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Tumor growth is absolutely dependent upon angiogenesis. Prior to the development of breast cancer, the breast tissue in many women undergoes progressive changes which include proliferative breast disease and carcinoma in situ. We examined these pathologic changes for the level of vascularity and the presence of angiogenic growth factors in the hopes of identifying targets for chemoprophylaxis. To date, we have shown that angiogenesis begins in the earliest stages of progression. In addition, even normal breast tissue contains numerous angiogenic factors; however, these are insufficient to induce angiogenesis. Using an in vitro organ culture system of normal breast tissue, we found that exogenous angiogenic factors were unable to stimulate endothelial cell proliferation. Furthermore, under non-stimulated conditions, endothelial cell proliferation was restricted to the adipose tissue and perilobular connective tissue. The endothelium within the fibrous stroma could almost never be induced to proliferate. We are now examining normal breast epithelium and stroma for the presence of angiogenic inhibitors. Angiogenic potential in this tissue appears to be the result of an interplay of angiogenic stimulators and inhibitors.

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FOREWORD

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Response to Previous Report Critiques In response to the annual report for this project in 1996, I received two quite different critiques. Reviewer 1 made specific helpful suggestions regarding format and content which have been incorporated into this document. Reviewer 2, on the other hand, raised scientific issues regarding the value of documenting the presence of numerous growth factors in pre-invasive breast disease and questioned the ability of the organ culture system to be a model system for angiogenic growth. Reviewer two also asked me to provide an alternative approach if the organ culture system does not work. Since I share some the concerns raised by reviewer two, I shall begin this report by specifically addressing these concerns. Because this is a response, in this section I shall assume knowledge of the project and specific details of the proposal.

Technical Issues:
Under "Technical Issues" reviewer two made the statement that the project is "overly ambitious". Furthermore, reviewer two indicated that little information would be gained in documenting the growth factors and proteinases in pre-invasive breast disease relative to what is already in the literature. In response, clearly the documentation of growth factors immunologically can be accomplished. Furthermore, if one reviews the literature, there is little-to-no information on the expression of these factors in proliferative breast disease or in normal breast tissue and only scant information in in situ carcinoma. In personal discussions at meetings, I have spoken with individuals who I would have expected to know the field (for instance I spoke with Dr. Hal Dvorak regarding the expression of vascular permeability factor, VEGF, in breast); in all cases no one had looked earlier than in situ carcinoma. Therefore, while descriptive in nature, we continue to believe that the cataloguing of angiogenic factors is a worthwhile goal, since these are all potential therapeutic targets. With regard to the organ culture system, we are testing new criteria for identifying angiogenic growth. Clearly we will not be able to perform studies using every growth factor identified. Therefore, we present in this report our approach and the factors which we, in consultation with others, have determined to be most important.

Reviewer two also is concerned with the feasibility of the organ culture system and requests more detail regarding these experiments. As stated from the beginning, this system was chosen because it is complex, containing all the interacting elements which may influence angiogenic growth. At the beginning, we did not know to what extent we would be able to induce angiogenesis. Since the initial experiments, we have become more sophisticated in our analysis and have begun to analyze our data with respect to the specific steps of angiogenesis which can be identified (eg. endothelial cell proliferation, pericyte motility, vascular sprouting, endothelial cell migration, expression of specific adhesion molecules, etc.) which were not part of our original proposal. We now know that we can induce endothelial cell proliferation, pericyte proliferation, and early vascular sprouting. To date we have been unable to form a complete vascular network. However, we are beginning to understand that this is regulated by the surrounding matrix of the breast, since proliferation and sprouting are much more likely to occur in adipose tissue or

adjacent to lobular epithelium, than within fibrous stroma. The assay systems we are now using will allow us to determine which of the angiogenic factors will induce the proliferative and sprouting responses. Since the matrix appears to be so important in regulating vascular growth we will look carefully at proteinase and matrix production in a few isolated cases. Details of these studies are presented below. Therefore, we agree with reviewer two that the organ culture project is ambitious and that examining the effects of all growth factors is unrealistic; however, we are learning about the regulation of vascular growth in the breast and believe that we will learn what some of the sources of in situ inhibitors of angiogenesis are from the proposed experiments. We anticipate that these data will be important in designing experiments to inhibit angiogenesis in vivo.

As indicated by reviewer two, we began our VEGF experiments using A431-conditioned media rather than purified VEGF. This was done for two reasons. First, we had not purified VEGF at the time. Second, A431 cells are known to produce large quantities of all forms of VEGF, as well as other angiogenic factors. These cells are angiogenic in CAM assays; therefore, we felt that conditioned media would be a positive control for our assay, in that it would present the tissue with a complex array of activated factors, including VEGF. Since the time of our last report we have also used purified commercial VEGF in our assay system. The results of these studies are presented below.

Finally, reviewer two believes that studies using PKC and PKA inhibitors are premature. I disagree. In the field of angiogenesis, PKC in particular has been studied extensively and phorbol esters are potent angiogenic agents. Folkman has often emphasized in his lectures that the control of angiogenesis is due a complex interplay of factors which both stimulate and inhibit specific aspects of vessel formation. PKC is thought to be a critical signaling pathway in the switch to the angiogenic phenotype. Therefore, we have every reason to expect that regulation of PKC may give us effects that isolated growth factors do not. Our studies with PKC and PKA inhibitors to date may have yielded the most interesting data in this complex system, in that it has highlighted the fact that normal tissue from pre- and post-menopausal women differ in vitro (see enclosed manuscript). As a result of these studies, we are going back to our earlier tissues to see whether the expression of an angiogenic phenotype also differs with patient age.

<u>Specific discrepancies, recommendations, and/or technical assistance:</u>
In this section reviewer two made the recommendation that if technical difficulties persist, we should examine our archival tissue for fewer factors, but more in depth. We have encountered a number of difficulties, particularly with commercial reagents which do not have the advertised specificity. To overcome this problem, when possible we have used multiple antibodies to the same growth factor. In addition, we have used either in situ hybridization or RT-PCR on either archival or freshly isolated tissue to confirm antibody specificity. We have already completed the vast majority of the immunologic studies and are at the point of checking these data with a few in situ hybridization experiments. In a few cases, such as with uPA and tPA, we have not found reagents which work in paraffin tissue. At present we are putting these studies on hold, while attempting to identify and request reagents from private sources.

Finally, reviewer two states that "the organ culture is risky" and "the PI may benefit from reevaluating the feasibility of this aim and developing an alternate approach if the model system does not work". I think that the critical term here is "does not work". Currently, our data indicate that we shall not ever get a complete vascular network by the simple addition of growth factors to the organ cultures; therefore, the organ culture system is probably not going to yield a high through-put screening assay for inhibitors of angiogenesis. On the other hand, it is extremely important to know why we can get endothelial cell proliferation, pericyte proliferation and vascular sprouting, but not endothelial migration into the stroma, and why even the proliferative response is dependent upon the adjacent stroma. Isolated endothelial cells from almost any source are capable of forming tubules. In our system, nutrient deprivation strongly stimulates the early but not the late phases of vascular growth. Now we need to find out why the response is inhibited. Potent angiogenic agents also have limited effect. This could be due to an overwhelming amount of angiogenic inhibitors or to the presence of potent angiogenic agents already present in the tissue. Therefore, we agree that the organ system is "risky" for the development of a definitive angiogenic assay, but we think that what we will learn from this system will allow us to design future studies to more fully understand the regulation of angiogenesis in this tissue.

INTRODUCTION:

Breast Cancer Breast cancer is the most common incident cancer among women (1). This year alone, nearly 50,000 women will die of this disease in the U.S.(2). With a steep rise in incidence among older women and a progressively older population base, the incidence of breast cancer is expected to continue to rise (3). Despite intensive efforts towards early detection, through self examination and mammographic screening, the mortality rates for this disease have not changed significantly over the past two decades (4).

The problem is further compounded by epidemiologic studies which have not identified a dominant risk factor for breast cancer (5,6). However, women with a family history of breast cancer and/or proliferative breast disease are at increased risk of developing breast cancer (7). Recently, the average stage of breast cancer at diagnosis has decreased; smaller, less advanced tumors are being detected through screening modalities (6). In addition, many breast biopsies are performed which detect *in situ* carcinoma and proliferative breast disease. Unfortunately, the biology of these pathologic conditions has not been predictable, and therefore the clinical utility of identifying patients with these conditions is controversial (4). No prognostic marker has yet been identified which can effectively stratify tumors with similar histopathology into subgroups that identify those at greatest risk for developing invasive disease (7). Because of the clinical difficulty in counseling patients with pre-malignant breast disease (proliferative breast disease) and our current inability to target patients with *in situ* carcinoma of the breast for individualized treatment according to prognosis, we have decided to focus on developing prognostic factors for this early stage of disease. This proposal focuses on an early event in the tumorigenic cascade, angiogenesis.

The process of tumorigenesis in breast epithelium occurs in a defined series of morphologically identifiable steps. On the molecular level, specific alterations in the expression of proto-oncogenes and the loss of or aberrant expression of tumor suppressor genes accumulate as the epithelium progresses from a benign proliferation to an invasive growth of malignant cells, with metastatic potential. The transition from benign, albeit atypical, proliferation of clearly abnormal cells to a bonafide *in situ* carcinoma is defined by careful morphologic criteria by pathologists. However, the biochemical and phenotypic markers which define these transitions are poorly understood. Atypical proliferative lesions of the breast and, to a greater extent, *in situ* carcinomas have a prognosis which is easily defined for a population of women, but which is difficult to apply on a case-by-case basis. For instance, atypical hyperplasia is associated with a 4.5 fold increase in risk for developing invasive carcinoma; however, this diagnosis does not inform an individual woman that she will or will not develop cancer. The poor predictive value of these tests is reflective of an incomplete biochemical understanding of what we can define histopathologically. Clearly, some women with atypical hyperplasia will progress to potentially fatal invasive carcinoma of the breast. Other women will survive with their proliferative epithelium, and even in some cases with *in situ* carcinoma, and never develop invasive disease. Therefore, it is imperative that we dissect this transition from benign

proliferation to early pre-invasive cancer in order to understand which women are in danger of invasive disease.

Angiogenesis Early in the process of tumorigenesis a nutrient supply must develop. Proliferating cells induce a blood supply by a process termed angiogenesis. In the adult animal, angiogenesis occurs only during reproductive events in women or in pathologic conditions, such as cancer or wound repair (8). Angiogenesis is required for tumor growth (9). Since the production of new vessels occurs primarily during disease processes, therapeutic modalities which target new vessels selectively inhibit tumor growth without harming normal physiologic processes. A plethora of soluble growth factors and extracellular matrix proteins regulate the process of angiogenesis in different systems. Angiogenic growth factors function as soluble, diffusible proteins. They also become sequestered in the extracellular matrix (ECM); proteolytic enzymes, produced by the endothelium or tumor cells, dissolve the ECM allowing the released growth factors to stimulate vessel production (10). In addition, some growth factors, such as transforming growth factor β (TGFβ), are secreted in a latent form, which require proteolytic digestion for activation (11). Despite an extensive knowledge of cellular products which induce angiogenesis *in vitro* and *in vivo*, for any one tumor type the mechanism by which angiogenesis is controlled remains unknown. In particular, little information exists regarding the events which occur when an epithelial proliferation of cells converts from a pre-angiogenic growth phase to a rapidly growing population of cells which induces angiogenesis. The definition of these cellular events is critical to designing novel treatment strategies.

Microdissection studies show that angiogenesis begins in venules (12). Angiogenesis occurs by the coordinated regulation of numerous cellular events, including dissolution of the basement membrane, endothelial cell migration from a venule to form a sprout, alignment of emigrating cells to form a solid cord, formation of a lumen by changing the three-dimensional shape of the aligned cells, and investment of this new vessel with a basement membrane and adjacent pericytes (8). The initial stages of angiogenesis involve specific changes in the types of adhesive interactions which an individual endothelial cell recognizes. The cell which is destined to form a new vessel must depart from adjacent endothelial cells to which it is normally adherent, pass through the basement membrane, and migrate into the interstitial space, which may contain a variety of ECM components. For migration to occur, the shape of the cell changes, undergoing elongation and contraction, as adhesive interactions with the surrounding substrate are alternately made and broken. The extracellular matrix proteins made by the endothelium during this process also change. No one knows how specific growth factors or proteolytic enzymes regulate this complex process.

