 19, data not shown). Immunoreactivity for Fas was almost absent, with the exception of a few scattered positive stromal cells with the cytology of macrophages and leukocytes (Figure 5b, and data not shown). After delivery and during lactation, few Fas-positive glandular epithelial cells were found, while FasL immunoreactivity was present in almost all of the glandular epithelium, including ducts (data not shown).

Localization of FasL and Fas expression during mammary gland involution. Postlactational involution of the mammary gland during weaning is characterized by cell death of portions of the mammary epithelium. To determine how Fas and FasL expression is related to mammary gland involution we stained paraffin sections from mouse mammary glands obtained consecutive days after weaning. The number of glandular epithelial cells expressing FasL and Fas increased dramatically between day 1 and 2 after weaning (Figure 4, c and d; Figure 5, c and d). By day 4, the tissue was characterized by the presence of mainly ducts and clusters of epithelial chordae surrounded by adipocytes. Although FasL was still present, it was expressed in fewer cells than on previous days (Figure 4f).

Presence of apoptotic cells in the mammary epithelium of normal mice, but not in the Fas-deficient lpr mice. To determine if the expression of Fas and FasL is related to the apoptosis of the mammary epithelium characteristic during involution, we performed histological analysis by using the TUNEL assay on paraffin sections derived from mammary glands at days 1, 2, 4, and 12 of involution in normal BALB/c mice. Similarly, we compared the presence of apoptotic cells in the involuting mammary gland of MRL mice, the Fas-deficient MRL/*lpr* mice, as well as of C3H and C3H/*gld/gld* (FasL-deficient) mice. When we analyzed DNA fragmentation in mammary glands from MRL/*lpr* or C3H/*gld/gld* mice, no apoptotic cells were detected during the first 3 days of involution (Figure 6, c and e). However, apoptotic cells were present at day 4 after weaning in the MRL/*lpr* mice (Figure 6d) and in the C3H/*gld/gld* mice (Figure 6f). In contrast, DNA fragmentation (shown as blue nuclei) was present at day 1 of involution in MRL (Figure 6a) and C3H mice and increased to a substantial number at day 2 of involution, reaching a relative maximum at day 4 after weaning (Figure 6b).

In vitro hormonal regulation of Fas and FasL expression in mammary epithelial cells. To establish an in vitro model to study the role of the Fas/FasL system in mammary epithelial cell-mediated apoptosis, we performed studies using either the normal mouse epithelial cell line HC-11 or the normal human epithelial MCF-10A cells (data not shown).

Effect of hormone withdrawal on Fas and FasL expression by normal mouse HC-11 mammary epithelial cells. To study the hormonal involvement in Fas/FasL expression during involution of the mouse mammary gland, we attempted to reproduce in vitro conditions resembling

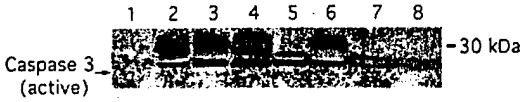

Figure 3
Expression of caspase 3 in mouse mammary tissue. Western blot analysis for caspase 3 was performed with protein samples obtained as described in Figure 1. Increase in the expression of the active form of caspase 3 was detected 24 hours after weaning; expression remained high during days 2 and 4 of involution, decreasing afterward. Lane 1, virgin; lane 2, lactation 10 days; lane 3, day 1 AW; lane 4, day 2 AW; lane 5, day 3 AW; lane 6, day 4 AW; lane 7, day 8 AW; lane 8, day 12 AW.

normal involution after weaning. The mouse mammary glandular epithelial cell line HC-11 was cultured in DMEM-F12 with 10% FCS and containing dexamethasone (1 μM), insulin (5 μg/ml), and prolactin (5 μg/ml) to induce lactogenic differentiation as described previously (30). We used a JAM or a MTT assay to evaluate the effect of hormone and serum withdrawal on Fas and FasL expression. Both methods gave similar results. As shown in Figure 7, HC-11 cells grown in normal conditions express only very low levels of Fas protein (Figure 7a, lane 1) and do not express FasL protein (Figure 7b, lane 1). By 1 hour after removal of the serum and hormones from the media (SM), there was a fourfold increase in Fas protein levels, and they remained high for up to 24 hours (Figure 7a, lanes 2–5). Interestingly, FasL protein expression increased only after 2 hours of incubation in SM and reached its highest level measured after 24 hours (Figure 7b, lanes 3–5).

Effect of SM treatment on caspase 3 expression. Fas/FasL-induced apoptosis is mediated by the activation of a proteolytic system involving proteases called caspases. The activation of caspase 3 is an important component of this cascade. To further confirm the involvement of the Fas/FasL system in the induction of apoptosis of mammary epithelial cells, we studied caspase 3 expression in HC-11 cells after removal of serum and hormones. As shown in Figure 7c, HC-11 cells maintained in normal conditions express only the inactive form of caspase 3 (Figure 7c, lane 1); incubation in SM induced the activation of caspase 3 in HC-11 cells after 1 hour (corresponding to Fas and FasL expression). As apoptosis progressed, the amount of the inactive form of caspase 3 decreased (Figure 7c, lanes 2–5).

Correlation between Fas/FasL expression and apoptosis in mammary epithelial cells. To compare the in vitro effect of hormonal deprivation on mammary epithelial cells' Fas and FasL expression and activation of caspase 3 with apoptosis, we performed annexin V and JAM assays. Cells were treated with SM as described above followed by incubation with FITC-labeled annexin V. Cells were then observed under a fluorescent microscope using 4,6-diamidino-2-phenylindole (DIP) as marker for the presence of cells in the culture. As shown in Figure 8, numerous annexin V–positive cells were present after 24 hours (Figure 8d) and 48 hours (Figure 8f) in SM, but not in the control group (Figure 8b). To quantify the number

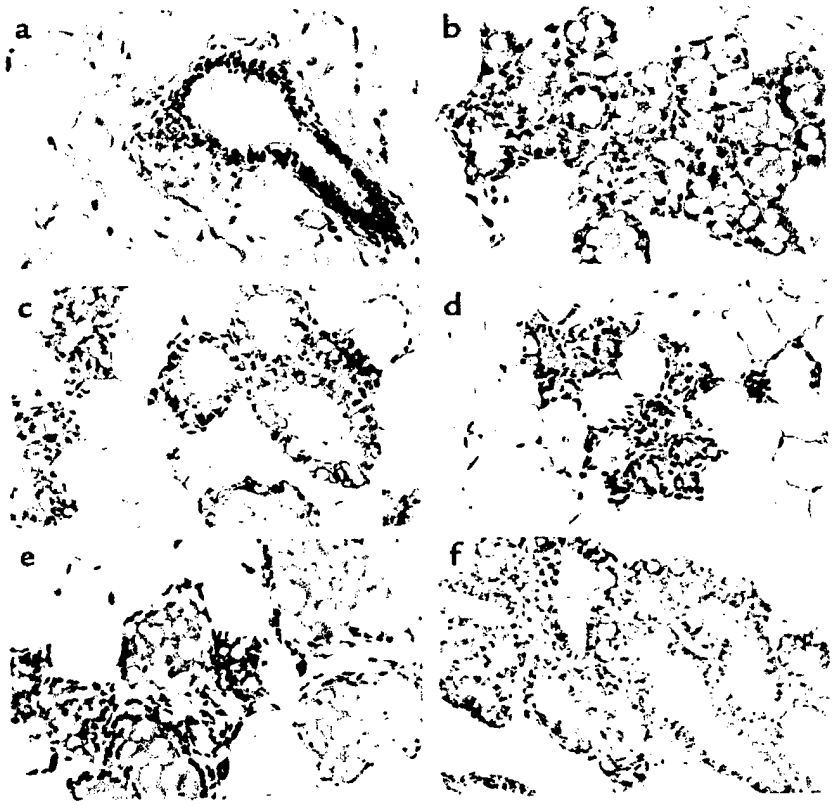

Figure 4
Localization of FasL expression in mouse mammary tissue during pregnancy, lactation, and involution. Paraffin sections of normal breast tissue were stained with anti-mouse FasL Ab. (**a**) Virgin mouse breast, showing immune negative for FasL. (**b**) Day 15 of pregnancy, abundant positive FasL immune reactivity was shown in the glands. (**c**) Day 1 of involution, FasL immunoreactivity is present in glandular epithelial cells surrounding milk accumulation. (**d**) Day 2 of involution, the number of FasL-expressing cells increased. (**e**) Day 3 of involution. (**f**) Day 5 of involution, characterized by presence of ducts and epithelial chordae surrounded by adipocytes with localization of FasL in a few cells. ×400.

of apoptotic cells that were the result of culture in SM, we used the JAM assay. We found 40% (± 12.9) and 43% (± 6) of apoptotic cells after 24 hours and 48 hours, respectively, in SM (Figure 9). Furthermore, to demonstrate the role of the Fas receptor in the regulation of HC-11 SM-induced apoptosis, cells were treated with anti-FasL (NOK-1 mAb) at the beginning of the culture. By blocking Fas/FasL interaction the apoptotic effect of hormonal removal in HC-11 cells was decreased (Figure 9). The control Ab, IgG1 isotype control immunoglobulin (clone 107.3; PharMingen) had no effect.

