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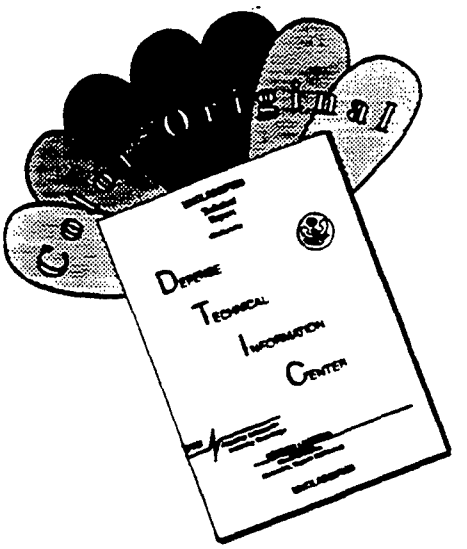

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Breast tumor growth and development is dependent upon the induction of neovascularization. The specific aims of this proposal define an approach to determine the stage of breast tumor progression in which angiogenesis is induced, to define cellular products which initiate vascular growth, and to develop and test an in vitro model system in which to define the role of multiple cell types in the angiogenic process. In the first two years of this proposal, we examined human tissues at multiple stages of tumor development and quantified vessel formation at each stage. These studies showed that epithelial vascularization begins in the earliest stages of epithelial proliferation. In addition, the vascularity of normal epithelium was greater in cancerous breasts than non-cancerous breasts. We have now examined these tissues by immunohistochemistry and in situ hybridization for numerous angiogenic factors and correlated changes in these factors with the initiation of vessel growth. Finally, we have begun developing the in vitro organ culture system. To date we have defined optimal media conditions and discovered that serine/threonine kinase activity alters epithelial differentiation. We are now in the process of isolating specific cell types from these cultures to examine the regulation of growth factor production in vitro.

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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 explant 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). However, little information exists regarding angiogenesis in pre-invasive lesions. For instance, it is unknown whether *in situ* lesions are more angiogenic than atypical proliferation, or that *in situ* lesions may be categorized prognostically by different levels of angiogenesis. In this specific aim we propose 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.

Specifically, we shall **1.1** Quantify angiogenesis in tissue sections of atypical hyperplasia and *in situ* ductal carcinoma by counting vessels immediately adjacent to these proliferative lesions on von Willebrand factor immunostained slides; **1.2** Investigate the utility of detecting the expression of c-ets-1, a transcription factor expressed in endothelial cells during angiogenesis, as a predictor for early vessel growth; C-ets-1 mRNA will be detected by *in situ* hybridization; and **1.3** Correlate these data retrospectively with patient outcome.

Work Accomplished in Specific Aim 1:

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

All of Task 1 is complete and published. The galley proof is attached. 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 (31). All of these data are consistent with the concept that angiogenesis is an early phenotypic marker of cells in the process of neoplastic transformation (32).

The completion of this aim has delayed our studies of Specific Aim 2 (below) because in the initial review of our manuscript, one of the reviewers requested that all of the in situ carcinoma data be re-evaluated according to new diagnostic classification systems. Our original data correlated vascularity with in situ carcinoma as defined by the U.S. consensus criteria (33), which are widely used in the U.S. and have significant prognostic implications for invasive disease (34). Since these new diagnostic criteria are now being tested in the U.S. and Europe for their prognostic significance, we thought that correlation of vascularity with these diagnostic criteria may yield histopathologic information regarding prognosis. Therefore, the PI learned the diagnostic criteria and re-analyzed all the in situ slides. While time-consuming, the result is interesting. As

shown in our paper, vascularity correlates very well with the "old" classification system, but has no correlation with either of the new classification systems. Until further studies have been published, we do not know whether these new systems will have prognostic significance for invasive disease.