While many soluble and insoluble factors mediate specific cellular functions during the angiogenic process, recently one growth factor, VEGF, has received prominence as an angiogenic factor in tumors and during development (13,14). Unlike all other mitogenic, angiogenic factors identified thus far, VEGF induces mitosis only in endothelium (15). VEGF-specific receptors are tyrosine kinases (16-18). The regulation of these receptors in

tumors and in embryos suggests that they regulate angiogenic events, occurring only in endothelial cells undergoing angiogenesis. VEGF contains a signal peptide allowing its secretion (19); several tumors which are highly angiogenic produce this growth factor (15,20). All other factors (soluble and contained within the ECM) are present in many tissues in the absence of an angiogenic response. This lack of specificity does not diminish their role as potential regulators in the angiogenic process; however, it makes them less likely candidates for the initiating angiogenic "promoter". As with specific ECM proteins, these growth factors, such as bFGF, TGFα, EGF, TGFβ, and PDGF may be permissive in their role, required for or augmenting specific aspects of the angiogenic process, but not sufficient for initiating the event.

Control of angiogenesis occurs through both positive and negative regulators. The process of angiogenesis resembles the process of early tumor invasion (21). Angiogenic agents may be thought of as oncogenes and angiogenesis inhibitors, as tumor suppressor genes (22). For instance, Noel Bouck has cloned a tumor suppressor gene, whose protein product was found to be an inhibitor of angiogenesis (23). Therefore, it is plausible that the elaboration of angiogenic agents by tumors may serve as an autocrine stimulus leading to invasion, or that other factors produced by tumors may cause the endothelium to produce agents which alter the phenotype of *in situ* epithelial neoplasms, allowing them to invade. Numerous growth factors affect both epithelial and endothelial cells (24). In addition, the same proteolytic enzymes which are produced by invading epithelial cells are produced by invading vessels (25). Therefore, understanding the basic mechanism of tumor-induced angiogenesis in breast cancer will have an impact on our understanding of the early events in tumor invasion.

Understanding the dynamic process of tumor-induced angiogenesis requires a model which provides multiple cellular elements. Epithelial cells, stromal cells (fibroblasts, smooth muscle cells, pericytes), and endothelial cells each produce and respond to growth factors in specific ways. For instance, basic fibroblast growth factor induces angiogenesis, but it is also mitogenic for most cells (26). Transforming growth factor alpha (TGFα) and the insulin-like growth factors (IGF I and II) stimulate DNA synthesis in many cell types (27). Other angiogenic agents, such as platelet-derived growth factor (PDGF), induce cellular migration (chemotaxis)(28). Proteolytic enzymes which are required for cellular invasion, both of tumor cells and endothelium, function by dissolving ECM protein. Growth factors released through this process include the heparin-binding growth factor family, acid and basic fibroblast growth factor (aFGF and bFGF), and transforming growth factor beta (TGFβ), all of which induce angiogenesis. TGFβ also regulates the synthesis of ECM proteins by endothelial cells (29). Because of this complicated array of proteins to which more than one cell type responds, a model, in which one can manipulate the expression of these proteins to understand the cellular interactions, must contain each cellular component in its natural surroundings. Animal models of tumorigenesis exploit this feature of normal cellular associations. However, these models possess the well-recognized disadvantage of inter-species variability.

My laboratory has been working on developing an organ culture system of normal breast tissue, which includes epithelial, stromal, and endothelial components. The organ culture system consists of organ cultures of normal breast tissue, derived from mastectomy specimens in which no residual tumor is found, or from reduction mammoplasties. The adipose tissue which comprises most of the specimen is translucent under bright light allowing identification of large vessel which retain their red color due to enclosed red blood cells. Ducts and lobules can be identified grossly upon dissection and dissolution of the fat. This organ culture system allows us to investigate the dynamic nature of tumor-induced angiogenesis. By introducing purified angiogenic agents or cells which produce factors hypothesized to elicit angiogenesis, we can study the response of capillaries and venules, as well as the response of epithelial and stromal elements within the tissue. Hormonal manipulation and nutrient level can also be studied. We believe that this model system will allow us to understand the complex cellular interactions which occur during angiogenesis. Most importantly, once we understand this process, we will be able to use this model to test strategies to inhibit the process of angiogenesis at its inception, when tumor cells are just beginning to grow.

HYPOTHESIS/PURPOSE:

The hypothesis to be tested is that angiogenesis is induced by breast carcinoma prior to becoming invasive, and that by documenting the degree of angiogenesis, one can predict future tumor behavior. Furthermore, we propose that by understanding the mechanism of angiogenesis at its inception, novel treatment strategies may be developed.

TECHNICAL OBJECTIVES:

The development of a malignant neoplasms in solid tissues represents the cumulative effect of multiple genetic mutations. In some forms of cancer, such as breast and colon carcinoma, these accumulated mutations are visible. Histopathologic examination of excised tissue shows that the morphology of the epithelium changes from a small defined area of proliferation, to a growth confined by a basement membrane (in situ carcinoma), to an invasive growth which is capable of metastasizing to distant sites during the tumorigenic process. Angiogenesis, the growth of vessels in the surrounding tissue, is one the earliest "signs" of the transition from a benign proliferative growth to a malignant, uncontrolled proliferation. Growth of a tumor beyond a few microns in diameter requires the invasion of vessels into the tumor (22). The signals which cause adjacent vessels to undergo the process of angiogenesis in breast carcinoma remain unknown. Here we propose to examine the relationship of angiogenesis to tumor growth in the very earliest stages of tumorigenesis. Inhibition of vessel growth, by definition, must inhibit tumor growth. Therefore, we are proposing to define the soluble and insoluble factors which induce angiogenesis in breast cancer patients, and therefore, may be targets of therapeutic intervention. Finally, we are proposing to develop an organ culture model system in which to examine the angiogenic effect of these factors, and to define mechanisms for inhibiting their production or action in breast cancer patients. These specific aims are as follows:

1. Identify and quantify angiogenesis in the earliest stages of breast carcinoma, and correlate these data with clinical outcome.

Rationale: The correlation of angiogenesis with prognosis of invasive breast cancer has been well characterized in breast carcinoma patients (30). Prior to beginning this work, little information existed regarding angiogenesis in pre-invasive lesions. For instance, it was unknown whether *in situ* lesions are more angiogenic than atypical proliferation, or whether *in situ* lesions may be categorized prognostically by different levels of angiogenesis. In this specific aim we proposed to quantify angiogenesis induced by pre-invasive epithelial proliferative disease in human breast tissue. Quantification of vessels requires that endothelial cells be identified by von Willebrand factor immunostaining. Because of the statistical variability in vessel distribution, we will also explore new techniques for detecting angiogenesis at its inception.

Task 1: Quantify angiogenesis in in situ and early proliferative breast lesions, Months 1-12.

Work Accomplished: All of Task 1, *as originally proposed*, is complete and published (31). In summary these data support the following: 1. Epithelial vascularity increases at the earliest stages of tumor progression, i.e. during simple epithelial hyperplasia, 2. Vascularity increases with tumor progression, 3. Histopathologic indicators of relative risk for invasive disease correlate directly with vascularity, 4. Normal epithelium from cancerous breasts has greater vascularity than normal epithelium from non-cancerous breasts. The latter data was not expected, but correlates nicely with the work of Jensen et al. in which normal epithelium from cancerous breasts was found to be more angiogenic than that from non-cancerous breasts in an *in vivo* rabbit angiogenesis assay system (32). All of these data are consistent with the concept that angiogenesis is an early phenotypic marker of cells in the process of neoplastic transformation (33).

Task 2: Detect c-ets-1 expression in breast tissue, Months 6-18.

Work Accomplished: The goal of this task was to evaluate c-ets-1 as a marker for angiogenic endothelium. As shown in the previous report, we cloned the c-ets-1 cDNA by RT-PCR of tumor necrosis factor-treated human umbilical vein endothelial cell RNA into a vector for production of riboprobes (PGEM-3Z, Promega) and confirmed the construct by sequencing. The antisense probe identifies the two predicted messages. In situ hybridization with these probes indicates that many cells in the stroma produce c-ets-1 mRNA making the identification of small vessel endothelium extremely difficult. In the past year we have repeated these studies at very high stringency and shown that the smooth muscle cells of arterioles highly express this transcription factor but there was little to no expression in small vessel endothelium, even in tissue which was highly angiogenic. In addition, we identified a commercial antibody for c-ets-1 (Santa Cruz), which works in formalin-fixed tissue. Unfortunately, the antibody cross reacts with a tissue antigen (probably a blood group antigen or MHC locus) making the use of this reagent useless for

the detection of angiogenic vessels, since it identifies all vessels in many patients. We have conferred with Dr. Fagin (34) who used this antibody successfully to identify epithelial expression of c-ets-1 and found that they also noted the positive reaction with blood components, but being interested in the epithelial compartment only, this cross reactivity did not confound their studies. Therefore, to date we have been unable to use c-ets-1 by in situ hybridization or by immunohistochemistry as a marker for angiogenic vessels.

Newly Proposed Studies For Specific Aim One: 1. Characterize the vessels adjacent to breast epithelium for some of markers identified specifically in angiogenic vessels during the past couple years, such as the Vascular Endothelial Growth Factor (VEGF) receptor, KDR, and the integrins, $\alpha v\beta 3$ and $\alpha v\beta 5$. 2. Determine the relationship of vascularity in each form of pre-invasive breast disease with epithelial area.

Rationale: Based on numerous discussions with others in the field of angiogenesis, we have continued working on these tissues to ask the following questions: 1. Are the vessels which are spacially increased in these tissues clustered because of angiogenesis or tissue remodeling? 2. Is the increase in vascularity with progression from normal-to-proliferative-to-in situ carcinoma-to-invasion due to the specific expression of one or more angiogenic factors or is it simply related to the greater concentration of epithelium in these more advanced lesions? The first question is based on the recent work from Weidner's group in which there appeared to be insufficient vascular proliferation to explain the increase in vessels adjacent to some tumors (35). The second question is driven by the observation that normal tissue induces vascular growth with epithelial expansion; therefore, one would like to know whether the increase in vascularity is due to "normal" tissue responses to increased epithelial density or due to some "aberrant" expression of an angiogenic factor or loss of an angiogenic inhibitor.

The question of angiogenesis vs. tissue remodeling can be addressed, in part, by identifying features which are unique to angiogenic vessels. Task 2 (identification of c-ets-1) was our original attempt at such a marker, but these studies have been very disappointing. Since angiogenic endothelium is uniquely proliferative, as discussed in our last report, we are reexamining our most vascular tissues using double labeling for von Willebrand factor (to identify endothelium) and Mib-1 (an antibody which recognizes the same epitope as Ki-67, a marker of cells in the cell cycle). A second such feature is the unique expression of two integrins within the endothelium upon stimulation with either VEGF or Basic Fibroblast Growth Factor (bFGF). These integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) have been studied extensively by David Cheresh (36). We contacted Dr. Cheresh to get his antibodies as well as those which are commercially available. However, in our hands and in his, these antibodies do not work in formalin fixed, paraffin embedded tissue. Although we are not able to use these reagents in our archival analyses, these reagents have proven to be very useful in our organ culture experiments, as will be discussed below. Finally, angiogenic endothelium has increased expression of the VEGF receptor, KDR (37). Although the antibodies to KDR we have tested to date do not work in archival tissue, by in situ hybridization we have shown specific expression of these receptors in our archival tissue, as shown in **Figure 1.** In this figure we show the in situ results for KDR in normal

breast (negative) and in comedo carcinoma (positive), as well as in invasive breast cancer, which is our positive control. While not yet complete, we are going back to the normal, proliferative, atypical, in situ, and invasive tissues which were the most vascular to document up-regulation of this receptor in vessels. Because of the low level of mRNA remaining in these tissues, we have be forced to abandon "non-radioactive" methods. In addition these studies are somewhat long-term in that the hybridized slides must be exposed to emulsion for nearly three months prior to development, as was the case in **Figure 1**. Thus, to date, our "proof" of angiogenesis in the archival studies is endothelial proliferation, performed by immunohistochemistry, and KDR expression performed by in situ hybridization.