Fas-induced apoptosis: Further confirmation that Fas expressed by mammary cells is active was demonstrated by in vitro experiments using SM media and the agonist anti-Fas mAb. Mammary epithelial cells (MCF-10A and HC-11) were treated with increasing concentrations of anti-Fas mAb for 24 hours, and the Fas-mediated apoptosis was determined by either JAM or MTT assay. Upregulation of Fas expression after treatment with SM media resulted in increased responsiveness toward Fas-mediated apoptosis (Figure 10).

In conclusion, culture of either HC-11 or MCF-10A cells in SM induced the rapid expression of Fas and FasL, which is followed by activation of caspase 3 and the induction of apoptosis in more than 40% of the cells. Furthermore, addition of DEVD-CHO (caspase inhibitor) protected mammary epithelial cells to the apoptotic effect of Fas Ab or SM (data not shown). DEVD-CHO is a pseudosubstrate that mimics the caspase-3 cleavage site and thus inhibits its protease activity.

Discussion

Mammary involution, which entails the physiological removal (apoptosis) of breast cells after lactation, is a fundamental process involved in normal development of the mammary gland and cancer prevention. In the present study we have characterized the expression of Fas and FasL during the normal mammary gland development and have shown a correlation between their expression and PCD of the involving mammary epithelium.

The normal development of mammary glands during pregnancy is characterized by a period of epithelial cell

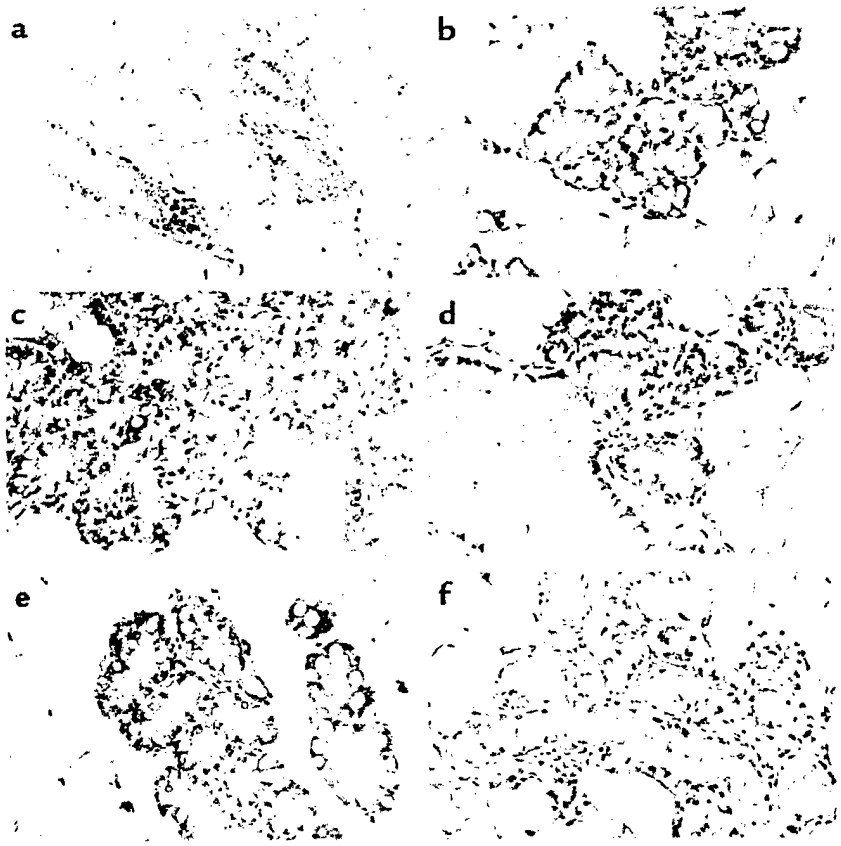

Figure 5
Localization of Fas expression in mouse mammary tissue during pregnancy, lactation, and involution. Immune staining of paraffin sections from mouse breast tissue is shown. (**a**) Virgin mouse breast, showing Fas-positive immune reactivity. (**b**) Day 15 of pregnancy, Fas-positive immune reactivity was absent except for some scattered positive stromal cells. (**c**) Day 1 of involution, strong Fas-positive immune reactivity was found in part of the glandular epithelium. (**d**) Day 2 of involution, the number of Fas-expressing cells is significantly increased. (**e**) Day 3 of involution. (**f**) Day 5 of involution, showing mainly ducts and epithelial chordae surrounded by adipocytes with localization of Fas in a few cells. ×400.

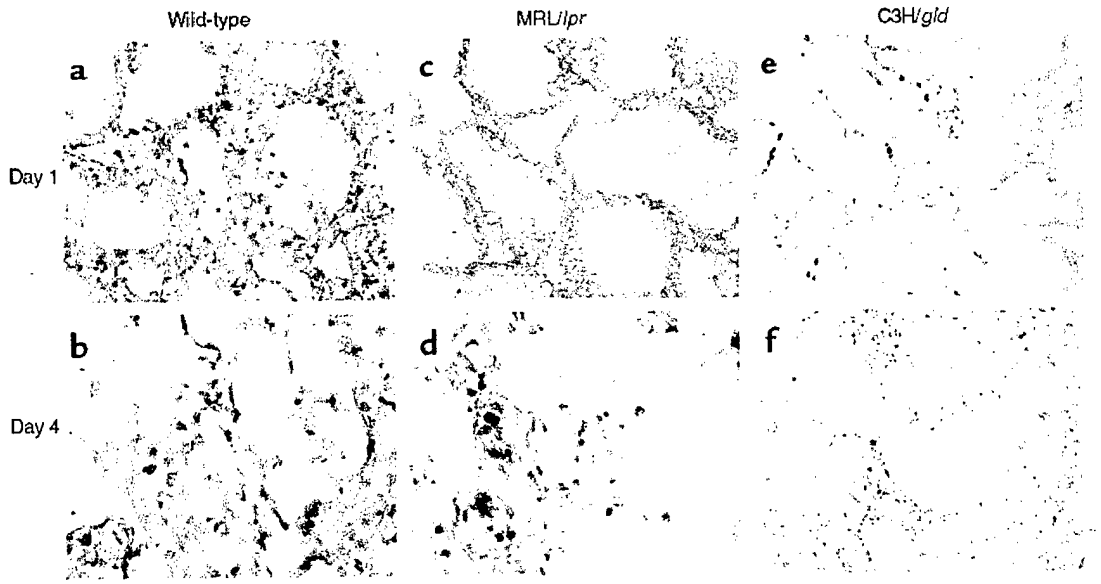

Figure 6
Localization of apoptosis in mouse mammary tissue during involution. Apoptosis in the involuting mammary gland was monitored by TUNEL on 5-μm paraffin sections derived from mammary glands of the MRL and C3H mouse, the Fas-deficient MRL/*lpr* mouse, and the FasL-deficient C3H/*gld* mouse. (**a**) Wild-type MRL mouse, day 1 of involution, showing numerous apoptotic cells (positive nuclei are detected as blue spots) in glandular structure. (**b**) Wild-type MRL mouse, day 4 of involution; the number of apoptotic cells is significantly increased. (**c**) MRL/*lpr* mouse, day 1 of involution, showing no evidence of apoptosis on intact glandular structure. (**d**) MRL/*lpr* mouse, day 4 of involution, showing increased number of apoptotic cells. ×400. (**e**) C3H/*gld* mouse, day 1 of involution, showing no evidence of apoptosis on intact glandular structure. ×200. (**f**) C3H/*gld* mouse, day 4 of involution, showing apoptotic cells. ×200.

proliferation followed by the differentiation of milk-producing cells after parturition. Several investigators have suggested that this differentiation is terminal and commits the differentiated cells to undergo PCD (apoptosis). Walker and coworkers have demonstrated that in the mouse and rat, mammary tissue involution is accompanied by the cleavage of chromatin into oligonucleotide fragments (31), a characteristic of apoptosis (32–34). These and other studies (35, 36) provide strong evidence that cell loss during involution occurs by PCD. The occurrence of such remodeling is supported by experimental manipulations that have shown that after litter removal, lactation can be maintained and involution impeded by injection of hormones similar to those that we removed in our in vitro experiments (37, 38).