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

The goal of this task was to evaluate c-ets-1 as a marker for angiogenic endothelium. 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. Figure 1 shows a Northern confirming the specificity of the antisense probe. As indicated, the antisense probe identifies the two predicted messages. However, in situ hybridization with these probes does not allow sufficient discrimination to identify endothelial cells which are angiogenic. Many cells in the stroma are positive specifically with the antisense probe, such that we have been unable to identify the endothelium unequivocally. Because of the possibility that a higher stringency may allow better discrimination, we plan to repeat these studies a few more times on several tissues with known angiogenesis. 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 in many patients. In summary, to date we have been unable to use c-ets-1 by in situ hybridization or by immunohistochemistry as a marker for angiogenic vessels.

However, one other characteristic of angiogenic endothelium is its ability to divide. We have worked out the technique for double labeling our tissues 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, 35). An example of such a double label is shown in Figure 2. These data will help us evaluate not just the level of endothelial proliferation, but also the proliferative state of the adjacent epithelium.

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 propose to determine the presence and cellular distribution of angiogenic factors using the same tissues in which we have quantified vessel growth, above. Angiogenic factors to be tested include those factors which have been identified in breast tissue and/or breast carcinoma. Since proteolytic enzymes induce angiogenesis, we will also examine these tissues for proteolytic enzymes and their inhibitors (36,37). Recent investigations indicate that VEGF is an excellent candidate for initiating angiogenesis in tumors (15). In addition, bFGF and TGFα are likely to participate. Therefore, for these three angiogenic factors, we shall examine the tissue for both the growth factor and their receptors. These data will be correlated with the clinical outcome. In patients with concurrent invasive disease, we shall correlate these data with prognostic factors known to be of predictive value.

Specifically, we propose to: **2.1** Examine tissue sections of early proliferative lesions and *in situ* carcinoma for the presence of known angiogenic growth factors and cytokines by immunohistochemistry or in situ hybridization. **2.2** Examine tissue sections of early proliferative lesions and *in situ* carcinoma for proteolytic enzymes and their inhibitors known to be involved in angiogenesis; **2.3** Correlate the presence and cellular origin of these factors with the degree of angiogenesis and clinical outcome.

Work Accomplished in Specific Aim 2: We have also made substantial progress in Specific Aim II. The Statement of Work pertaining to this aim is as follows:

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

As with c-ets-1, we designed oligonucleotides to make cDNA probes for VEGF and its receptors, KDR and flt. VEGF was cloned from the epidermoid carcinoma cell line A431. The primers allowed detection of all splice variants (38). The largest of these variants has now been cloned into pT7-blue (Novagen) by TA cloning and is now being subcloned into PGEM. In addition, we have cloned the receptor, KDR, into PGEM-3Z, confirmed the construct by sequencing, and performed initial studies by in situ hybridization. The other VEGF receptor, flt, has also been cloned from term-placental RNA and is under evaluation. An example of the RT-PCR results for VEGF, KDR, and flt are shown in Figure 3. Figure 4 shows confirmation of specificity of the KDR probe by Northern analysis. Antibodies for flt and KDR recently became commercially available; they do not work in formalin-fixed

paraffin-embedded tissue, but can be used for immunocytochemistry in vitro.

As we have completed our immunohistochemical analysis (Task 4), we have added to our list of confirmatory probes. For instance, cDNAs for TGFα and its receptor EGFR were obtained from ATCC and subcloned into PGEM. We have begun in situ hybridization using the TGFα riboprobe labeled with digoxigenin. We began our studies with TGFα because this angiogenic factor is found in normal breast epithelium, primarily in myoepithelial cells, by immunohistochemistry. In situ hybridization was used to confirm that myoepithelium is the cell of origin. An additional probe we are currently producing by PCR is the bFGF receptor, flg.