The second question addresses whether the vascularity we have measured is due to normal physiologic processes which induce vascular growth when tissue density increases or due to aberrant expression of angiogenic agents. We have answered this question in part by our catalogue of angiogenic agents in these tissues (see Specific Aim 2). In addition, I currently have a visiting scientist in my laboratory who is interested in this question. He is now doing morphometric analysis of our archival tissues, beginning with all of the slides of in situ carcinoma, to compare vascularity with epithelial volume. This work involves producing a computer file of each duct involved in disease and measuring the vascular area, the epithelial area within the duct, and the total area within 50 μm of the duct, estimated to be the diffusion distance in dense fibrous tissue. We hope to finish analysis of at least the in situ data within the next year.

2. Localize to specific cellular subsets known angiogenic factors in *in situ* carcinoma and atypical hyperproliferative lesions of the breast.

Rationale: As described above, many growth factors and cytokines have been implicated in the angiogenic process. Epithelial cells produce some of these angiogenic factors; others are produced by stromal cells. In the latter case, the epithelium may secrete a factor which induces synthesis of angiogenic agents in the surrounding stroma. In addition, many angiogenic factors are secreted in a latent form, requiring proteolysis for activation. Others, sequestered in the ECM, are released by proteolytic digestion of the matrix. The mechanism of angiogenesis in the very early stages of tumorigenesis remains obscure. To investigate this mechanism, we proposed to determine the presence and cellular distribution of angiogenic factors using the same tissues in which we have quantified vessel growth, above.

Task 3: Preparation of *in situ* hybridization probes, Months 6-18.

Work Accomplished: As shown in last year's report, we subcloned VEGF and its receptors, KDR and flt, Transforming Growth Factor alpha (TGFα), and Epidermal Growth Factor Receptor (EGFR). These are in a PGEM-3Z vector for riboprobe production.

Task 4: Examination of breast tissue for angiogenic growth factors, Months 13-24.

We have currently stained all of the proliferative and in situ carcinoma tissues used in the angiogenesis project in Specific Aim 1 for TGFα, EGFR, VEGF, Insulin-Like Growth Factor (IGF) I and II, bFGF, Transforming Growth Factor beta (TGFβ), and Platelet Derived Growth Factor (PDGF). In these studies we analyzed data for each growth factor on each slide with respect to luminal epithelium, myoepithelium, endothelial cells in vessels which touch the epithelial basement membrane, adjacent stromal cells, and infiltrating leukocytes. These data are shown in **Tables 1 and 2**. **Table 1** shows the analysis of epithelial growth factor expression as indicated by the intensity of staining on a scale of 0-4 in normal epithelium (Bn), all forms of proliferative disease (Prolif), atypical hyperplasia (Atypia), in situ carcinoma (In Situ), and invasive cancer (Invasive). Listed are the mean and standard deviation for all tissues in each diagnostic category and the percent positive cases within each category. **Table 2** shows similar data for stromal staining for those factors which showed some diffences among diagnostic categories. None of the other categories, such as endothelial, myoepithelial or leukocyte staining, showed statistically significant changes with disease progression.

From **Table 1** we conclude the following: 1. Normal breast epithelium and stroma contain a wide array of potent angiogenic factors, despite the lack of known angiogenesis in many of these tissues. 2. VEGF is present in normal tissue, showing slight increases with disease progression and a marked increase with the onset of invasion. 3. Basic FGF, TGFα, IGF2, and PDGF are uniformly present throughout disease progression in nearly 100% of cases and do not show an increase with invasion. 4. IGF1 shows a marked increase in production, both in quantity and number of positive cases, at the level of in situ disease. 5. TGFβ shows the earliest progressive increase, showing a statistically significant increase as early as proliferative breast disease and continuing to increase until the onset of invasion. Note that this is an increase in staining intensity, not number of positive cases, since TGFβ expression is nearly ubiquitous in all epithelium.

From **Table 2** we conclude that 1. TGFβ is essentially not found in the stroma. 2. Stromal IGF2 increases with disease progression, both in intensity and number of positive cases. IGF1 also increases, although the increase is not statistically significant. 3. Stromal VEGF varies very little with disease progression. 4. On the other hand, stromal bFGF increases significantly, particularly with onset of invasion. 5. Stromal TGFα increases slightly with progression but is markedly decreased with the onset of invasion.

We have also completed the analysis of TGFα immunoreactivity in the endothelium. In these studies we examined the endothelial cells touching the basement membrane of the epithelium of each diagnostic type (Near) vs. small venules and capillaries a few millimeters away (Far). **Table 3** shows the results of this analysis. With the exception of "Atypia" in which there were a small number of cases, all the categories showed a greater expression of TGFα in the "near" vessels than the "far" vessels ($p<0.05$). These immunological data are now being confirmed by in situ hybridization. Analysis of the receptor (EGFR) is also nearly completed.

Since one intent of these data was to begin to apply the knowledge learned from the in vivo situation to the in vitro model, we are most interested in studying TGFβ. These data indicate that this is the growth factor which changes the earliest with disease progression. TGFβ is an interesting factor because of its pleotropic effects on tissues and cells depending on the cell type and cell density. We did not expect TGFβ to be involved so early in disease progression, so we did not use it early in our organ culture studies (see below). Therefore, to date we have completed the analysis on only one experiment using this growth factor.

Task 5: Examination of breast tissue for expression of metalloproteinases and their inhibitors, Months 13-24.

Work Accomplished: As of the end of last year we had not found reliable reagents to complete this task. We have now identified two antibodies which work well in archival tissue and have examined these samples for Matrix Metalloproteinases (MMP) 2 and MMP9. The results of these studies are shown in **Table 1**. Surprisingly, MMP2 and 9 were almost never expressed in the stroma, nor were they found in endothelium of small vessels, including those surrounding comedo carcinoma and invasive disease. However, as noted in **Table 1** there were statistically significant increases in both of these proteases within the epithelium, particularly at the level of in situ carcinoma and invasion. As noted before, all available reagents for uPA and tPA work poorly in archival material. As suggested by reviewer 2, we are putting the examination of these proteases on hold, pending the production of new reagents or obtaining a cDNA clone.

3. Develop an *in vitro* breast organ culture system and test whether the angiogenic agents identified in Specific Aim 2 induce angiogenesis in this system.

Rationale: Over the past two decades numerous models of angiogenesis have been described. These models include whole animal studies, as well as *in vitro* cultures of endothelium in ECM gels. All of these models have a role in the investigation of specific questions; all, are highly criticized for poorly replicating the conditions in patients. My laboratory has used a model for angiogenesis which relies on the ability of endothelial cells to produce tubular structures in ECM gels (38). This model has allowed the identification of numerous cell-specific events which occur during angiogenesis. Nonetheless, it is a poor model for elucidating the events which occur in a complex tissue, such as breast. To replicate the *in vivo* situation as closely as possible, we have developed the model system in which breast tissues from surgical specimens are cultured *in vitro* as organ cultures, ie. the culture retains its orientation with respect to epithelium, stroma, and vessels. The strength of this system is that it allows us to directly test the function of agents identified in patient specimens in a model of "normal" breast tissue. This system has all of the cellular components found in normal breast (epithelium, stroma, and endothelium). We have performed preliminary studies, described below, that document the angiogenic response of the vessels in these cultures to various culture conditions. Therefore, we can now examine the dynamic intercellular interactions which occur during angiogenesis,

define the angiogenic agents which function in breast tissue, and test reagents which inhibit the angiogenic process.

Our initial studies and the results of numerous investigators who have examined breast cancer tissue for the presence of growth factors led us to question the dogma that if an angiogenic factor is present in a soluble form, it must be functioning. Many tissues in which no angiogenesis is seen contain angiogenic factors. These data may be explained in one of two ways. First, the presence of angiogenic factors is permissive, ie. their presence is required, but not sufficient. Second, these angiogenic factors serve other functions in these tissues, unrelated to angiogenesis. The proposed model system allows us to choose between these explanations. In this system combinations of angiogenic factors and proteolytic enzymes can be examined. In addition, novel factors produced by purified breast carcinoma epithelial and stromal elements can be identified by introducing these agents into the organ culture system.

To utilize this model to its fullest potential, we propose to: **3.1** Characterize the model in detail with regard to media requirements, long-term viability, and proliferative capacity of individual elements; **3.2** Examine the effect of angiogenic agents identified in Specific Aim 2 with regard to the production of angiogenesis and the induction of other angiogenic agents by each cellular component within the model; **3.3** Test isolated breast carcinoma cells and tumor stromal elements in co-culture with the organ culture for the presence of angiogenic factors not identified by the immunologic screening performed in Specific Aim 2. These experiments will allow us to characterize the epithelial and/or stromal proteins which regulate angiogenesis in the earliest stages of breast carcinoma. Once we have identified and characterized these proteins, we will develop strategies for altering their angiogenic potential.

Task 6: Develop culture conditions in which explant cultures give reliable angiogenesis, Months 18-24.

Work Accomplished: Briefly, the organ cultures are obtained from reduction mammoplasties or mastectomies within one or two hours of removal. The tissue is finely minced (1-2mm^2) and cultured floating in media or embedded in Matrigel. Test agents are added to the media every other day or are included in a diffusion vector (gelfoam or Elvax beads). BrdU/Fldu is added 24-48 hours prior to analysis. At each time point the tissue is washed, fixed in buffered formalin and paraffin embedded. If cells have grown out of the tissue as a monolayer on the bottom of the dish, these are isolated by trypsin/EDTA, washed, spun into a gel (Cytoblock, Shandon, Pittsburgh, PA), and paraffin embedded. 20 3μm serial sections are cut from each block for analysis. One experiment may contain up to 30 blocks.

In addition to morphologic analysis of vascular density, we define a vessel as angiogenic by one or more of the following criteria: 1. The endothelium is proliferating, 2. The endothelium expresses αvβ3, 3. The endothelium expresses αvβ5, or 4. The endothelium expresses the VEGF receptor, KDR. Since the integrin assays can not be performed on

formalin-fixed tissue, within the last year we have performed replicate experiments, fixing and embedding half the tissue for morphology and freezing the other half for analysis of integrins and in situ hybridization. **Figure 2** shows the results of immunologic studies for these two integrins in frozen breast tissue. Compare the negative immunoreactivity in the normal tissue with the markedly positive immunoreactivity in the invasive tumor for both integrins.

To date we have performed and analyzed 59 organ culture experiments. The form used to analyze these experiments was presented in last year's report. As indicated in the enclosed manuscript (39) we have been successful in maintaining partial to complete viability of all cell types for up to 8 weeks. Our routine is now to end cultures after one month, since we don't seem to increase our angiogenic phenotype beyond that point. Also as explained last year, upon analysis of these data we discovered that the epithelium of pre and postmenopausal women behaved differently in culture, the latter undergoing spontaneous squamous metaplasia (39).

In addition to getting some idea about the effects of various medias on specific cell types and the relative viability of certain cell types in these cultures, we have gained some interesting insights into the process of angiogenesis. In our cultures we consistently observe a sequential "activation" of vascular cell types. The pericytes are often induced to proliferate and migrate almost a week prior to endothelial proliferation. The endothelial cells appear to follow the lead of the migrating pericytes. The pericyte as a precursor to vascular sprouts is an old concept that is not well appreciated. We have preliminary data that when fully analyzed we believe will support the idea that pericyte function is critical for angiogenesis. This type of information supports the importance of using a model system which has multiple cell types.