Apoptosis is influenced by a wide variety of stimuli. Among the known "death receptors" (TNF-R1, DR-3, TRAIL-R1 and -R2), Fas is one of the most important death-domain receptor. Interaction of Fas with its ligand, FasL, induces receptor trimerization, which in turn results in the recruitment of the adapter protein FADD and activation of caspases (39), which leads to irreversible cell damage and death (40).

In the present studies we demonstrate that although Fas protein is present during normal breast development, it is absent during pregnancy and lactation, only to return after weaning. Interestingly, Fas mRNA is present through the entire period. This phenomenon has been described in other cases and is referred to as "illegitimate transcription" (41–43). On the other hand, FasL

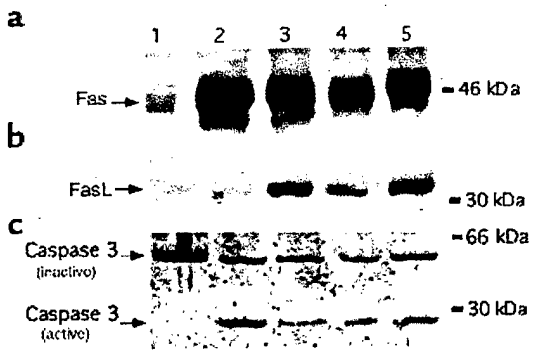

Figure 7
Expression of Fas/FasL and caspase 3 in mouse mammary glandular epithelial cell line HC-11. Western blot analysis for Fas (**a**) and FasL (**b**) was performed with protein extracted from in vitro culture of HC-11 cells. Note the increase of Fas protein expression after 1 hour in hormone- and serum-deprived media (SM). FasL protein was expressed after 2 hours in SM and reached the highest level after 24 hours. (**c**) Western blotting for caspase 3 was performed with the same protein as above. Presence of SM converted the inactive form of caspase 3 to an active form corresponding to Fas and FasL expression. Lane 1, culture in normal media; lane 2, 1 hour in SM; lane 3, 2 hours in SM; lane 4, 6 hours in SM; lane 5, 24 hours in SM.

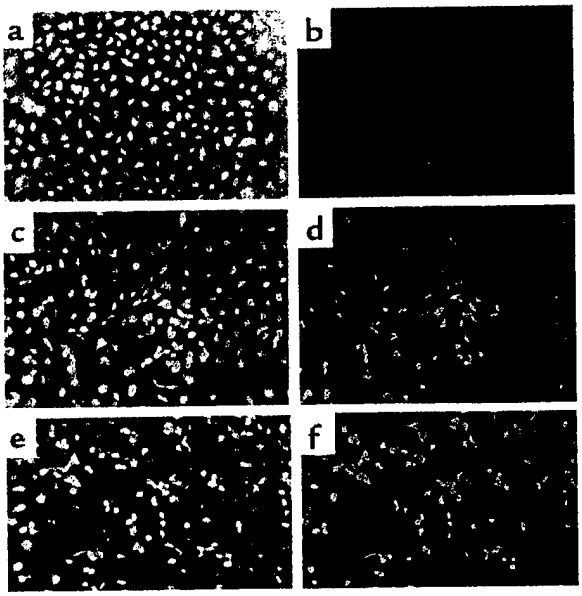

Figure 8
In situ detection of apoptotic cells in in vitro culture of mammary glandular epithelial cells. MCF-10A and HC-11 cells were treated with SM for 24 and 48 hours. Apoptotic cells were detected by annexin V (**b**, **d**, **f**). The figures are a representative experiment using HC-11 cells. (**b**) Annexin V staining of cells in complete media. (**d**) After 24 hours in SM. (**f**) After 48 hours in SM. (**a**, **c**, **e**) DAPI staining was used to prove the presence of cells in **b**, **d**, and **f**, respectively. ×400.

protein is present during pregnancy, lactation, and weaning, but not in the virgin mouse (Figures 1–4). The overlapping expression of Fas and FasL during involution is accompanied by apoptosis of the mammary epithelium. Apoptosis was monitored using the TUNEL assay on paraffin sections derived from mammary glands, where we also studied Fas and FasL expression. Apoptotic cells were detected at day 1 after weaning, reaching a relative maximum at day 4 of involution (Figure 6, a and b). The presence of apoptotic cells at as early as 24 hours of involution is in accordance with numerous studies showing cell death in the first 4 days of involution and tissue remodeling later on (38, 44).

We further evaluated the role of Fas and FasL in mammary gland remodeling using the Fas-deficient MRL/*lpr* mice, in which the Fas gene is interrupted by an early transposable element and carries a point mutation in the death domain (24, 45). Lack of Fas or FasL expression in the MRL/*lpr* and C3H/*gld* mice, respectively, prevented apoptosis of mammary cells during the first 3 days of involution (Figure 6, c and e). However, apoptotic cells were found at day 4 of involution (Figure 6, d and f), suggesting that the Fas/FasL system may play an important role in early stage of involution. The timing of the differences in mammary apoptotic cells in the MRL/*lpr* and C3H/*gld* mice is crucial in light of the two-stage model of involution proposed by Lund and coworkers (46). The authors proposed that, postlactational involution of the mammary gland is characterized by two distinct phases. The first phase of involution is characterized by rapid induction of proapoptotic genes within the epithelium (days 1–4) (46, 47). This is the period when Fas and FasL are active based on the pattern of apoptotic cells found in the MRL/*lpr* and C3H/*gld* mice (Figure 6). The second phase of involution is characterized by the induction of genes encoding proteases within stroma cells that result in the remodeling of the gland (46). This phase is Fas/FasL independent as shown by the presence of apoptotic cells in the MRL/*lpr* and C3H/*gld* mice.

This chronology is consistent with Fas and FasL being expressed in the cell surface during the first phase, resulting in the "suicide" of the secretory epithelium. Cells escaping the first phase are then removed by secondary mechanism that is Fas independent. At this time, stroma cells, including macrophages, may induce cells death of the epithelium to ensure the removal of secretory cells (48). IL-1β–converting enzyme (ICE) and p53, two important apoptotic genes, have also been shown to be expressed during the first phase (23, 46, 49). It is noteworthy that these two genes are related to the mechanism of action by which Fas induces apoptosis in normal cells (50–54).

Cells undergoing neoplastic transformation are characterized by the complete or partial loss of posttranslational Fas expression, which leads to resistance to Fas/FasL–induced apoptosis (55–57). While Fas resistance has been reported in breast carcinoma cell lines (58), until now the normal physiological regulation of Fas in breast tissue was unknown. In the present study, we show for the first time that the molecular regulation of Fas and FasL in the mammary gland is sensitive to the hormonal microenvironment, with the expression of Fas and FasL varying according to the stage of development. Thus, during pregnancy, a time of high levels of estrogen and progesterone and very active mammary epithelial cell proliferation, Fas expression is inhibited while FasL protein levels are increased (Figure 2).

Stimulation of mammary cells with sex hormones or lactogenic hormones normally result in cell growth and differentiation (59). These hormones also synergize to promote cell survival by enhancing the production of growth factors and cytokines and the upregulation of antiapoptotic cellular proteins. Although estrogen has been proven to be a hormone that promotes cell survival, stimulation of mammary cells through the estrogen receptor leads to increased cell-surface expression of FasL, an apoptotic gene (27). This estrogenic effect demonstrates that during cell growth and differentiation there is a tight connection between signaling pathways that support cell survival and those that culminate in apoptosis.

We further characterized those cells that express Fas and FasL using immunocytochemistry. Positive immunoreactivity was localized primarily in the glandular epithelium. Moreover, we found not only changes in levels of expression, but also in the intracellular distribution; for example, during pregnancy FasL staining

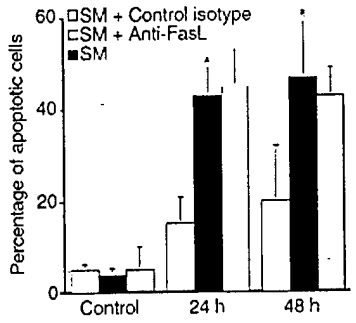

Figure 9
Fas dependence of SM-induced apoptosis. Mammary glandular epithelial cells MCF-10A (human) and HC-11 (mouse) were labeled with [^{3}H]-thymidine for 24 hours and treated with SM for 24 hours and 48 hours in the presence or absence of anti-FasL (NOK-1 mAb, 100 ng/ml). As control we used the purified mouse IgG1 isotype control from PharMingen. The percentage of apoptosis was calculated as described in the text. Data represent the mean ± SD of three independent experiments. $^AP < 0.001$, $^BP < 0.05$.

was predominantly in the cytoplasm, while during involution both Fas and FasL had a primarily membranous localization. The immunostaining for Fas and FasL was not homogeneous, even in the same gland or duct (Figures 4 and 5). Some cells were positively stained ,while other nearby cells were stained negatively, suggesting differences in remodeling stages between cellular districts of the glands and ducts. This heterogeneity seems to be related to differences in remodeling stages between cellular districts of the glands and ducts. Moreover, this heterogeneity of apoptosis (milk shut-down) provides a survival advantage since the entire function of the gland is not lost immediately when sucking stops. Rather, apoptotic factors (e.g., Fas/FasL) trigger graded PCD and decreased milk production, allowing for sucking to be restarted if necessary. If pups resume suckling, the hormonal microenvironment, which constitutes a survival factor, inhibits apoptosis and promotes restoration of milk production. However, if suckling is not restored, the second phase of involution takes place, promoting disruption of basement membrane and extracellular matrix, resulting in a complete remodeling of the gland to a state resembling the mature virgin (47).