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, EGF, VEGF, IGF I and II, bFGF, TGFβ, and PDGF. The PI has analyzed all of the slides and is currently entering all of the data into the statistical package. While the statistical analysis is still incomplete, preliminary data, as shown in Table 1, have already led to some interesting conclusions. Table 1 shows the analysis of four growth factors for intensity of staining on a scale of 1-4 in normal epithelium (Bn), all forms of proliferative disease (prol), in situ carcinoma (IS), and invasive cancer (inv). Listed are the mean and standard deviation for all tissues in each diagnostic category and the cell type examined. These include luminal epithelium (epithel), myoepithelium (myoep), endothelial cells in vessels which touch the epithelial basement membrane(endo), adjacent stromal cells (stroma), and infiltrating leukocytes (lymph). In some cases ductal and lobular epithelium have been analyzed separately. Note that "myoepithelium" listed under invasive disease actually represents stromal cells within the tumor mass, many of which are α-smooth muscle actin positive, but do not necessarily represent basaloid differentiation. Despite the preliminary nature of this analysis, the following conclusions can be made: 1. Several angiogenic growth factors are found in normal breast epithelium and do not increase with progression to invasive disease, 2. VEGF appears to be an exception to this, increasing with progression. These data will have to be confirmed by in situ hybridization, 3. Stromal cells and invading leukocytes are important contributors of angiogenic agents even in normal tissue. We have not yet analyzed our data with respect to the presence of invasive cancer elsewhere in the breast, nor do we know yet whether specific growth factors in individual cases correlate with especially aggressive vascularization. However, the work remaining in this task is

mostly statistical and answering specific questions by in situ hybridization.

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

This is the task with which we have had the most experimental difficulty. Antibodies listed in the original proposal were evaluated and used in many of the tissues. However, upon analysis we discovered that the results were inconsistent over time, with loss of reactivity in the last tissues examined. Therefore, we are now evaluating new antibodies, which have become commercially available in the past year. Many of these have been tested and they do not work in paraffin-embedded tissues. These studies will be completed as appropriate reagents are identified.

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 (39). 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 exogenous angiogenic factors. Therefore, we have identified a new model of human tissue in which to 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.

Work Accomplished in Specific Aim 3:

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

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 (paper or 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.

Early in our analysis of these data, it became clear that we had overestimated our ability to define angiogenesis and to catalogue

the types of information which we would get from our analyses. Therefore, in collaboration with Mr. Gary DeVoe and Mrs. Mary Ann Miller in the Department of Pathology, we set up a database for analysis of these cultures. Figure 5 shows an example of the data sheet filled in by the PI when examining each slide. Despite moving our tissue culture laboratory to a new building in the past year, we have performed 39 organ cultures for periods of a week to up to a couple months. Agents tested in this system include Matrigel, bFGF, cell culture conditioned media containing VEGF, PMA, staurosporine, calphostin C, forskolin, IMX, tamoxifen citrate, and ECGF. A complete listing of conditions is seen in Table 2. 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). In addition, we have performed co-culture with tumor cells and normal fibroblasts.

The primary focus of these experiments is to induce angiogenesis. Since vessels are ubiquitous, we had to develop reagents to define new vessels and identify cell types in vitro which may be producing angiogenic factors. During the course of these experiments we have developed the markers listed in Table 3. Currently, sections from each block from each experiment have been double labeled for von Willebrand factor and BrdU. In these slides we can accurately assess endothelial cells which are in the cell cycle. An example of such a stain is shown in Figure 6. Since normal endothelial turnover is rare, the presence of endothelial mitosis is a marker for angiogenesis (41). In addition to cell division, new vessels have a unique extracellular matrix (40). We have begun to examine serial sections of these organ cultures for laminin, fibronectin, and collagen IV. At this point it is clear that we need to enter these data into a computer and overlay the images to identify the matrix composition of individual sprouts. This past summer the PI took a course in image analysis to begin working out the details of these studies, and via a grant from the Ohio Board of Regents, was able to purchase the necessary software. In addition, we are exploring the possibility of using thick section confocal microscopy of immunofluorescent images to define endothelial sprouts. These studies will be performed in the departmental confocal core facility.