In our original series of experiments reported last year, we had included 10% fetal calf serum (FCS) in our cultures because these conditions best preserved the viability of all cell types. Unfortunately, we had numerous experiments in which we saw no endothelial proliferation. Therefore, in the past year we repeated many of these experiments with 2% FCS, knowing that our cell viability, particularly of the epithelium, would decrease markedly. **Table 4** shows a summary of the differences in proliferation of the endothelium (Endo), pericytes (Peri), a particular type of pericyte which is pointed out from the vessel (Tips), and stromal cells immediately adjacent to a vessel (Fibro). The data are reported as the mean proliferative index (on a scale of 0 to 4) and the standard deviation. In all cases only venules and capillaries are included in this analysis. The mean age of patients in each series and average time in culture are also reported. Note that the 2% data show a mean time in culture of 2 weeks because the longest time points, usually at 5 weeks, are not yet in the database. Even so, there is a marked increase in the 2% FCS experiments in the proliferation of the endothelium, pericytes, and tips ($p<0.05$). We interpret these data in one of two ways. First, either nutrient deprivation is a strong stimulus for proliferation; in monolayer cultures nutrient deprivation usually causes cells to become quiescent, but in mixed cell cultures this is often not the case. Second, something in the serum is inhibiting proliferation. That "something" is usually thought to be TGFβ; however, based on our

results described in Specific Aim 2, in which TGFβ was strongly associated with disease progression and angiogenesis, we believe that TGFβ may have a stimulatory effect. The results of a single experiment using this growth factor are shown in **Table 5**. Being an extremely complex material, we are not going to analyze the serum effect further, but continue to keep the level at 2% for our additional experiments. Less than 2% FCS does not permit extended epithelial or endothelial cell viability.

Task 7: Isolate VEGF from HL60 cells, Months 24-32.

As previously suggested, commercially available sources of VEGF are now available and have been used in several organ culture experiments.

Task 8: Test the growth factors and metalloproteinases discovered in Task 4 for their angiogenic potential in this system, Months 25-36.

a. Add angiogenic factors to the explant using permeabilized latex microbeads and assess angiogenesis histologically.
b. Assess response of tissue

As explained in our previous report, in collaboration with Mr. Gary DeVoe and Mrs. Mary Ann Miller in the Department of Pathology, we set up a database for analysis of the profuse amount of data obtained from our cultures. We have now performed 59 organ cultures for periods of a week to up to a couple months. Agents tested in this system include Matrigel, bFGF, VEGF, EGF, TGFα, TGFβ, cell culture conditioned media containing VEGF, PMA, staurosporine, calphostin C, forskolin, IMX, tamoxifen citrate, and ECGF. Many of these agents have been tested as media supplements, as well as in directed diffusion, ie. the agent is either placed on the tissue in pieces of blotting paper or in Elvax polymer beads (see below). Most recently we have begun to use gelfoam. In addition, we have performed limited co-culture with tumor cells and normal fibroblasts.

Work Accomplished: This task has been the main focus of our activity this year. As noted above, we switched serum concentrations and repeated some of earlier experiments for two reasons. First, with some notable exceptions, many cultures showed no endothelial proliferation. Second, we needed to acquire frozen tissue for integrin analysis and in situ hybridization. The formalin fixed, paraffin embedded tissue is also necessary to identify sprouting of vessels by morphology. Summary data for these experiments are presented in **Tables 5-9.** Again the data are the mean and standard deviation of number of proliferation cells determined by immunohistochemistry (Mib-1) on a linear scale from 0-4.

Growth Factor Data Conclusions:
VEGF (**Table 5**): Endothelial cell proliferation is the same with VEGF whether in 10% or 2% serum. In 10% serum, VEGF has no effect on proliferation of any cell type. In 2% serum, VEGF increases the proliferation of Tips, only.

Basic FGF (**Table 6**): Endothelial cell proliferation is the same with bFGF whether in 10% or 2% serum. In 10% serum, bFGF has no effect on proliferation of any cell type. In 2% serum, bFGF increases the proliferation of adjacent stromal cells, only.

TGFα (**Table 7**): These experiments were done in 2% serum only. TGFα had no effect on the proliferation of any cell type.

EGF (**Table 8**): These experiments were done in 2% serum only. EGF had no effect on the proliferation of any cell type. Note overall these experiments are much more proliferative than the others; being our most recent experiments, we are still investigating why this may be so.

One of our early objectives was to add growth factors in a small defined are of the culture rather than in the media in general. Our first attempts used Elvax beads and bFGF. As shown in **Table 9**, the beads containing bFGF had the same cell proliferation as beads containing bovine serum albumin, a protein control.

While these data seem to indicate that purified angiogenic factors have no effect in our model system, these data leave out three important pieces of information which we are just now beginning to analyze. First, all of the proliferative indices are based on an estimate of the total number of a given cell type in a specimen. The reported values are a mean of the entire specimen. We have noticed that endothelial proliferation occurs primarily in the adipose tissue and adjacent to ducts and lobules, but almost never in the extralobular fibrous stroma. Therefore, the amount of endothelial proliferation is prejudiced by the relative amount of adipose tissue and epithelium. The most interesting aspect of this is why there is no endothelial cell proliferation in fibrous tissue, when other cell types proliferate there. This question we have begun to address, as noted in the next task. Second, our analyses are based on the means of all experiments, whereas some wells of tissue are far more angiogenic than others; we are now in the process of pulling out the organ cultures which showed exceptional endothelial and pericyte proliferation for more detailed evaluaton. Third, proliferation is just one aspect of angiogenesis. We have preserved most of the cultures performed in 2% as snap frozen tissue and in paraffin and will be analyzing these for the expression of integrins and KDR, as described above.

Finally, the archival tissue studies indicate that while numerous angiogenic factors are present in normal tissue, they are not sufficient to drive angiogenesis. TGFβ appears to correlate with increased angiogenesis. As shown in Table 5 our one experiment showed a significant effect of TGFβ on proliferation. These studies are being repeated now.

Task 9: Examine tumor epithelium and tumor stroma for angiogenic factors, Months 37-48.

a. Co-culture explant with fibroblasts and tumor cells derived from patient tumors to look for angiogenesis.
b. Examine fibroblast and tumor cell conditioned media for angiogenic agents

c. Fractionate the conditioned media for characterization of novel angiogenic factors.

Work Accomplished: Upon observing that endothelial cell proliferation did not occur in the vessels within the fibrous stroma, we performed organ cultures in which we co-cultured isolated normal breast fibroblasts. The fibroblasts were grown as a monolayer culture under the floating organ cultures. The results of these studies are shown in **Table 10**. All three experiments were performed in 10% FCS. Despite the low overall proliferation, there is a marked decrease in endothelial cell proliferation in the co-culture experiments. These experiments are now being repeated in 2% FCS and will be analyzed for proliferation, integrin expression, and KDR.

As a further test for whether fibroblasts inhibit angiogenesis, we have isolated normal breast fibroblasts and will test them in more conventional angiogenesis assays, i.e. endothelial cell mitogenesis using breast microvascular endothelial cells (HMVECS) (from Clonetics) which are routinely cultured in our laboratory and endothelial tubulogenesis in Matrigel, again using HMVECS.

In consultation with Mina Bissell we have also begun to produce epithelial organoids from normal breast (40). These are being cultured in a three-dimensional matrix to look for the production of angiogenic agents using our well-characterized angiogenic model system (38). Because epithelial organoids have been well-characterized in the Bissell lab, we look at these cultures as an adjunct to the organ culture system, in order to sort out which cell-cell interactions may regulate production of angiogenic growth factors.

Since we are also ultimately interested in tumor angiogenesis, we have begun to isolate breast tumor fibroblasts and early passage tumor epithelium. We also have a large collection of breast tumor cell lines, but cell lines in general have undergone adaptive changes in vitro which increase their production of angiogenic agents such as bFGF.

Work Proposed:
We interpret our previous experiments as follows: 1. Normal breast tissue, particularly the epithelium, produce numerous angiogenic factors, and yet angiogenesis is still in the "off" state. 2. In vitro the addition of more angiogenic agents has no effect on endothelial cell proliferation, although we are not yet sure about other angiogenic phenotypes such as expression of $\alpha v\beta 3$ and $\alpha v\beta 5$ or up-regulation of KDR. 3. We can induce endothelial cell proliferation by serum deprivation, but the growth factors still do not change endothelial cell proliferation. 4. Disease progression to proliferative breast disease and in situ carcinoma is uniquely associated with increases in TGFβ. Furthermore, we have one preliminary study which indicates that this factor may have an angiogenic effect in vitro. 5. As expected there is also an increase in metalloproteinase production with disease progression. Production or addition of proteinases to organ cultures may activate factors which lie quiescent in the tissue. We have no data in this regard as yet.

To follow-up on these observations we plan to: 1. Examine the effects of TGFβ on our organ culture system, measuring proliferation, integrin expression, and KDR. 2. Examine the effects of fibroblasts in co-culture and as conditioned media in 2% serum on our organ culture system. 3. Determine whether normal breast fibroblasts alter HMVEC mitogenesis or tubule formation in Matrigel alone and in the presence of agents which are extremely angiogenic in these assays, bFGF and VEGF. 4. Examine the effects of normal breast organoids in co-culture and as conditioned media in 2% serum on our organ culture system. 5. Determine whether normal breast organoids alter HMVEC mitogenesis or tubule formation in Matrigel. 6. Examine the effect of metalloproteinase addition on organ culture angiogenesis, adding first combined proteinases to cultures in Elvax, and using individual proteinases if we see an effect. (Proteinases are available from James Quigley at SUNY, and we are in the process of obtaining these reagents). 7. Perform experiments in which organ cultures are co-cultured with either tumor epithelium or tumor fibroblasts. In these studies the effects of normal epithelium and fibroblasts will be compared directly with those from tumor.

If fibroblasts (from any source) prove to be producing angiogenic inhibitors, we shall pursue the nature of this agent(s). First, obvious experiments to determine size (dialysis) and chemical nature (heat denaturation and trypsin digestion) will be performed. Second, we shall look for obvious inhibitors such as thrombospondin, angiostatin, and endostatin. Once we have a feel for these, we shall begin fractionation, the nature of which depends on the size and nature of the inhibitor.

If normal organoids are angiogenic, we shall pursue the nature of these agents. We are currently testing freshly isolated organoids for the angiogenic growth factors tested in our archival studies.