To verify the role of Fas and FasL in postlactational involution, we designed an in vitro system employing the normal human MCF-10 cells and the mouse epithelial cells HC-11. HC-11 is an excellent model to study lactogenic differentiation and involution since these cells retain important features of normal mammary epithelial cells as evidenced by their ability to differentiate and synthesize the milk protein β-casein after exposure to lactogenic hormones (60). Removal of lactogenic hormones from these mammary epithelial cells induces the expression of Fas and FasL, followed by caspase 3 activation and cellular cell death (Figures 7 and 8). Quantification of cell death using the JAM assay showed that 40% of the cells were apoptotic after 24 hours of treatment (Figure 9) corresponding to the levels of Fas and FasL expression. The expression of these two proteins had a time pattern similar to the presence of apoptotic cells determined by an annexin V and JAM assay (Figure 8 and 9). Hence, both assays demonstrate a correlation between the presence of apoptotic cells and the expression of Fas and FasL. Furthermore, the activation of caspase 3 after SM treatment (Figure 7), the induction of apoptosis with anti-Fas mAb (Figure 10), and the partial blocking of apoptosis with anti-FasL mAb (Figure 9) further suggest the involvement of Fas and FasL as the mediator of apoptosis in mammary epithelium.

The expression of Fas and FasL during the involution of breast tissue, as well as the presence of apoptotic cells, further supports our hypothesis that the regulation of cell proliferation and cell death by the Fas/FasL system is not confined to cells of the immune system (26, 61). In immune as well as in nonimmune cells numerous evidence suggests that the Fas/FasL system operates downstream of other cellular control mechanisms, such as p-53 (50), c-Myc (54), or Ras (52), to protect against neoplastic transformation (20, 62, 63). Specifically, the death of mammary epithelium during the period of involution may be a physiologic means of eliminating mutated or otherwise transformed cells.

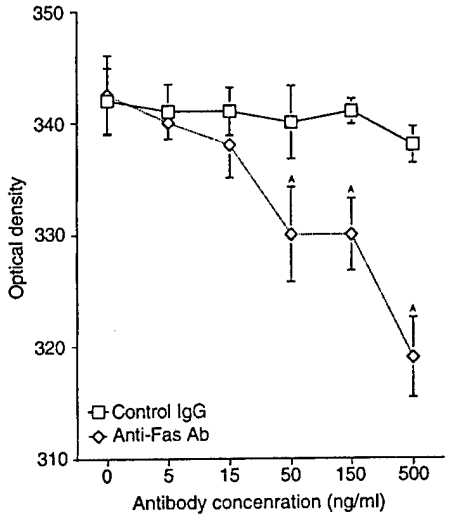

Figure 10
Effects of the anti-Fas mAb on HC-11 and MCF-10A cells. Normal mammary glandular epithelial cells MCF-10A and HC-11 were cultured with different concentrations of anti-Fas IgG mAb for 24 hours (ranging from 5-500 ng/ml). MTT assay was performed to evaluate cell viability. As control, cells were treated with a mouse IgG1 mAb at concentrations similar to the anti-Fas Ab. $^AP < 0.05$. Results shown are representative of at least three independent experiments done with HC-11 cells.

Weaning after breast feeding, which constitutes a crucial factor for involution in the mammary gland, significantly increases Fas protein expression and mammary epithelium apoptosis.

In conclusion, the present study further links the Fas/FasL system and hormones such as estrogen and lactogenic hormones to the biology of the normal mammary gland and the development of breast cancer. We have shown previously how estrogen regulates FasL expression in breast cancer cells to produce an immunologically privileged site that allows the progression of breast cancer without rejection (27). Supporting this hypothesis, we showed that the estrogen receptor antagonist tamoxifen blocks the expression of FasL, which may help to explain it's beneficial role in preventing or treating breast cancer (27). The present report extends our studies to the prevention of breast cancer, suggesting that breast remodeling after lactation could be mediated also by the Fas/FasL system and may play a physiological role in eliminating potentially cancerous cells of the mammary epithelium.

Acknowledgments

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METHYL-TESTOSTERONE INHIBITS AROMATASE ACTIVITY IN JAR CHORIOCARCINOMA CELLS AND MACROPHAGE-LIKE THP-1 CELLS IN CULTURE

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Running title: Methyl-testosterone is an aromatase inhibitor

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ABSTRACT

17α-methyl-testosterone is a synthetic androgen with affinity for the androgen receptor. Methyl-testosterone is used widely as a component of hormone replacement therapy. Previous reports have indicated that contrary to testosterone, methyl-testosterone is not aromatized. However, methyl-testosterone still could affect local estrogen formation by regulating aromatase expression or by inhibiting aromatase action. Both possibilities have important clinical implications.

To evaluate the effect of methyl-testosterone on the expression and activity of aromatase, we tested the choriocarcinoma Jar cell line, a cell line that express high levels of P450 aromatase and the macrophage-like THP-1 cells, which express aromatase only after undergoing differentiation.

We found that in both cell lines methyl-testosterone inhibits aromatase activity in a dose-related manner. The curve of inhibition parallels that of letrozole and gives 86% inhibition at 10^{-6} methyl-testosterone, determined by the tritium release assay. Methyl-testosterone does not have detectable effects on aromatase RNA and protein expression by JAR cells. Undifferentiated THP-1 cells had no aromatase activity and showed no effect of methyl-testosterone, but differentiated THP-1 (macrophage-like) cells had a similar inhibition of aromatase activity by methyl-testosterone to that seen in JAR cells. The Lineweaver-Burke plot shows methyl-testosterone to be a non-completive aromatase inhibitor.

Our results show for the first time that methyl-testosterone acts as a non-competitive aromatase inhibitor. These findings are relevant for understanding the effects of methyl-testosterone as a component of hormone replacement therapy. Methyl-testosterone may, as a functional androgen and inhibitor of endogenous estrogen production, also offer special possibilities for the prevention/treatment of hormone-sensitive cancers.

KEY WORDS: Aromatase inhibitor, methyl-testosterone, androgen, hormone replacement therapy, breast, cancer

INTRODUCTION

The clinical significance of *in situ* estrogen production remains controversial, especially in regards to the development and progression of estrogen-dependent neoplasms [1, 2]. In addition to ovarian follicle cells, estrogens are synthesized from androgens by a variety of human cells, including testicular Sertoli cells, the syncytiotrophoblast, breast stromal and gland/duct cells, fibroblasts, fat cells and neurons in the brain (review see [3], [4]). The aromatase enzyme, a product of the CYP19 gene, is responsible for the conversion of C19 steroids (androstenedione, testosterone and 16-α-hydroxyandrostenedione) into estrone, estradiol-17ß and estriol, respectively [4]. In breast cancer, recent clinical observations suggest that there may be a correlation between intra-tumoral aromatization, tumerogenesis and tumor response to inhibition of estrogen synthesis in patients treated with aromatase inhibitors [3, 5]. The evidence indicates that local production of estrogen by stromal cells[6] and local macrophages [7] could have an important role in the regulation of tumor proliferation [3]. Androgens have been shown to induce aromatase expression in several organs [4, 8]. However, steroidal aromatase inhibitors also have been described [9].

Local formation in the breast and breast cancer is a major estrogen source in both pre- and postmenopausal women [3, 7]. Recently, we have reported that this may include estrogen effects on breast macrophages: In addition to regulating cytokines and growth factors, estrogen also regulates expression of FAS ligand, thereby affording an immune sanctuary to breast lesions [10, 11]. Macrophages express aromatase and estrogen receptors [10], raising the possibility that these estrogen actions are the products of an autocrine loop based upon local estrogen synthesis. We believe that the expression and regulation of aromatase is of especially great importance and clinical relevance during the postmenopausal period when circulating estrogens are low.

The development of aromatase inhibitors and their increasing use in the treatment of breast cancer has given further insight into another aspect of the regulation of this enzyme. Selective inhibition of aromatase constitutes a powerful tool to decrease the production of estradiol without interfering with the production of other essential steroid hormones formed

earlier in the steroid biosynthetic pathway [6, 12, 13]. This carries special importance in the case of lesions that may or may not themselves contain aromatase or estrogen receptors but are affected by estrogen formation and action on e.g. macrophages [3, 13].