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. While we have found that numerous culture systems appear to generate vascular sprouts, it is too early in the analysis of the data to be certain that we have significant angiogenesis. However, we have consistently observed a sequential "activation" of vascular cell types in these cultures. 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 addition to evaluating angiogenesis, the organ culture experiments have given us some insight into epithelial growth and differentiation under specific circumstances. First, in premenopausal women, the preservation and response to growth factors may be a function of the time of the menstrual cycle in which the tissue was removed. This is an unexpected finding, which will require some cooperation on the part of surgeons to address. Second, it is clear that myoepithelium is much more resilient in culture than ductal or lobular epithelium. Our experiments using stimulators and inhibitors of the PKC and PKA signaling pathways are aimed at understanding the effects of these agents on both angiogenesis and the preservation of epithelium.

One surprising finding from these experiments is the observation that staurosporine induces squamous metaplasia in myoepithelium (Figure 7). Staurosporine is a somewhat non-specific inhibitor of the PKC pathway. Others have shown that stimulation of PKA, such as with IMX and forskolin, will also induce squamous metaplasia (41). We have examined the effect of staurosporine on epithelial differentiation multiple times and found a consistent complete metaplasia to squamous cells. Furthermore, there is a complete absence of epithelial outgrowth from explants using this reagent. However, other PKC inhibitors, such as calphostin C and tamoxifen citrate, do not cause any squamous differentiation. Neither does the long-term treatment with phorbol esters, which downregulates many isozymes of PKC, induce this change. As expected from the literature, IMX and forskolin also induce squamous metaplasia in our culture system, but the response is less complete than with staurosporine. In addition, like staurosporine, IMX and forskolin inhibit epithelial outgrowth. We are now in the process of preparing these data for publication.

Another unexpected result of these organ cultures has been the delineation of culture conditions which lead to outgrowth of specific cell types. We have been particularly interested in the preservation and culture of myoepithelial cells versus luminal epithelium. We have developed markers to differentiate these types of epithelium, as described in Table 3. For instance, Figure 8 shows cells which were grown as a monolayer, isolated from the organ culture dishes, and mixed to form a cell pellet. These cells have been double labeled for α-smooth muscle actin

(brown) showing myoepithelium and keratin 19 (red) showing luminal epithelial cells. When fully analyzed, these data should allow us to be more efficient in isolating purified populations of cells from normal breast tissue.

In summary, our results are very preliminary, but to date we have "discovered" that a PKC antagonist and PKA agonists will induce myoepithelial squamous metaplasia, that pericyte proliferation precedes endothelial activation, and that we can by our current methods induce vascular sprouting. Technically, we have worked out methods to embed and cut serial sections, we have developed numerous markers to determine cell type and matrix production, and we have developed mechanisms to measure cell-specific proliferation by using double labels. Further development of this system will proceed over the next two years.

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

We have already performed preliminary studies using conditioned media from A431 cells which produce high levels of VEGF and are easier for us to grow with our current incubator configuration than HL60 cells. We are currently testing the angiogenic capacity of this media in our in vitro angiogenesis assay using isolated microvascular endothelial cells cultured in a three-dimensional matrix (39).

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

One of the technical difficulties in the organ culture system is to provide stable long-term exposure to angiogenic factors in vitro. We explored the Elvax bead approach (44) and have shown that bFGF placed in these beads is able to support proliferation of human umbilical vein endothelial cells. Table 4 shows the data from such an experiment. We have used these beads once in an organ culture. However, the beads we have made to date are still too large, so we are now exploring new technologies to make smaller beads to be used for injection into the tissue.