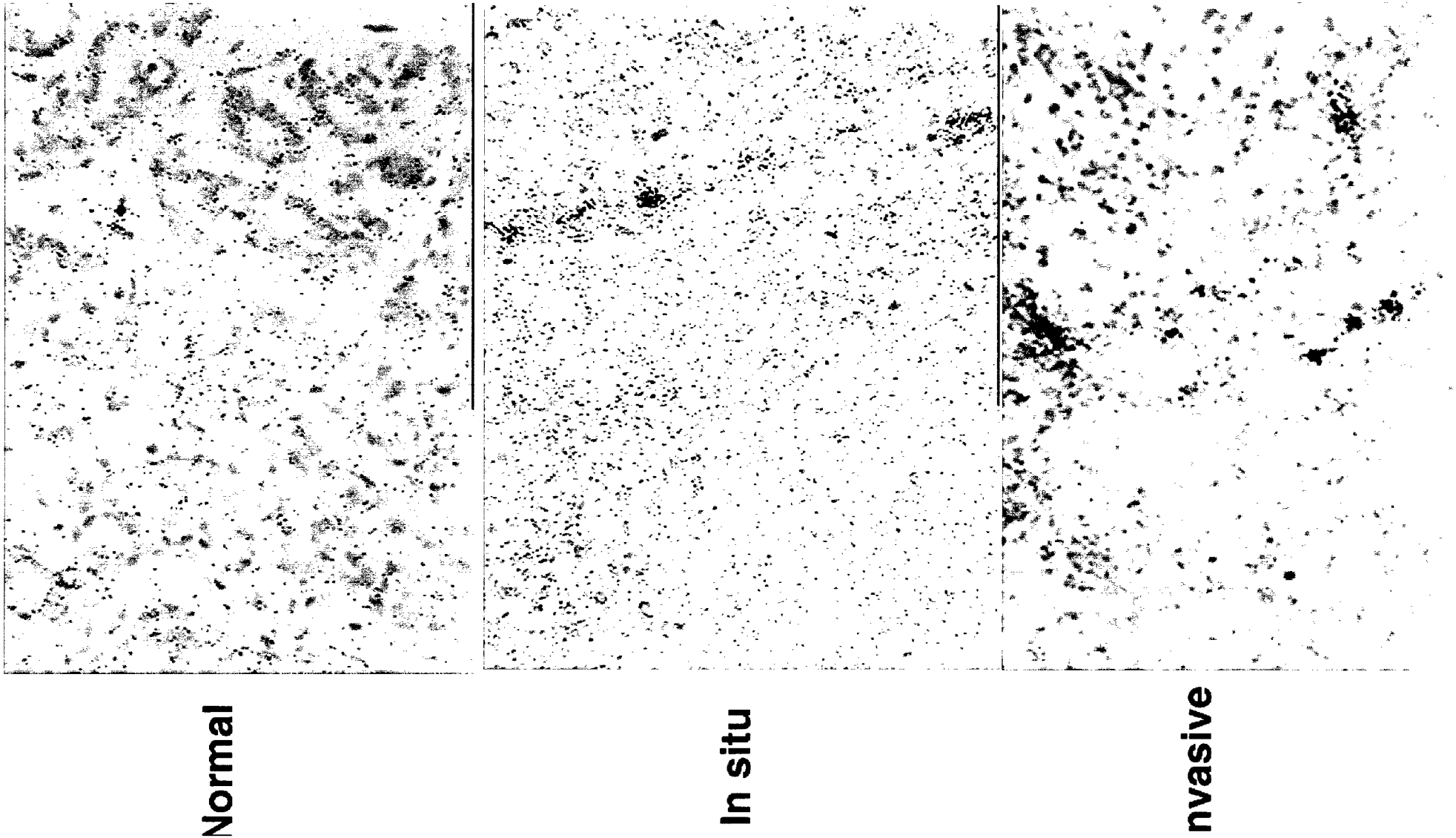
If metalloproteinase are required for angiogenesis in the organ culture system, we shall identify which are most effective and whether they work optimally in combination with exogenous angiogenic agents.

Conclusions: Many women with pre-invasive breast disease go on to develop invasive carcinoma. However, only a few of these pre-invasive pathologies are actually precursor lesions to invasive disease. We proposed to study the vascularity of these pre-invasive diseases for two reasons: First, at some stage in this progression, angiogenesis is initiated to provide nutrients to the growing tumor. The point at which angiogenesis is initiated is unknown. Second, we know that inhibiting angiogenesis in invasive tumors stops their growth. If pre-invasive breast diseases are angiogenic, then angiogenic inhibitors are a potential method for chemoprophylaxis. In addition to studying vascularity, we proposed to examine these tissues for angiogenic agents which may be potential chemoprophylactic targets. Finally, we proposed to set up a model system in which to examine the regulation of angiogenesis in these tissues.

To date we have discovered that angiogenesis begins at the earliest point of progression, i.e. at the point of proliferative breast disease. Furthermore, the degree of vascularity

correlates with histopathologic features which predict progression to invasion. We have also shown that even normal breast tissue contains numerous angiogenic factors, even though angiogenesis is essentially "off". During disease progression, increases in TGFβ are seen very early, and levels of TGFβ progressively increase until invasion is initiated. IGF1 also increases, but not until the development of in situ carcinoma. Levels of other angiogenic factors are relatively stable throughout progression. Finally, we have developed an organ culture system in which we can induce endothelial cell proliferation and sprouting. In this system, the growth factors which are commonly found in normal breast do not alter endothelial cell proliferation. We are now testing their effects on other aspects of the angiogenic phenotype, such as integrin expression and expression of the VEGF receptor, KDR. Finally, we have discovered that fibrous stroma inhibits endothelial cell proliferation. We are now pursuing the effects of both isolated stromal fibroblasts and normal breast epithelium on the regulation of angiogenesis in this tissue.

Figure 1. In Situ Hybridization for KDR in Formalin Fixed, Paraffin Embedded Tissue. Riboprobes were produced from the complete KDR cDNA cloned into PGEM-3Z and labeled with ^{35}S. Shown are antisense hybridizations with histologic sections of normal breast, in situ carcinoma, and invasive breast tumor. Normal breast shows no hybridization. Note the silver grains over the vessels immediately adjacent to the in situ carcinoma. Vessels within the invasive carcinoma are also specifically labeled. All hybridizations with the sense probe were negative.



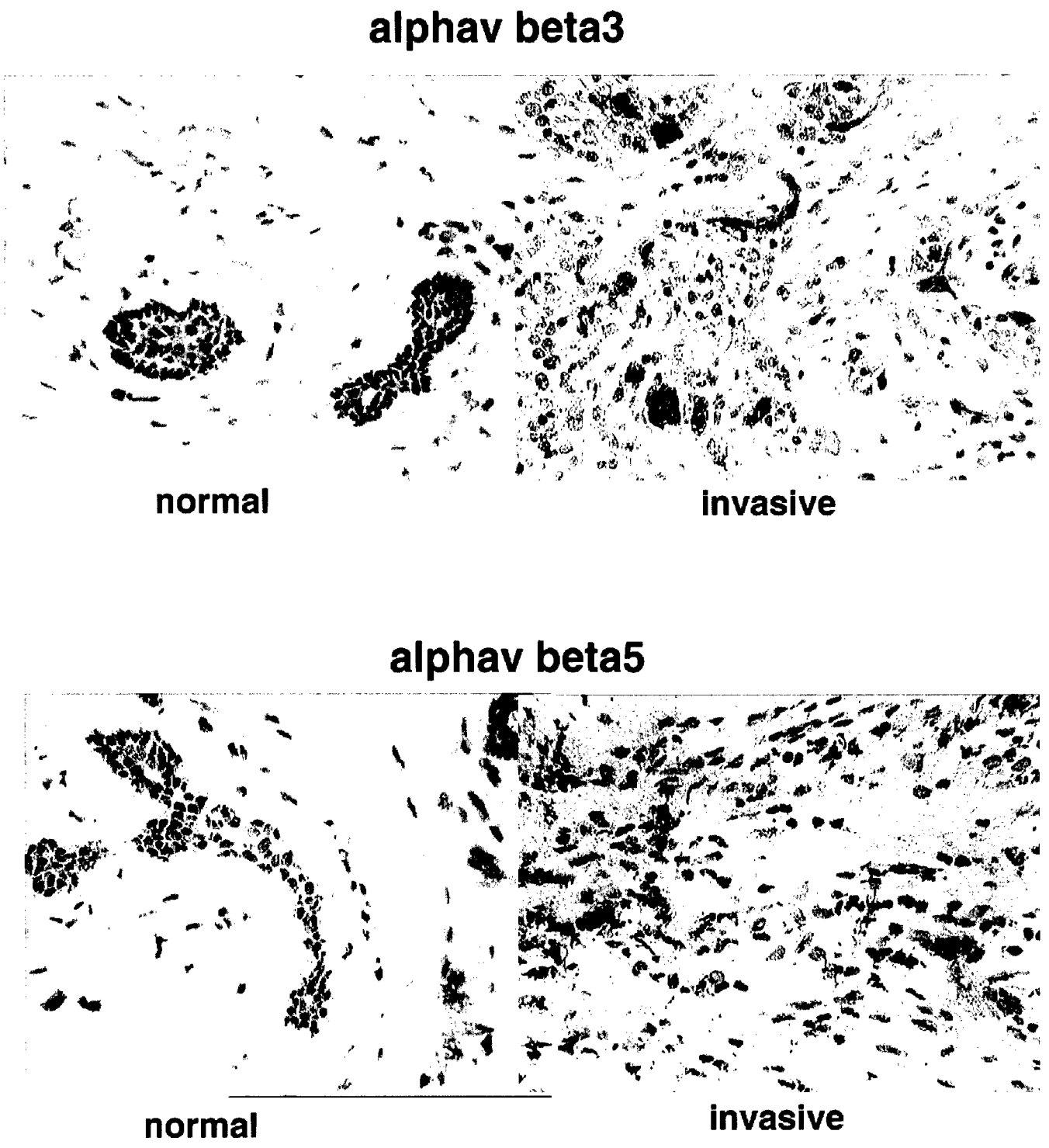


Figure 2. Immunohistochemical Detection of αvβ3 and αvβ5. Frozen normal breast tissue and invasive breast tumor were incubated with monoclonal antibodies specific for αvβ3 and αvβ5, followed by incubation with a secondary antibody conjugated to horse radish peroxidase. Positive immunoreactivity is seen using the peroxidase substrate, diaminobenzadine. Note that only the vessels within the tumor are immunoreactive, indicating the presence of angiogenesis.

Table 1. Epithelial Immunoreactivity

		VEGF	bFGF	TGFα	TGFβ	IGF1	IGF2	PDGF	MMP2	MMP9
Normal	Mean	2.26	2.82	2.44	1.28	0.51	1.44	1.11	0.30	0.03
	S.D.	1.06	0.76	1.02	1.84	0.70	1.31	0.95	0.67	0.82
	% pos.	95	100	96	97	40	65	74	22	19
Prolif.	Mean	2.34	2.95	2.31	**1.84***	0.52	1.57	1.33	0.52	0.40
	S.D.	0.71	0.78	0.79	0.87	0.63	1.35	0.94	0.87	0.82
	% pos.	100	98	97	100	45	71	80	36	24
Atypia	Mean	2.63	2.73	2.57	1.70	0.44	1.70	1.58	0.75	0.50
	S.D.	0.52	0.79	0.98	0.48	0.73	1.25	1.24	1.04	0.76
	% pos.	100	100	100	100	33	70	83	50	38
In Situ	Mean	2.72	2.55	2.06	**2.15***	**1.22***	1.97	1.21	**1.09***	**0.66***
	S.D.	0.79	1.05	1.12	0.86	1.18	1.60	1.11	0.97	1.00
	% pos.	100	92	87	100	66	71	67	69	44
Invasive	Mean	**3.17***	3.00	2.06	**2.73***	0.88	2.06	1.10	**1.42***	**1.00***
	S.D.	0.64	0.63	1.02	0.96	0.96	1.61	1.06	0.64	0.85
	% pos.	100	100	94	94	56	81	60	92	73

* indicates a statistically significant difference relative to normal epithelium ($p<0.05$)

Table 2. Stromal Immunoreactivity

		VEGF	bFGF	TGFα	IGF1	IGF2
Normal	Mean	0.50	0.77	0.24	0.07	0.00
	S.D.	0.66	0.88	0.80	0.26	0.00
	% pos.	42	54	23	7	0
Prolif.	Mean	0.45	1.07	0.20	0.07	0.04
	S.D.	0.65	0.97	0.45	0.26	0.19
	% pos.	37	66	17	7	4
Atypia	Mean	0.50	0.91	0.11	0.00	0.00
	S.D.	0.53	0.30	0.33	0.00	0.00
	% pos.	50	100	11	0	0
In Situ	Mean	0.79	1.20	0.53	0.16	0.26
	S.D.	0.92	0.98	0.70	0.45	0.96
	% pos.	51	71	42	13	9
Invasive	Mean	0.79	**1.50***	**0.06***	0.31	**0.50***
	S.D.	0.98	1.24	0.24	0.61	1.21
	% pos.	50	77	6	25	19

* indicates a statistically significant difference relative to normal epithelium ($p<0.05$)

Table Three. TGFα in the Endothelium.