Methyl-testosterone is a synthetic androgen with relatively weak affinity to the androgen receptor, which is used as an adjunct to conventional hormone replacement therapy (HRT) during menopause. At present methyl-testosterone is the only FDA-approved androgen in estrogen-androgen HRT treatment in the United States. Its clinical indication is stimulation of libido in women who have undergone ovariectomy [14]. However, it has been shown that methyl-testosterone inhibits estrogen-induced sexual receptivity in the rat and that this effect is androgen receptor-independent [15, 16]. Thus, there is much to learn about the action of this interesting compound.

In the present study we evaluated the effects of methyl-testosterone on two cell lines known to express the enzyme aromatase and found that methyl-testosterone has an inhibitory effect on aromatase activity that is not due to alterations in expression of aromatase at the transcriptional level.

EXPERIMENTAL

Cells and Chemicals

THP-1 myelocytic leukemia cell line cells and choriocarcinoma cell line Jar cells were purchased from the American Type Culture Collection (Rockville, MD). Testosterone and estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{3}H] Androstenedione (24.5 Ci/mmol) and [^{3}H] thymidine (20 Ci/mmol) were purchased from NEN Dupont (Boston, MA). Letrozole was a gift from Novartis and 17α-methyl-testosterone from Solvay Pharmaceuticals, and were used without further purification. All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparations of microsomes from Jar cells

The Jar choriocarcinoma cells were plated in T-75 culture dishes using DMEM medium until they reached 90% confluence. After washing with PBS, cells were scraped from the culture dishes and homogenized with 0.25 M sucrose containing 10mm Tris-HCL (pH 75) and 1 mM EDTA. Microsomal fractions were prepared by successive centrifugation as described by Harada and Omura [17].

Macrophage differentiation

Cells of the human pre-myeloid cell line THP-1 cells were differentiated from their monocyte-like state into macrophages as previously described [7, 18]: In short, $1x10^{-6}$ THP-1 cells were treated with 10-ng/ml the phorbol ester phorbol myristate acetate (PMA) for three days until cells adhered to the flask and exhibited macrophage-like morphology. For experiments, cells were allowed to reach 70-80% confluence and treated as below.

Radiometric Aromatase assay: Tritium Release Assay.

Aromatase activity was measured using the tritiated water release assay as previously described [5, 7]. Briefly, cells were incubated for 1h in the presence of [^{3}H] androstenedione and

0.5 μM unlabeled androstenedione substrate at 37 ° C for one hour under 5% CO_2. Following incubation, 1ml aliquots of the supernatant were transferred to a test tube and 2 ml of chloroform added to each tube to extract unconverted radioactive substrate and other steroids. The samples were mixed by vortex and centrifuged. The aqueous phase was collected and treated with 2.5% activated charcoal suspension in phosphate buffered saline (PBS) to bind residual labeled and unlabeled steroids and centrifuged. The supernatant was decanted, mixed with scintillation fluid and counted in a beta-spectrometer; thus, tritiated water formed during the aromatization of [1^3H] androstenedione to estrogen was determined by measuring the radioactivity in the supernatant. We used boiled Jar cells and undifferentiated THP-1 cells that do not express aromatase [7] as negative controls. Pretreatment with letrozole was used as a positive experimental control since letrozole blocks aromatase activity.

Hormonal treatment of cell lines

All *in vitro* experiments were done when the cell cultures reached 80% confluence. Prior to any treatment the cells were incubated in serum and phenol red-free medium for 24h. Hormones and letrozole were freshly prepared using the same culture media as a diluent to obtain the doses described below.

RT-PCR and hybridization for aromatase mRNA

Total RNA was prepared from 2 X 10^6 Jar- or THP-1 cells, using TRIzol reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. RT-PCR was performed using the RT-PCR kit from Pharmacia BioTech (Piscataway, NJ) according to the manufacturer's protocol. cDNA synthesis was carried out with 0.2 μg of pd(N)6 and 5 μg of total RNA. The primers used for amplification of aromatase mRNA have been described [19]. The PCR product was then separated by electrophoresis and transferred to a blotting membrane by capillary elution. Southern hybridization was performed with the ECL 3'-oligolabelling and detection system (Amersham, UK) according to the instructions of the manufacturer.

Western Blot Analysis

Total protein was isolated with TRIzol reagent according to the manufacturer's instructions. Proteins were separated by SDS-PAGE using 12% polyacrylamide gels and transferred to nitrocellulose membranes. Immuno-blotting was performed after blocking of non-specific binding to the membranes with buffer containing 5%-powdered milk. The primary antibody (aromatase polyclonal antibody [20]) was used at 1:2000 dilution. The labeling antibody (peroxidase labeled goat anti-rabbit, Vector, Burlingame, CA), was developed with the TMB Peroxidase substrate kit (Vector, Burlingame, CA). For quantification, the protein was loaded and transferred to a membrane, Ponsou Red was applied followed by antibody incubation. The signals were quantified by a densitometer, standardized against the amount of loaded protein in each lane.

Analysis of data

Statistical analysis was done using Student's T-test or ANOVA analysis. Analysis of data and graphic utilized GraphPad PRISM software (version 2.0) (San Diego, CA).

RESULTS

Effect of methyl-testosterone on aromatase activity in JAR choriocarcinoma cells.

In order to examine the effect of methyl-testosterone on aromatase activity, we treated Jar cells with methyl-testosterone. Jar cells grown in 6-well plates were incubated in serum-free media for 24h followed by treatment with methyl-testosterone for another 24h. Aromatase activity measured as release of tritium-labeled water from substrate androstenedione by Jar cells in culture was determined as described in Material and Methods. In the initial study, methyl-testosterone (10^{-8} M) induced a 60% inhibition of aromatase activity in Jar cells (data not shown). Therefore, the effect of methyl-testosterone was further characterized by dose- and time responses (below).

Boiled JAR cells and undifferentiated THP-1 cells were negative method controls and as expected showed no release of tritiated water.

Dose response: Jar cells cultured in serum-free media for 24h were treated with different concentrations of methyl-testosterone for 24h. As showed in figure 1, methyl-testosterone administrated in different concentrations (10^{-6}, 10^{-8}, 10^{-10}, 10^{-12} M) showed a dose-response inhibitory effect on Jar cells' aromatase activity. Under these conditions inhibition of the enzymatic activity was 60%, at methyl-testosterone concentrations of 10^{-8} M. The substrate consisted of 300 nM unlabeled androstenedione and 30nM ^{3}[H]-androstenedione. Triplicate assays were performed two times and the results expressed as percentage of the control.

Time course: To explore the mechanism of aromatase inhibition by methyl-testosterone in Jar cells, we performed kinetic analysis of inactivation of the enzyme. Incubation with methyl-testosterone (10^{-8} M) had a maximal inhibitory effect by 3h of treatment that lasted at least 24 h (Fig 2). In fact, the same levels of aromatase activity were present at 48h (data not shown) indicating the possibility of a prolonged, non-competitive action of methyl-testosterone, see below. While these results indicated a direct effect of methyl-testosterone on aromatase activity, gene expression was evaluated to confirm this.

Effect of methyl-testosterone on aromatase gene transcription.

Jar cells were treated with different concentrations of methyl-testosterone and for different time intervals. Total RNA and protein were extracted and aromatase expression was evaluated by RT-PCR and Western blot analysis. Treatment of Jar cells with methyl-testosterone did not significantly affect aromatase mRNA expression (data not shown).

These results were confirmed at the protein level. Western blot analysis did not show changes in Jar cells' aromatase protein concentration following treatment with methyl-testosterone (data not shown).

Thus, while methyl-testosterone inhibited aromatase activity, it neither increased nor decreased the transcription of the gene or affected the amounts of aromatase protein present in Jar cells.

Inhibitory effect of methyl-testosterone on aromatase activity of macrophage-like THP-1 cells

We have shown previously that monocytes, upon differentiation with PMA into tissue macrophages, express aromatase and have aromatase activity [7]. We therefore evaluated the effect of methyl-testosterone on differentiated THP-1 cells. Using PMA at a concentration of 10ng/ml the myeloid leukemia cell line THP-1 was induced to differentiate into macrophage-like cells in culture. After three days in culture, differentiated THP-1 cells (+PMA) were incubated in the presence of various concentrations of methyl-testosterone. As in our experiments with the Jar cells, aromatase activity in **differentiated THP-1 cells** was inhibited in a dose dependent fashion (figure 3). A 50%, 45%, 14%, 29% inhibition of aromatase activity was observed at methyl-testosterone concentrations of 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12} M, respectively. A similar experiment was carried out using undifferentiated THP-1 cells, which have previously shown not to express aromatase.