As mentioned in Task 6 and Table 2, we have already tested bFGF and VEGF, two very potent angiogenic agents, in our organ culture system. The results show an effect on vessel sprouting, but we do not yet have linkage of vessels within the tissue. Based on these data, and the fact that isolated endothelial cells form vessels in a variety of in vitro matrices, and in fact aortic rings will sprout vessels into the appropriate matrix, we believe that

normal tissues contain potent angiogenesis inhibitors. Recently it was discovered that thrombospondin is just such an inhibitor, which is under the control of the tumor suppressor gene, p53 (45). Other tissue inhibitors of angiogenesis have been hypothesized and include such agents as TIMPS. Therefore, as a means of addressing this problem, we are developing reagents to identify the known inhibitors in our organ culture system, in the hopes of overcoming this type of inhibition in future experiments. In addition, since it is now known that different angiogenic agents induce angiogenesis by different mechanisms (46), some angiogenic agents may be blocked by tissue inhibitors and some may not. These experiments will continue into the next year.

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.

To date we have performed one organ culture using conditioned media from normal breast fibroblasts and done one co-culture of normal breast with tumor explants. As indicated above, the most we can detect is vascular sprouting, so we will continue these studies after we have investigated the issue of tissue inhibitors.

As a means of identifying which cells make growth factors and how stroma may regulate this process, in consultation with Mina Bissell we have begun to produce epithelial organoids from normal breast (47). These are being cultured in a three-dimensional matrix to look for the production of angiogenic agents using our well-characterized angiogenic model system (39). 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.

Summary of Work Accomplished:

Technical:

1. Completion and publication of Task 1.
2. Production of most of the probes for in situ hybridization: Tasks 2 and 3.
3. Completion of the immunohistochemical staining to identify angiogenic factors: Task 4.
4. Re-evaluating reagents for metalloproteinases: Task 5

5. Characterized culture conditions allowing a minimum of 2 months viability for organ cultures; induction of vascular sprouting in organ cultures with angiogenic agents.
6. Tested several angiogenic agents and developed approach for long-term delivery: Task 8
7. Production of organoid cultures and isolates of specific cell types: Task 9

Scientific:
1. Epithelial vascularity begins at the earliest stages of epithelial proliferation.
2. Vascularity correlates with histologic predictors of disease progression.
3. Normal epithelium from cancerous breasts is more vascular than from non-cancerous breasts.
4. Numerous potent angiogenic factors are present in normal epithelium.
5. Tissue leukocytes and stromal cells are also sources of angiogenic agents.
6. Agents which increase cAMP, as well as, the kinase inhibitor staurosporine induce squamous differentiation.
7. Angiogenic agents induce vascular sprouting in organ cultures but not complete vessel formation indicating that these tissue may contain matrix inhibitors of angiogenesis.

Specific Methods

Vascularity of pre-invasive breast disease: as in attached manuscript.

Probe preparation for in situ hybridization: Probes for the two VEGF receptors, KDR and flt-1, were prepared as follows: Oligonucleotides allow RT-PCR amplification of the first 900 nucleotides of the KDR gene (17); this sequence includes 5' untranslated sequence with no homology to the other known receptors. The 5' oligo is ACTG(T/C)A(A/G)GTACCCTTGTTATCC-AAGCGGCA; a Pst 1 site is inserted in the 5' end. The 3' oligo is CATGACGATGGAC(A/T)AGTAGC-CTGTCTTCAG with an Spe 1 site in the 5' end. These oligonucleotides yield a 900 base pair product from RT-PCR of human umbilical vein endothelial cell RNA. The PCR product were directly cloned into PGEM-3Z (Promega). Similarly, flt-1 sequences from the 3' untranslated region were used as reported (18). Oligonucleotides are CTATGGAAGATCTGATTTCTTACAGT ending at nucleotide 3248 and GATGTCGACGGTATAAATACACATG ending at 4288. These oligonucleotides permit a SalI/BglII insertion into PGEM. *VEGF probes* are as used in (20): GGGCTCTAGATCGGGCCT-CCGAAACCAT and GGGCTCTAGAGCGCAGAGTCTCCTCTTC. Confirmation of this sequence uses the internal probe GGGCTCTCGAATGAACTTTCTGCTGTCTTGGGT. In addition, cDNA sequences

were confirmed by dideoxy sequencing. TGFα and EGFR cDNAs were purchased from ATCC and directly subcloned into PGEM.