		Far	Near
Normal	Mean	0.66	1.73
	S.D.	0.75	0.93
Prolif.	Mean	0.59	1.66
	S.D.	0.71	0.88
Atypia	Mean	0.88	2.33
	S.D.	0.78	0.87
In Situ	Mean	0.83	2.11
	S.D.	1.11	0.89
Invasive	Mean	0.53	1.71
	S.D.	0.72	0.92

Table 4. 10% FCS vs. 2% FCS

	Age[1] (yr)	% Pos.[2]	# Days[3]	Endo. Prolif.*	Peri. Prolif.*	Tips Prolif.*	Fibro. Prolif.
10% FCS	49	48	23.15 ± 11.87	0.35 ± 0.68	0.23 ± 0.49	0.10 ± 0.34	0.06 ± 0.26
2% FCS	36	100	14.67 ± 9.00	1.36 ± 1.31	1.15 ± 1.11	0.54 ± 0.69	0.27 ± 0.73

1. Patient age at time of tissue removal.
2. Percent of cases positive for endothelial proliferation.
3. # of Days is mean ± standard deviation of time in culture.

* Indicates statistically significant increase in 2% compared to 10% serum ($p<0.05$), reported as mean ± standard deviation.

Table 5. Organ Cultures with VEGF.

		VEGF				Control			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
10% FCS	Mean	1.30	0.40	0.80	0.80	1.50	0.10	0.40	0.90
	S.D.	0.67	0.52	0.79	1.23	1.08	0.32	0.52	0.88
2% FCS	Mean	1.88	0.12	0.94	1.82	1.76	0.12	0.71	1.53
	S.D.	1.41	0.33	0.83	1.29	1.44	0.33	0.85	1.07

Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2 in 10% serum and 5 in 2% serum.

Table 6. Organ Cultures with Basic FGF.

		bFGF				Control			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
10% FCS	Mean	0.80	0.50	0.10	0.10	0.60	0.30	0.00	0.00
	S.D.	1.30	0.71	0.32	0.32	0.70	0.48	0.00	0.00
2% FCS	Mean	1.73	1.45	0.91	0.45	1.55	1.82	0.82	0.00
	S.D.	1.42	0.93	0.83	0.93	1.21	1.08	0.87	0.00

Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2 each, 10% and 2% serum.

Table 7. Organ Cultures with TGFα.

		TGFα				Control			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
2% FCS	Mean	0.50	0.33	0.17	0.00	1.33	0.83	0.67	0.33
	S.D.	0.84	0.67	0.45	0.29	1.15	1.08	0.75	0.67

Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2

Table 8. Organ Cultures with EGF.

		EGF				Control			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
2% FCS	Mean	2.00	1.00	1.00	0.50	3.00	1.50	0.50	0.50
	S.D.	1.20	1.20	1.20	1.00	1.60	1.00	0.60	0.60

Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2.

Table 9. Organ Cultures with bFGF Beads.

		bFGF				BSA			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
2% FCS	Mean	0.46	0.18	0.09	0.00	0.45	0.18	0.00	0.00
	S.D.	0.82	0.40	0.30	0.00	0.69	0.40	0.00	0.00

Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels.

Table 10. Organ Cultures with Fibroblast Co-Culture.

		Co-cult.				Control			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
10% FCS	Mean	0.18	0.24	0.06	0.06	0.56	0.44	0.13	0.27
	S.D.	0.39	0.44	0.24	0.24	0.51	0.51	0.52	0.59

Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 3.

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APPENDIX 1

Acronym and Symbol Definition:

aFGF and bFGF	Acidic and basic fibroblast growth factor
BrdU	bromodeoxyuridine
DOD	Department of Defense
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylene-diamine-tetraacetic acid
EGF	Epidermal growth factor
FCS	fetal calf serum
HMVECS	human microvascular endothelial cells
IGF I and II	Insulin-like growth factors
Il-1	Interleukin 1
Il-6	Interleukin 6
PDGF	Platelet-derived growth factor
PKC	Protein Kinase C
PMA	phorbol myristate acetate
SNOMED	Systemic Nomenclature of Medical Diagnoses
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor βeta
TIMP	Tissue inhibitor of metalloproteinase
TPA	Tissue plasminogen activator
UC	University of Cincinnati
UPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor

ADDENDUM TWO

Staurosporine-Induced versus Spontaneous Squamous Metaplasia in Pre- and Post-Menopausal Breast Tissue

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5 tables; 3 figures

This Manuscript Has Been Accepted With Revision.

Missing Figures Are In Preparation.

Abstract:

Breast cancers from pre- versus post-menopausal women display unique characteristics which may be related to differences in epithelial differentiation between these two populations. In addition to lobular development, lactational changes, and involution, breast epithelium can undergo metaplastic alterations, often in association with carcinoma. Since protein kinase C (PKC) regulates differentiation and proliferation in many cell types, we asked whether modulation of PKC activity could define biochemical differences in breast epithelium from pre- versus post-menopausal women. Organ cultures of normal human breast were treated with PKC agonists and antagonists. Epithelial differentiation was evaluated based on morphologic criteria and the expression of cell-type specific proteins. Staurosporine, a non-specific but extremely potent inhibitor of PKC, induced squamous metaplasia in 8/8 cases within 2 weeks of treatment. Other inhibitors of PKC, such as calphostin C and tamoxifen, had no effect on epithelial differentiation. Long-term treatment with phorbol esters also did not induce squamous metaplasia. However, stimulation of cAMP levels by forskolin and isobutyl-methyl-xanthene (IMX) rapidly induced squamous metaplasia, as has been previously reported. Surprisingly, squamous metaplasia occurred in 10/12 cultures derived from post-menopausal women in the absence of exogenous agents. Untreated cultures derived from pre-menopausal women never developed this type of epithelium (0/11). Therefore, breast epithelium from pre- and post-menopausal women responded differently to in vitro culture. Forskolin/IMX or staurosporine can reproduce these conditions, acting independent of menopausal status. Since staurosporine's action was unique among PKC inhibitors, staurosporine may induce squamous metaplasia of breast epithelium by a PKC-independent mechanism.

Introduction:

Breast cancer is a heterogeneous disease with unique characteristics in pre- versus post-menopausal women. Differences include expression of estrogen receptors, clinical aggressiveness, and response to therapeutic agents (Nixon et al, 1994). The reasons for these differences are not clear. However, unlike most cancers, breast cancer development may differ over the lifetime of an individual because the normal tissue is continuously changing its state of differentiation in response to hormonal cues (Russo et al, 1990).

Our experimental approach was based on the hypothesis that as breast epithelium changes over a lifetime, its response to signal transduction events will also change. Therefore, we examined the changes in differentiation of breast organ cultures from pre- versus post-menopausal women when exposed to altered cell signaling, focusing on the pathways which function through protein kinase C. Several studies have implicated PKC in the response of breast epithelial cells to estrogen (Cho and Katzenellenbogen, 1993). In breast tumors the expression of PKC is inversely correlated with expression of the estrogen receptor (Borner et al, 1987) and some of the effects of the anti-estrogen, tamoxifen, on breast tumor growth are now thought to be mediated by its ability to directly inhibit PKC (O'Brien et al, 1986). In addition, PKC is in the signaling pathway of many growth factors known to function in normal breast epithelium. In particular, PKC regulates the expression and release of neu differentiation factor or heregulin (Burgess et al, 1995). This growth factor is capable of inducing growth arrest and differentiation with production of milk proteins (Peles et al, 1992; and Bacus et al, 1992). PKC is also in the pathway for the heregulin receptor, neu (Peles et al, 1991; and Stancovski et al, 1992).

Gomez et al (1993) showed that PKC played a regulatory role in specifying the maturation of breast stem cell-like progeny. In their system total PKC activity decreased with breast cell maturation. In breast carcinogenesis, PKC expression is increased (O'Brien et al, 1989), and PKC is thought to modulate various tumor phenotypes such as resistance to chemotherapy and metastatic potential (Blobe et al, 1994; and Takenaga and Takahashi, 1986). Finally, the studies of De Vente et al, 1995, implicate PKC in pathways regulating the switch from proliferation to apoptosis.

To address the function of PKC in normal breast, we used an organ culture system. This approach was chosen to maintain the normal epithelial/stromal relationships, thereby avoiding artifacts induced by monolayer culture (Lochter and Bissell, 1995). The organ cultures were treated with the PKC agonist, phorbol ester, and three known PKC inhibitors, staurosporine, tamoxifen, and calphostin C. Phorbol ester treatment resulted in the maintenance of a well-differentiated glandular phenotype. Staurosporine alone among the PKC inhibitors led to rapid squamous metaplasia. Neither PKC stimulation nor inhibition regulated epithelial proliferation. Finally, the epithelium in organ cultures from post-menopausal women, underwent squamous metaplasia in vitro in the absence of exogenous agents. This never occurred in tissues from pre-menopausal women. Therefore, these studies highlight a fundamental difference in the epithelium from these two populations of women.

Materials and Methods

Patient population: Specimens from 23 patients were cultured. 11 patients were premenopausal (< 50 years old) with a mean age of 28 years (range 19-47); 12 patients were postmenopausal (≥ 50 years old) with a mean age of 62 years (range 51-72). Patient diagnoses were as follows: for premenopausal patients, 7/11 had normal tissue or fibrocystic change and 4/11 had infiltrating ductal carcinoma; for postmenopausal patients, 1/12 had an intraductal papilloma, 5/12 had ductal carcinoma in situ, 1/12 had infiltrating lobular carcinoma, and 5/12 had infiltrating ductal carcinoma.

Organ culture: Fresh normal breast epithelium was obtained within two hours of surgical removal. Specimens included mastectomies and reduction mammoplasties. If invasive cancer was present, tissue was taken from non-involved quadrants. Fat was removed by dissection and the fibroglandular tissue minced with scalpel blades to 1-2mm^3 while bathed in RPMI, 50 units/ml penicillin, 50 μg/ml streptomycin. Three or four 1-2mm^3 pieces of tissue were floated on M/M media [3/4 Earle's MEM (Gibco) and 1/4 Waymouth's MAB 87/3 (JRH Scientific)] with 50 units/ml penicillin, 50 μg/ml streptomycin, 10mM Hepes, pH 7.4, and 10% fetal calf serum in each well of 24 well dishes. One ml of media per well was used to assure that the tissues floated freely and were not adherent to any solid surface. Culture was at 37°, 5% CO_2. Test reagents were added at the time of initial culture and in each sequential feeding twice weekly. 48 hours prior to fixation, each well was treated with 100 μM 5-bromo-2'-deoxyuridine (BrdU) and 10 μM 5-fluoro-2'-deoxyuridine (FldU) for subsequent histochemical detection of cell proliferation. Organ cultures from each well were then washed in phosphate buffered saline, pH 7.4, formalin

fixed for a range of 4-8 hours, and embedded into paraffin. Reagents tested included 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Calbiochem) [six patients], 10 nM staurosporine (Calbiochem) [eight patients], 50nM calphostin C (Calbiochem) [three patients], 20 uM tamoxifen (Sigma) [two patients], and 50 uM forskolin and 100 uM 3-isobutyl-1-methylxanthine (IMX) (Sigma) [three patients]. Tissues from all 23 patients were cultured with media in the absence of these test reagents as controls. Mean culture time was 38 days (postmenopausal, 37 days; premenopausal, 38 days).

Cell culture: MCF10A cells were the gift of Dr. David Hui (University of Cincinnati) and were cultured in XXXXX.

Morphology: Minced tissue was fixed in buffered formalin, paraffin-embedded, and sectioned at 4 μm intervals onto glass slides. Deparaffinized tissues were stained with hematoxylin and eosin for morphologic analysis. Fresh tissue from each specimen prior to culture was examined to assure normal epithelial morphology and content. Adequate epithelial density was considered to be ≥ five ductal or lobular units per histologic section, which included all of the tissue from a single well. Specimens from each culture condition were examined at roughly one week intervals. Squamous metaplasia of the ductal or lobular epithelium was defined morphologically by four criteria: the presence of cobblestone morphology, abundant eosinophilic cytoplasm, intercellular bridges, and keratin pearls. Slides were graded for squamous metaplasia on a linear scale of 0-5 as follows: a score of 5 would indicate that all of the epithelium in a tissue section was squamous vs. a score of 1, in which less than 25% of the epithelium was squamous and the

remainder was typical lobular or ductal epithelium. Early squamous differentiation, i.e. regions which contained two or three out of the four morphologic criteria, were confirmed or excluded based on the presence of the squamous marker, thrombomodulin (Raife et al, 1996). In addition, more mature regions of squamous differentiation were further characterized by the presence or absence of cytokeratin 10 (Ming et al, 1994).