Aromatase activity in **undifferentiated THP-1 cells** remained at background levels at all concentrations of methyl-testosterone. Similarly as with Jar cells, RT-PCR and Western blot analysis showed that methyl-testosterone had no effect on aromatase expression either at the RNA or protein level (data not shown).

Effect of methyl-testosterone and letrozole on aromatase activity

Letrozole is a triazole that has been shown *in vivo* as well as *in vitro* to suppress estrogen formation [3]. In order to test the specificity of our system we compared the effect of letrozole on Jar cells to the above described effects of methyl-testosterone. As shown in figure 4, at 10^{-12} M both compounds inhibited aromatase activity. However, letrozole had a superior inhibitory effect to methyl-testosterone at higher concentrations: In comparison with 100% inhibition with letrozole at 10^{-6} M, inhibition by methyl-testosterone at 10^{-6} M was 87.5%.

Lineweaver-Burk plot

In order to determine the type of inhibitory effect of methyl-testosterone, aromatase activity was measured using JAR microsomes and varying concentrations of [^{3}H] androstenedione (5 - 500 nM) in the presence of fixed concentrations of methyl-testosterone (10^{-6}M). The Lineweaver-Burk plot in figure 5 shows a classical non-competitive inhibitory effect of methyl-testosterone.

DISCUSSION

Aromatase is a cytochrome P450 enzyme complex that is responsible for the demethylation of androgens to form estrogens [3, 21, 22]. Methyl-testosterone is a clinically popular androgen that could affect estrogen formation in many organs. In these studies we showed that methyl-testosterone is a potent aromatase blocker, but does not affect aromatase gene expression in Jar or THP1 cells. Estrogen has been implicated in the function of almost all organs and the pathophysiology of numerous diseases, among which are breast and endometrial cancers, uterine leiomyomas, endometriosis, and gynecomastia [22, 23]. Therefore, the blockade of estrogen formation by methyl-testosterone has considerable potential clinical importance.

Although the safety and beneficial effects of methyl-testosterone administration in conjunction with estrogen has been documented [14, 24], little or nothing is known about methyl-testosterone's cellular mechanism of action and how it might differ from that of the parent compound, testosterone. Methyl-testosterone is an anabolic-androgenic compound used in combination with estrogen to improve symptom relief and long term effects of decreased sex steroids in menopausal women. Methyl-testosterone has also been used to treat complaints of decreased sexual motivation, fatigue, depression and headaches [24]. From the present data, it is unlikely that methyl-testosterone's salutory actions are via increased estrogen.

Aromatase consists of one polypeptide chain that is a product of a single gene, and displays similarities to members of the cytochrome P450 superfamily [25]. Aromatase has the property of catalyzing three chemical reactions converting androgens into estrogens [25]. In the present study we evaluated the effect of methyl-testosterone on aromatase activity and gene expression using the JAR choriocarcinoma cell line as an *in vitro* model. Jar cells express high levels of aromatase, which makes them a good model for studying estrogen biosynthesis. Thus, we first evaluated the effect of methyl-testosterone on aromatase activity by the tritiated water-release assay [26]. Jar cells were treated during 24 hours with methyl-testosterone (10^{-7} M) following by addition of radioactive labeled [^{3}H]-androstenedione. The choriocarcinoma cell line

had high levels of aromatase activity as previously reported, but had markedly decreased activity following methyl-testosterone treatment. We further characterized this effect by dose- and time response studies, confirming that methyl-testosterone specifically inhibits aromatase activity in a dose-dependent manner. The response was seen at the first time point examined (3h) after treatment and remained present for at least 48h, indicating that the methyl-testosterone might be bound to the aromatase, requiring the production of new protein before aromatase activity could resume, i.e. that methyl-testosterone is likely a noncompetitive inhibitor of aromatase.

Review of the literature indicates that others [27] did not find methyl-testosterone to be an aromatase inhibitor. While we cannot explain the apparent disparity in results, our positive findings (marked dose- and time-related aromatase inhibition) plus the use of boiled cells and aromatase negative cells as controls supports the present study. Moreover, the parallel with letrozole effect and the Lineweaver-Burke study using JAR cell microsomes further support methyl-testosterone's effect as a powerful, noncompetitive aromatase inhibitor. We plan *in vivo* studies to further support the present results.

We analyzed whether methyl-testosterone changed aromatase gene expression. Jar cells were treated with methyl-testosterone for 24h followed by RNA and protein extraction. Using specific primers for human aromatase [7] we analyzed aromatase mRNA expression by RT-PCR. No change in aromatase mRNA was detected after methyl-testosterone at different concentrations or times of incubations. We verified this result at the protein level using a polyclonal antibody for human aromatase in western blot analysis [7, 28, 29]. Again, no changes in the expression of the enzyme were detected by western blot. These findings are very interesting since methyl-testosterone has been shown to have considerable potency as an androgen [30], apparently via interaction with androgen receptors and might have been expected to increase aromatase expression [8]. Further studies of the mechanism(s) action of methyl-testosterone will be required to explain this apparent difference in action and rule out novel mechanisms of methyl-testosterone action.

In the present study we present evidence that methyl-testosterone inhibits aromatase activity in human choriocarcinoma Jar cells and differentiated, macrophage-like THP-1 cell lines. We found no evidence of methyl-testosterone-induced aromatase gene expression. It is fortunate that JAR cells have been reported to lack androgen receptors. This further clarifies the mechanism of methyl-testosterone to be direct, inhibiting the enzyme [8]. Thus far no reports have appeared regarding androgen receptors in THP-1 cells and it is a matter for future studies to assess methyl-testosterone action as an androgenic promoter of aromatase in androgen receptor-positive cells.

The inhibitory effect of methyl-testosterone on aromatase activity is not limited to Jar cells. We previously have shown that monocytes, after undergoing differentiation into macrophages express aromatase [7]. Therefore, we tested methyl-testosterone's effect on the monocyte/macrophage-like THP-1 cell line. Treatment of macrophages with methyl-testosterone inhibited aromatase activity with similar potency as described for Jar cells. Interestingly, methyl-testosterone did not induce aromatase in undifferentiated THP-1 monocytes; changes at the protein or mRNA levels were not found in methyl-testosterone-treated monocytes, or in macrophages. Since monocytes/macrophages and local estrogen formation figures prominently in our hypothesis of the relationship between estrogen and breast cancer these results raise a possible protective effect of methyl-testosterone on breast lesions [3].

The lack of a methyl-testosterone effect on aromatase protein expression suggests that methyl-testosterone's inhibitory effect on aromatase activity is not at the gene level. We therefore compared the effect of methyl-testosterone with that of letrozole, a highly selective competitive inhibitor of the aromatase enzyme [31]. Indeed, both letrozole and methyl-testosterone had inhibitory effects on aromatase activity in the tested cells (Fig. 4), confirming that methyl-testosterone's inhibitory action could be at the catalytic site of the enzyme.

Inhibitors of aromatase have been subdivided into two main groups according to their mechanism of action and structure: Type I inhibitors associated with the substrate-binding site of the enzyme and having an androgen structure; and type II inhibitors which interact with the

cytochrome P450 moiety of the system and are non-steroidal. Our evidence indicates that methyl-testosterone acts as a Type I inhibitor of aromatase activity, binding to the active site of aromatase. The specificity of these results are supported in our experiments with Jar and THP-1 cells where methyl-testosterone inhibited aromatase activity in a dose-and time-dependent manner. The inhibitory effect of methyl-testosterone was consistently maintained and independent of the pre-incubation time. Furthermore, the effect of methyl-testosterone on aromatase activity belongs to the non-competitive type of inhibition as demonstrated in figure 5. These results suggest that the compound is not easily removed from the cells. In that regard, methyl-testosterone appeared to be stable in Jar cell culture for at least 48h and inhibited any newly synthesized enzyme during that period.

Although estrogen's role in carcinogenesis remains unclear [2], the search for therapeutic modalities designed to block estrogen production is ongoing. So far aromatase inhibitors have had some success in controlling metastases and perhaps new tumor growth [3, 25]. Our data indicate that methyl-testosterone might also play such a role.

In summary, this work for the first time provides evidence that, in addition to its androgenic activity, methyl-testosterone acts as an aromatase inhibitor. These findings are relevant for understanding the effects of methyl-testosterone in hormone replacement therapy as a possible preventive or therapeutic agent against hormone-sensitive cancers.

FIGURE LEGENDS

Figure 1. Inhibition of aromatase activity by pretreatment of Jar cells with methyltestosterone. Jar cells grown in 6 well plates were treated with methyl-testosterone at concentrations of 10^{-6}, 10^{-8}, 10^{-10} and 10^{-12} M for 24 hours. Aromatase activity was evaluated by the water release assay. The results are expressed as mean ± SD of three experiments performed in duplicate.

Figure 2. Effect of methyl-testosterone on aromatase activity, time response. Aromatase activity was determined in Jar cells grown in 6 well plates treated with methyl-testosterone (10^{-8} M) during 3, 6 and 24 hours.