Immunohistochemistry: All immunohistochemistry was performed on 4 μm, paraffin-embedded sections using the Ventana ES immunostaining system. Following deparaffinization in xylenes and any required pre-treatments, slides were placed in the instrument which adds the primary antibody, the biotinylated anti-mouse secondary, and avidin-conjugated peroxidase or alkaline phosphatase as dictated by a bar code. Primary antibodies were incubated for 32 minutes at 37° C. All washes were performed by the instrument. The slides were either counterstained with hematoxylin on the instrument or with nuclear fast red, by hand. In all cases an irrelevant mouse immunoglobulin was used as a negative control. Preservation of the epithelium, its proliferative capacity, and its tendency for differentiation were examined using the following markers: for myoepithelium - smooth muscle actin (1A4, Dako), keratin 14 (ll002, Nova Castra); for luminal epithelium - keratin 19 (RCK108, Dako), Mucin-1 (Ma552, Nova Castra), luminal epithelial antigen (LEA.135, Dako), human milk fat globulin-1 (1:1.10.Fβ, Immunotech); for squamous epithelium - keratin 10 (LHP1, Nova Castra), thrombomodulin (1009, Dako); and for proliferation - bromodeoxyuridine (3D9, Oncogene Science). Reaction to each antibody was assessed by two observers (SCH and MAM) and graded for intensity on a scale of 0-5. Proliferation was assessed for each cell type using the immunologic

detection of BrdU. BrdU incorporation was valued on a linear scale of 0-5 based on the quantity of total epithelium stained (0=none, 5=100%).

PKC assay: PKC activity was determined using the mixed micelle assay (Gibco/BRL) using myelin basic protein as the kinase substrate. Confluent flasks of MCF10A cells were scraped into .....

Statistics: All data were stored in a comprehensive database (Microsoft Access). Data were compared using ranked statistics, Kruskall-Wallis and Dunn's, due to the non-parametric nature of the analysis. Statistics were performed using SigmaStat (Jandel Scientific).

Results:

Organ culture epithelium maintained a differentiated phenotype. The quantity of epithelium was determined in each sample as described in Materials and Methods. There were no significant differences in the amount of epithelium between pre- versus post-menopausal patients, nor among individual specimens from a given patient over time. Epithelial differentiation was identified in each specimen by immunologic detection of protein expressed in luminal cells (LEA, Muc-1, or HMFG-1) or myoepithelial cells (α-smooth muscle actin). In all cases these markers were preserved in the glandular epithelium for the duration of the cultures (mean 38 days).

Staurosporine induces squamous metaplasia in all cases tested. To test the role of PKC in breast epithelial differentiation, we treated each culture with the PKC agonist, PMA, and the PKC inhibitor, staurosporine. PMA leads to the chronic activation of most PKC isozymes (Rando and Kishi, 1992), but in some cell types can also cause downregulation of a subset of PKC isozymes over time (Kishimoto et al, 1989). Staurosporine, on the other hand, is an extremely potent inhibitor of PKC (Tanaoki and Nakano, 1990). Cultures treated by either PMA or staurosporine showed no significant effects on the in vitro preservation of glandular epithelial cell types or change in expression of proteins associated with luminal cell differentiation, such as LEA, Muc-1, or HMFG-1 (data not shown). However, staurosporine treatment uniquely led to the rapid development of squamous metaplasia in all cases tested (N=8). In all our in vitro cases, the squamous metaplasia was well differentiated with formation of a granular layer and keratin pearls. An example of the histology of this metaplasia is shown in **Figure 1**. **Table 1** is a case by case

documentation of the amount of squamous metaplasia induced in vitro by staurosporine. The earliest appearance of squamous epithelium occurred within 7 days of culture; the longest period of treatment required to induce metaplasia was 28 days with a mean of 12 days. Note that most cases did not develop a completely metaplastic phenotype (score<5), and those cases which became completely squamous did so only after several weeks of culture. Squamous metaplasia occurred independent of patient age or type of surgical specimen.

Thrombomodulin expression is seen early in squamous differentiation of the breast, whereas keratin 10 is up-regulated with keratinocyte maturation. After several weeks of culture, all cases contained regions which fit all four morphologic criteria for squamous metaplasia (see Methods). However, in order to characterize the cells which were beginning to differentiate toward squamous epithelium, we tested the tissues for expression of genes known to be found in stratified squamous epithelium from other tissue sites. Keratin 14 is an intermediate filament found in stratified squamous epithelium but also present in normal breast myoepithelial cells (Schnier et al, 1994). It was present in all our cases of squamous differentiation (refer to **Table 4**, below). In addition, thrombomodulin and keratin 10 are commonly expressed in stratified squamous epithelium (Raife et al, 1996; Ming et al, 1994). Our data confirmed that thrombomodulin is an excellent marker for squamous differentiation of the breast, being present in nearly all examples (42/43) of squamous metaplasia which had all four morphologic criteria (**Table 2**). In addition, there were several thrombomodulin-positive cases in which the epithelium looked metaplastic, but one or two of the four criteria were not certain. However, by the

next week in culture the epithelium was clearly squamous by all four criteria. Therefore, thrombomodulin can detect squamous differentiation, early in its development, before the formation of obvious keratin pearls. Keratin 10, on the other hand, became positive only in the later stages of squamous differentiation when the cells were encased in large amounts of keratin, consistent with the published reports that it is a marker for fully differentiated keratinocytes in the skin, but does not stain the basal layers (Ming et al, 1994). Neither thrombomodulin nor keratin 10 was identified in normal (glandular) breast epithelium. These data are summarized in **Table 2.**

PKC inhibitors, beside staurosporine, do not induce squamous metaplasia. Although a potent inhibitor of PKC, staurosporine is known to inhibit other kinases (Gong et al, 1994; Yanagihara et al, 1991; Friedman et al, 1990). Therefore, we tested organ cultures with two other PKC inhibitors, calphostin C (Kobayashi et al, 1989; Gopalakrishna et al, 1992) and tamoxifen (O'Brian et al, 1986). Both of these agents had a similar capacity to inhibit PKC activity in breast epithelium derived from fibrocystic change (MCF-10A cells) as shown in **Table 3**. In neither case did these agents induce squamous metaplasia (**Table 4**). In addition, long-term exposure to PMA to down-regulate specific PKC (Kishimoto et al, 1989; Rando and Kishi, 1992). In our cultures PMA was never associated with the development of squamous metaplasia (**Table 4**).

Staurosporine acts like agonists of PKA. Several investigators have described squamous metaplasia of the breast in vitro. A common feature of these reports is activation of protein kinase A (PKA) (Schaefer et al, 1983; Yang et al, 1987). To determine whether

the squamous metaplasia seen by staurosporine was similar in time and morphology to that induced by increases in cAMP, we treated organ cultures from three patients with the combination of forskolin, which stimulates adenylate cyclase (Monneron et al, 1987), and 3-isobutyl-1-methylxanthine (IMX), which inhibits cAMP phosphodiesterase (Beavo et al, 1970). A case by case documentation of the amount of squamous metaplasia induced by these agents is shown in **Table 5**. Under these conditions, squamous metaplasia also occurred very early (as early as 4 days) and involved large portions of the total epithelium. Therefore, the processes induced by staurosporine and cAMP-inducing agents appear to be similar (compare **Tables 1 and 5**). Although staurosporine can inhibit PKA with a K_i two-fold above PKC (Tamaoki et al, 1986), there are no reports of staurosporine acting as a PKA agonist.

In the absence of treatment, squamous metaplasia is unique to post-menopausal epithelium. While squamous metaplasia was a consistent feature of staurosporine and forskolin/IMX treatment, it was not limited to these conditions. Of the twelve organ cultures performed using tissue from postmenopausal women (≥50 years old) in which no kinase inhibitors were added (media controls), ten showed evidence of focal squamous metaplasia, in one case as early as 14 days. These data are shown in **Table 6**. This squamous metaplasia was unique to the cultured tissue because examination of the surgical specimens, fixed and embedded immediately after surgery, showed no squamous metaplasia in any of these patients. However, of the 11 media control organ cultures from premenopausal women (<50 years old) no cases of squamous metaplasia were found in the

absence of an exogenous inducing agent. Therefore, staurosporine and forskolin/IMX induce a process which occurs "spontaneously" in cultures from postmenopausal women.

Squamous metaplasia occurs independent of co-incident carcinoma. Since most breast specimens from post-menopausal women are mastectomies and from pre-menopausal women, mammoplasties, we considered that squamous metaplasia in the post-menopausal population was the result of co-incident growth with invasive cancer or ductal carcinoma in situ. Therefore, cultures were performed from mastectomies with co-incident cancer in pre-menopausal women. Therefore, four of the eleven patients tested had infiltrating ductal carcinoma. A summary of patient diagnoses is given in Materials and Methods. No squamous metaplasia was observed in these cases, making it unlikely that squamous metaplasia is related to the presence of breast cancer in the surgical specimen (data not shown).

Metaplastic cells express genes associated with both luminal and myoepithelial differentiation. To determine the cell of origin for the squamous metaplasia, slides containing squamous epithelium and all earlier time points were examined for luminal and myoepithelial differentiation. Keratin 14, a protein associated with both myoepithelium and squamous differentiation, was found in all cases (**Table 7**). Keratin 19 was often found in several patterns: encircling the squamous epithelium, focally positive within squamous pearls, and uniformly positive throughout the epithelium, the latter being the least common. Most cases showed a mixture of keratin 14 and 19 staining. In no case was smooth muscle actin expressed within this epithelium, although staining was occasionally

present focally at the periphery of affected ducts or lobules (data not shown). To confirm the presence of a luminal cell phenotype within these areas of metaplasia, we further tested these cultures for the expression of genes unique to luminal cells, such as Muc-1, LEA, and HMFG-1. All three markers were retained in the glandular epithelium throughout the course of each experiment. In addition, one or more of these proteins was present in all cases of squamous epithelium. Examples of these immunologic stains in squamous metaplasia are shown in **Figure 2**. Finally, there were no apparent differences in marker immunoreactivity between squamous metaplasia which was induced by staurosporine or forskolin/IMX vs. spontaneous.

Induced squamous metaplasia is more proliferative than spontaneous metaplasia. Despite having a similar expression of differentiation-specific proteins, there were subtle differences between induced and spontaneous squamous metaplasia. Spontaneous squamous metaplasia occurred most commonly after several weeks of culture, was less sustained over time, and involved fewer lobules per slide than that which was induced (compare **Tables 1, 5 and 6**). Furthermore, there was a significant difference in the level of BrdU uptake, the mean value in the spontaneous squamous epithelium being 1.0 ± 1.3 vs. 2.5 ± 1.6 in the stimulated (**Figure 3**). This is in contrast to the normal breast epithelium which had a similar proliferative rate independent of patient age or culture condition (data not shown).

Discussion:

The results of this study are two-fold. First, the kinase inhibitor, staurosporine, consistently induces squamous metaplasia in normal breast epithelium, independent of the menopausal status of the tissue donor. Other authors have documented this effect by increases in cAMP and prostaglandins (Schaefer et al, 1983); however, to our knowledge squamous metaplasia has never been reported in breast by use of staurosporine. Second, the breast epithelium of post-menopausal women is significantly more likely to undergo squamous metaplasia than the epithelium from pre-menopausal women in the absence of exogenous agents. Therefore, the culture conditions used for these studies highlight a fundamental difference in the breast epithelium from these two populations of women.

In vivo, squamous metaplasia of the breast may occur in a variety of benign pathologic conditions (Worgotz and Norris, 1990), most of which are not associated with an increased risk of malignant transformation. Malignant squamous differentiation usually occurs in the context of ductal carcinoma, but may be the sole malignant phenotype (Woodard et al, 1980; Eggers and Chesney, 1984; Shousa et al, 1984; Weigel et al, 1996). Although few in number, the mean age of patients with this tumor is peri-menopausal, about 50-55 years (Weigel et al, 1996). In our study ten out of twelve (83%) of tissues derived from post-menopausal women showed squamous differentiation, whereas it never occurred in cultures from eleven women under 50 years old. This difference cannot be explained by any known differences in culture technique between the two groups, nor by the presence of co-incident invasive breast disease.

The studies of Schaefer et al. also found a differential ability of breast epithelium to undergo squamous metaplasia among populations of women (Schaefer et al, 1983). They showed that cAMP-induced squamous metaplasia is most readily achieved during the first ten days of the menstrual cycle. They saw no spontaneous squamous metaplasia; however, their cultures were of much shorter duration than in this study. We did not observe a consistent difference in sensitivity to staurosporine-induced squamous metaplasia, and have no data regarding the menstrual history of our pre-menopausal patients. However, both of these studies indicate that the physiologic state of the patient prior to surgery, i.e. the menstrual or menopausal status, impacts the epithelial phenotype in vitro days to weeks later. This is consistent with the work of Russo et al. (1989) who show that the state of epithelial differentiation in vivo is reproduced in vitro.

We had not predicted that staurosporine would induce squamous metaplasia, because in the literature this phenotype is restricted to stimulation of PKA. Indeed the mechanism by which staurosporine induced squamous metaplasia is still not clear. Staurosporine is a commonly used inhibitor of PKC which inhibits the PKC catalytic domain by a non-competitive mechanism (Wark and O'Brien, 1991). Biochemical studies indicate that it inhibits specific PKC isozymes to different degrees (Seynaeve et al, 1994; Budworth and Gescher, 1995). However, it is often unable to inhibit complex cellular activities induced with phorbol esters (Seynaeve et al, 1994), and in fact, may elicit cellular phenotypes identical to those stimulated by phorbol esters, seeming to act as a PKC agonist (Jalava et al, 1992; Jalava et al, 1993). In order to test whether PKC was in the pathway for

staurosporine-induced squamous metaplasia, we evaluated other known inhibitors of PKC. Unlike staurosporine, calphostin C inhibits PKC by making an irreversible photo-adduct of the regulatory domain (Gopalakrishna et al, 1992). Therefore, calphostin C is a more specific inhibitor of PKC than staurosporine, but is less potent. Tamoxifen was chosen for these studies because of its known pharmacological action on estrogen receptors of breast epithelium, but also because it is a known inhibitor of PKC in breast cell lines (O'Brien et al, 1986). Finally, phorbol esters were used because in the short-term they activate most PKC isozymes, and in the long-term they cause down-regulation of specific PKC isozymes (Kishimoto et al, 1989; Rando and Kishi, 1992). Our studies show that staurosporine is unique among these PKC inhibitors in its ability to induce squamous metaplasia. These studies can be interpreted in one of two ways. Either staurosporine is acting by a mechanism independent of PKC, or staurosporine is preferentially inhibiting a unique subset of PKC isozymes. Our current studies cannot differentiate between these two possibilities.

The literature regarding squamous metaplasia of the breast in vitro and in vivo does not clearly define the cell of origin. Both myoepithelial cells and a progenitor cell for both myoepithelial and luminal cell differentiation have been implicated (Rudland and Barraclough, 1988; Petersen and van Deurs, 1988; Starzec et al, 1994). In our studies, the earliest forms of squamous differentiation often contained proteins associated with luminal cell differentiation, such as Muc-1, LEA, and HMFG-1 (Berry et al, 1985; Shimizu and Shaw, 1993). Furthermore, nearly all cases of squamous differentiation contained strong reactivity for one or more of these luminal proteins. In addition, in several cases

extracellular lumens were seen in the inner portions of keratin pearls, indicating that the cells were intermediate in their phenotype between squamous cells and luminal epithelium. This is consistent with the finding that squamous cell carcinomas of the breast are often cystic (Weigel et al, 1996). Although luminal cells may be entrapped in squamous epithelium derived from myoepithelium or a stem cell, we would not expect this to be true in nearly every case. In addition, as shown in **Figure 4** both the squamous marker, thrombomodulin, and the luminal marker, HMG-1, co-localize to the same cells. Therefore, we believe that squamous differentiation may not only be derived from stem cells and myoepithelial cells, but also from cells which already have a luminal phenotype.

Here we have shown for the first time that the kinase inhibitor, staurosporine, induces squamous metaplasia of breast epithelium from both pre- and post-menopausal women. More importantly, these cultures highlight a fundamental difference in the epithelium from these two populations of women. Tissue from post-menopausal women has a strong predilection to undergo squamous differentiation in the absence of exogenous agents, a property which could potentially be exploited in the treatment of post-menopausal breast malignancies. Further characterization of these cultures will be necessary to discover the mechanism underlying these differences.

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Table 1. Squamous Metaplasia Induced by Staurosporine Over Time

Case #	Age	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Case 1	72	nd	0	0	1	2	2	4
Case 2	28	nd	2	4	-	-	-	-
Case 3	72	nd	1	3	3	5	5	4
Case 4	66	1	1	3	2	3	4	-
Case 5	19	nd	0	3	5	4	3	4
Case 6	19	0	1	2	4	2	4	-
Case 7	19	0	nd	3	3	4	4	-
Case 8	19	0	0	3	4	4	-	-

Table1. Specimens from each patient were fixed, embedded, and examined histologically at weekly intervals for morphologic changes. The presence of squamous metaplasia was graded based on the proportion of total epithelium containing squamous features on a linear scale of 0-5. Criteria for defining squamous metaplasia are as described in Methods. Age is the patient's age at the time of surgery. (nd = cultures not examined; - = no cultures at those times.)

Table 2. Specificity of Thrombomodulin and Keratin 10 for Squamous Epithelium.

	Glandular	Squamous
TM	0/43	42/43
Ker-10	0/43	23/43

Table 2. Slides from each culture condition at weekly time intervals were examined for immunoreactivity in the normal ductal or lobular epithelium (glandular) and in the squamous epithelium. Listed are the number of positive samples in each epithelial category over the total number of samples tested.

Table 3. Inhibition of PKC Activity.

Table 4. Effect of Treatments on Squamous Metaplasia

Culture Condition	N	Squamous Metaplasia
Staurosporine	8	30/36
Calphostin C	3	0/18
Tamoxifen	2	0/9
PMA	6	0/27
Forskolin/IMX	3	15/17

Table 4 indicates the number of specimens in which squamous metaplasia was identified relative to the number examined. Note that squamous metaplasia never occurred in tissues treated with PMA, calphostin C or tamoxifen. N = number of patients tested.

Table 5. Squamous Metaplasia Induced by Forskolin/IMX Over Time

Case #	Age	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Case 8	19	1	1	3	1	2	1	-
Case 9	59	nd	0	1	1	3	2	2
Case 10	52	nd	0	1	3	4	2	-

Table 5. Specimens from each patient were fixed, embedded, and examined histologically at weekly intervals for morphologic changes, as in Table 1. Age is the patient's age at the time of surgery. (nd = cultures not examined; - = no cultures at those times.)

Table 6. Spontaneous Squamous Metaplasia

Case #	Age	Week 1	Week 2	Week 2	Week 4	Week 5	Week 6	Week 7
Case 1	72	nd	0	0	1	0	1	0
Case 3	72	nd	0	1	4	0	0	1
Case 4	66	0	1	0	2	4	0	-
Case 9	51	nd	0	0	0	0	0	0
Case 10	52	nd	0	0	0	1	0	-
Case 11	57	0	2	-	-	-	-	-
Case 12	53	nd	0	0	0	0	1	0
Case 13	56	0	0	0	0	0	0	-
Case 14	63	nd	0	0	0	2	-	-
Case 15	62	0	1	2	-	-	-	-
Case 16	66	0	0	0	0	1	-	-
Case 17	63	0	1	1	1	0	-	-

Table 6. Media control specimens from 12 post-menopausal patients were fixed, embedded and examined histologically at weekly intervals for morphologic changes, as in Tables 1 and 5. Squamous metaplasia was found in 10/12 patients; squamous metaplasia was not detected in cases 9 and 13. Age is the patient's age at the time of surgery. (nd = cultures not examined; - = no cultures at those times.)

Table 7. Expression of Cell-Type Specific Proteins in Glandular and Squamous Epithelium.

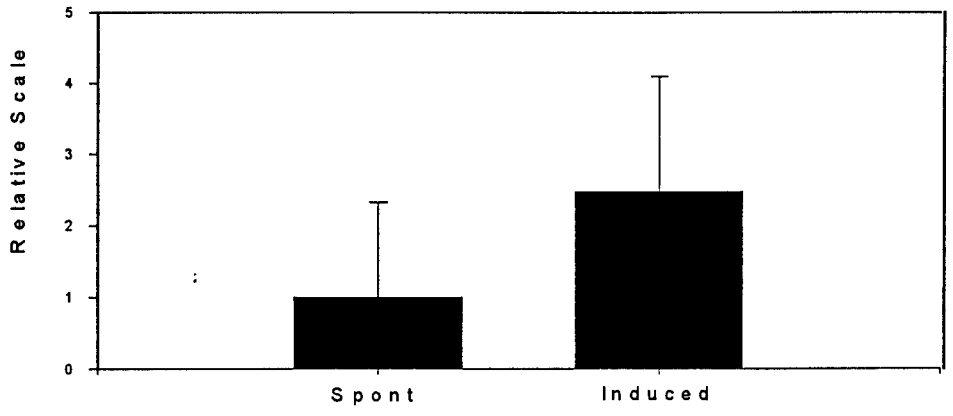
	Glandular	Squamous
Ker 14	110/111	43/43
Ker 19	108/111	36/43
Muc-1	103/105	37/40
LEA	68/97	29/40
HMFG-1	103/105	38/41

Table 7. Slides from each culture condition at weekly time intervals were examined for immunoreactivity in the normal ductal or lobular epithelium (glandular) and in the squamous epithelium. Listed are the number of positive samples in each epithelial category over the total number of samples tested.

Figure 1. Shown is a hematoxylin and eosin-stained slide of staurosporine-induced squamous epithelium. Note the presence of intercellular bridges (arrowhead) and a prominent granular layer (arrow). Bar = 500 μm.

Figure 2. All slides were examined for immunoreactivity against cell-specific protein. Shown are examples of squamous epithelium tested for expression of keratin 14 (a), smooth muscle actin (b), keratin 19 (c) , mucin-1 (d), luminal epithelial antigen (e), human milk fat globulin-1 (f), thrombomodulin (g), and keratin 10 (h). Keratin 14 (a) is ubiquitously positive in squamous epithelium, but also in myoepithelium, particularly in ducts. Smooth muscle actin (b) also is present in normal myoepithelium of ducts and lobules, but is not found in squamous metaplasia. Keratin 19, mucin-1, luminal epithelial antigen, and human milk fat globulin-1 (c-f) are normally expressed in luminal epithelial cells of ducts and lobules. Thrombomodulin and keratin 10 (g-h) are not found in normal breast epithelium but are expressed in squamous metaplasia. All photographs are at the same magnification. Bar (shown in a) = 500 μm.

Figure 3. Proliferation of Squamous Epithelium



Organ cultures were treated with Brdu and Fldu for 48 hours prior to fixation. Proliferation was assessed by the immunologic detection of Brdu on histologic sections. Incorporation was evaluated on a linear scale based on the number of positive nuclei (0=none, 5=100%). The data are plotted as the mean $\pm$ standard deviation. A statistical difference was found between the incorporation of BrdU in spontaneous (spont.) vs. induced squamous metaplasia ($p=0.006$).

Figure 4. Co-localization of Thrombomodulin and HMG-1 in Squamous Epithelium.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
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1 JUN 2001

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DAMD17-95-C-5078	ADB232057
DAMD17-95-C-5078	ADB242387
DAMD17-95-C-5078	ADB253038
DAMD17-95-C-5078	ADB261561
DAMD17-94-J-4433	ADB221274
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