Figure 3. Effect of methyl-testosterone on aromatase activity in THP-1 cells. THP-1 cells express aromatase only following differentiation into tissue macrophages. Methyl-testosterone inhibits aromatase activity on differentiated THP-1 cells. No effect of methyl-testosterone was found in undifferentiated THP-1 cells.

Figure 4. Aromatase inhibition by letrozole and methyl-testosterone in Jar cells. Jar cells were treated with letrozole or methyl-testosterone at different concentrations for 24h. Aromatase activity was determined by the tritiated water release assay.

Figure 5. Kinetic analysis of aromatase inhibition by methyl-testosterone in Jar cells homogenates. Lineweaver-Burke plot using [^{3}H] androstenedione in varying concentrations (5 to 500 nM) and constant methyl-testosterone concentration. The plot shows that methyl-testosterone has a non-competitive inhibitory effect on aromatase activity.

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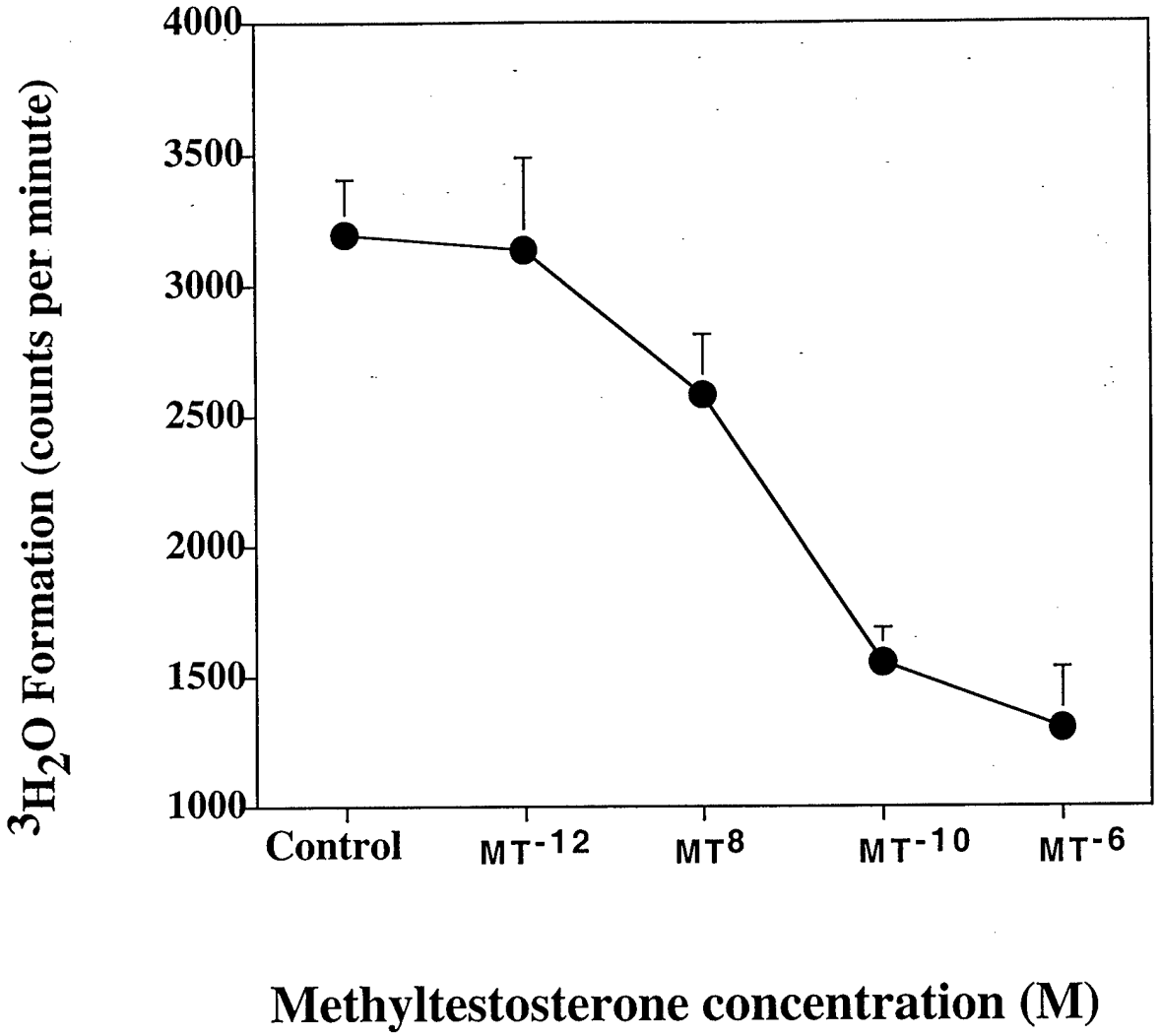
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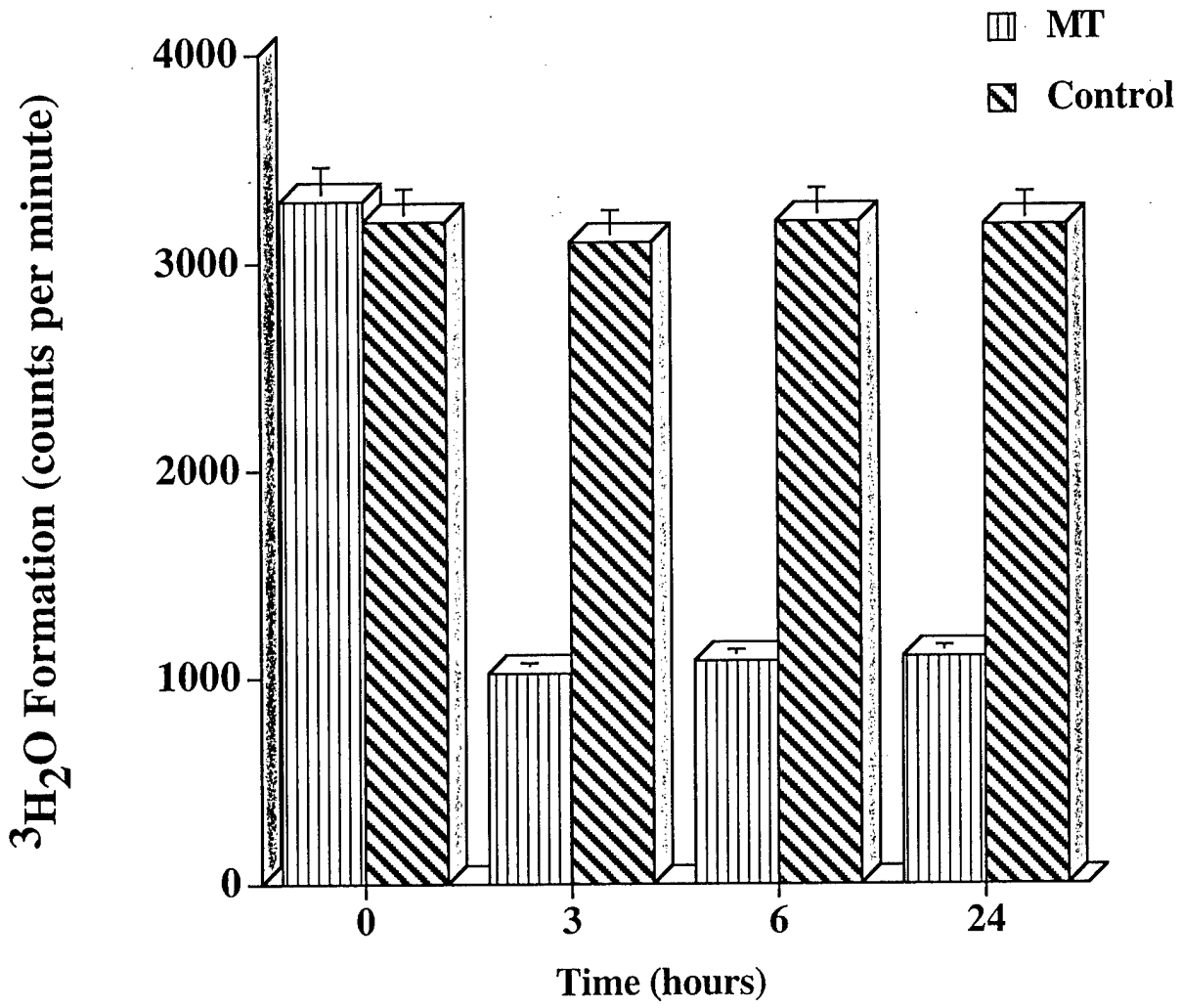
Methyltestosterone concentration (M)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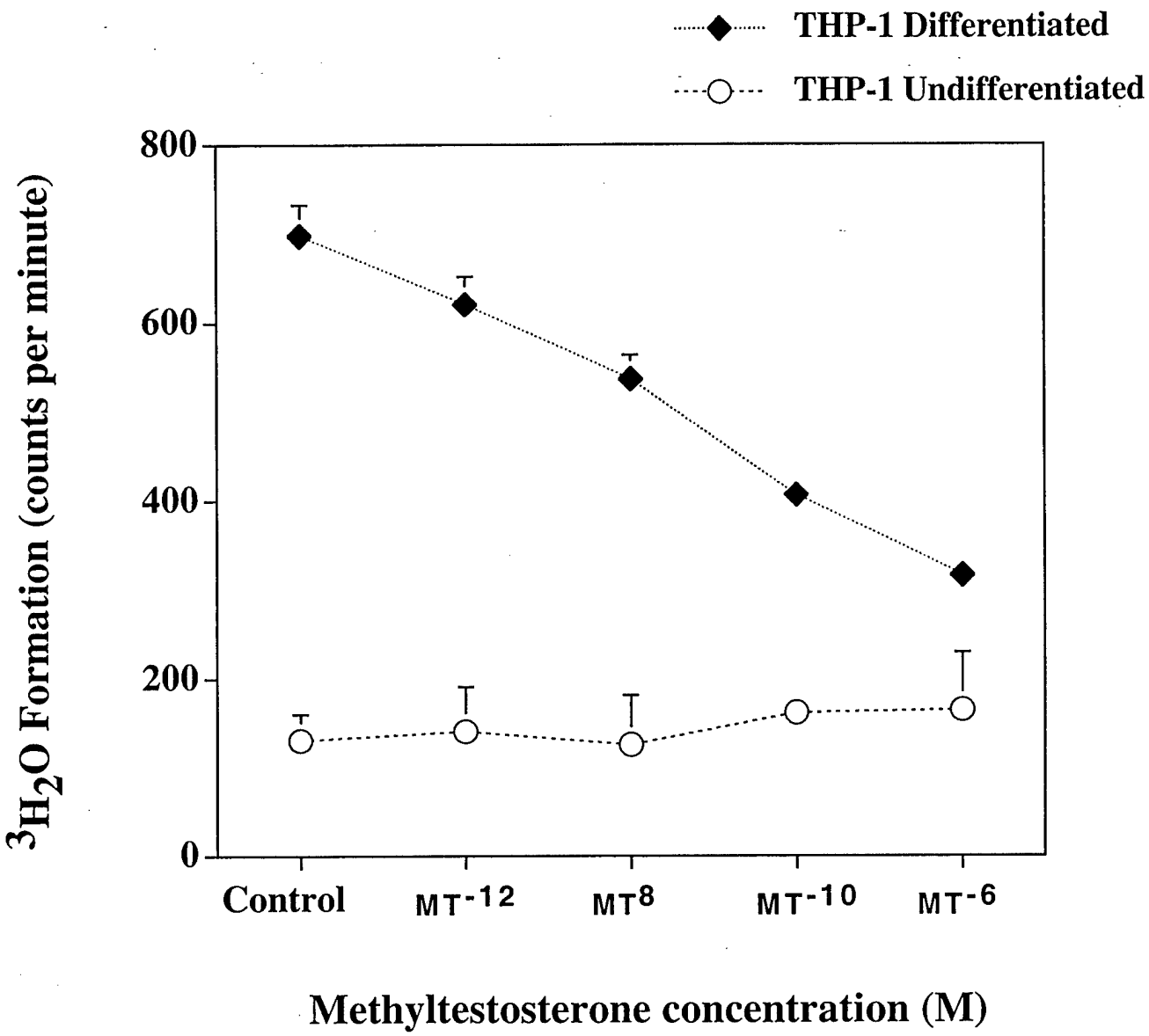
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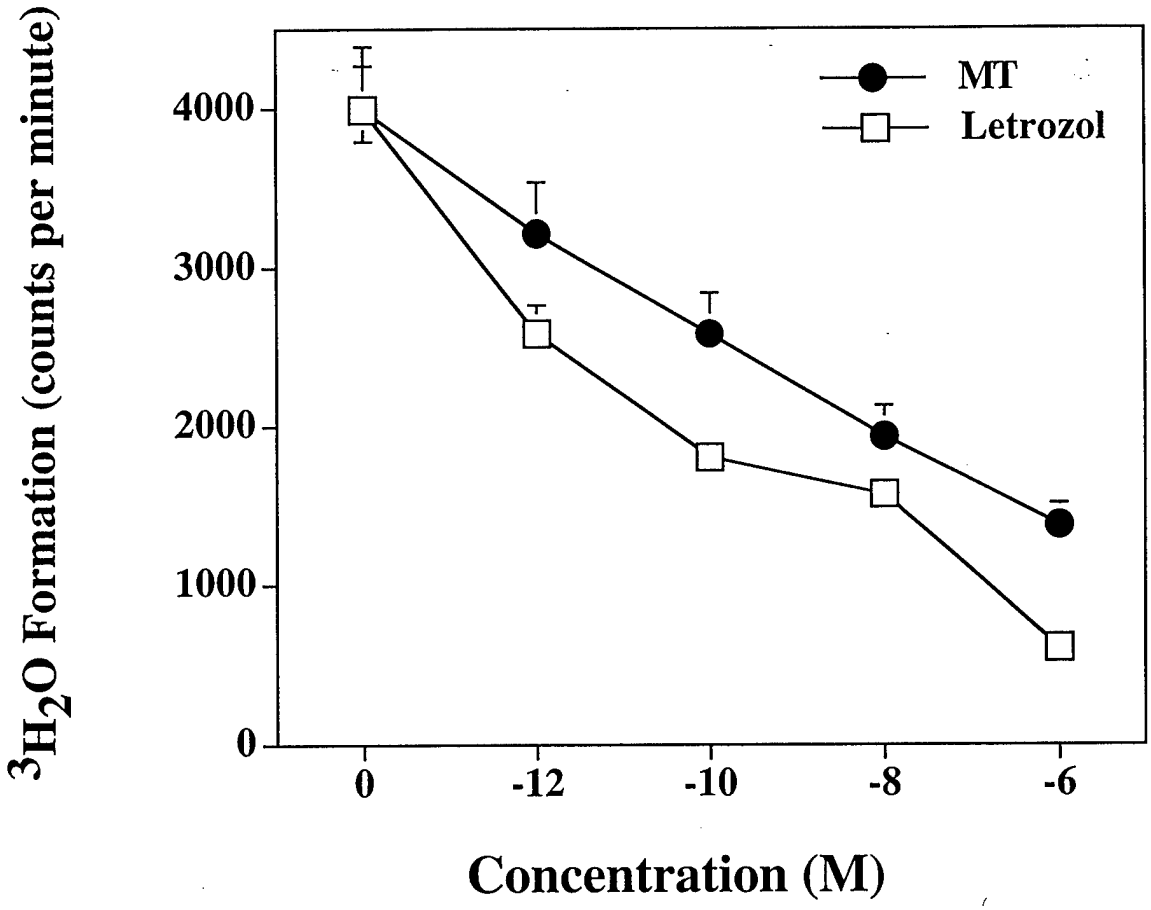
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Letrozol
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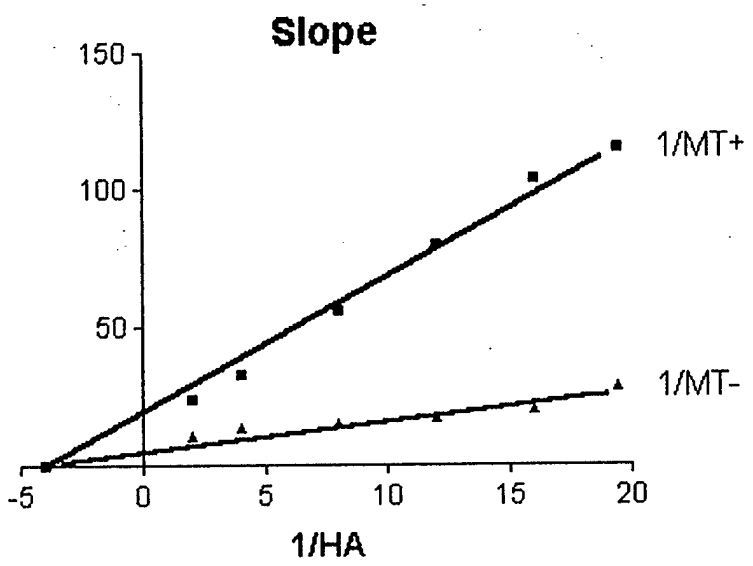
^3H_2O Formation (counts per minute)
Concentration (M)


Slope
150
100
50
-5
0
5
10
15
20
1/HA
1/MT+
1/MT-