Probe labeling: Sense and antisense strands are labeled by incorporation of digoxigenin using either the T7 or Sp6 promoter, as per Promega protocols. Following strand synthesis, the reactions are treated with RQ1 DNase and the RNA strands separated from unincorporated nucleotides using a quick spin column (Boehringer Mannheim) with tRNA as a carrier.

Hybridization conditions for *in situ* analysis on deparaffinized, hydrated tissue include: treat with 0.2N HCl for 20′ at room temperature and 0.3% Triton X-100 for 15′ at room temperature, digest with 50 μg/ml proteinase K in 50mM Tris HCl, 5mM EDTA for 30′ at 37°, refix in 4 % paraformaldehyde for 5′, and acetylate in 0.1M triethanolamine/acetic anhydride for 10′; sections are pre-hybridized in 50% formamide/2x SSC for 2 hours at 37°; heat denatured probe is added in 50% formamide, 0.3M NaCl, 20mM Tris HCl, 5mM EDTA, 10mM Na_x PO_4 pH 8.0, 1x Denhardt's, 0.5 mg/ml yeast RNA at 42° for 18 hours; washes include 2x SSC, followed by treatment with 20μg/ml RNase, 0.1x SSC and 0.1M Tris-HCl/0.15M NaCl.

Digoxigenin detection. Digoxigenin antibody is used (1:500) either at RT for 3 hours or overnight at 4°. Alkaline phosphatase-conjugated anti-mouse is then added for 1 hour at RT followed by the colorimetric substrate, NBT/BCIP. Color development is overnight in the dark at RT.

Organ culture. The basal media is 1/3 MAB (JRH Co.) and 2/3 Earle's minimum essential media. Cultures consist of 1 mm^3 dissected normal breast floating on media in 24 well plates under varying conditions. At specific time intervals these explant are labeled with bromodeoxyuridine (BrdU) at 100uM and FldU at 10uM for 24 hours prior to fixation, embedding, and sectioning. Proliferation assays on paraffin-embedded sections are performed by immunologic detection of the BrdU.

Organoid cultures. Microdissected breast epithelium is treated by Collagenase type I (900IU/ml, Sigma) for 24-48 hours and various sizes of organoids separated by differential centrifugation (47). Cultures are grown on non-polymerized collagen I, collagen I gels and Matrigel to characterize growth condition conducive to production of angiogenic agents. 24 hour conditioned media is used for the in vitro angiogenesis assays.

Examination of angiogenic agents. Growth factors, cytokines, or proteolytic enzymes are added to organ cultures and angiogenesis, quantified. Application of the angiogenic factor to the specimen via the media will be contrasted with application via Elvax

beads, such that diffusion into the specimen is limited; application of beads which have not been treated with the angiogenic agent will serve as controls. In our preliminary data using bFGF as an angiogenic agent applied to one side of the explant, we were able to easily differentiate the treated end of the explant from the untreated end. Application of a marker (India Ink) prior to embedding is used to assure proper orientation of the specimen during embedding. With some experience, we have identified the optimal size and shape for the explant, in order to clearly define a treated and an untreated side. In each experiment, multiple explants are left untreated to define basal vessel distribution. Angiogenesis is quantified on von Willebrand stained slides, as described above.

In vitro angiogenesis: Human microvascular endothelial cells (Clonetics) are cultured on matrigel in defined media (EBM, Clonetics) with and without angiogenic agents. Tubule formation is quantified by morphometry at 4, 6 and 24 hours.

Figure 1. Northern analysis of tumor necrosis factor-α-treated human umbilical vein endothelial cell RNA. Each riboprobe was labeled with digoxigenin. Hybridization was detected using anti-digoxigenin conjugated with alkaline phosphate. Sp6=antisense; T7=sense. The black lines indicate the expected message sizes of 6.8kb and 3kb.

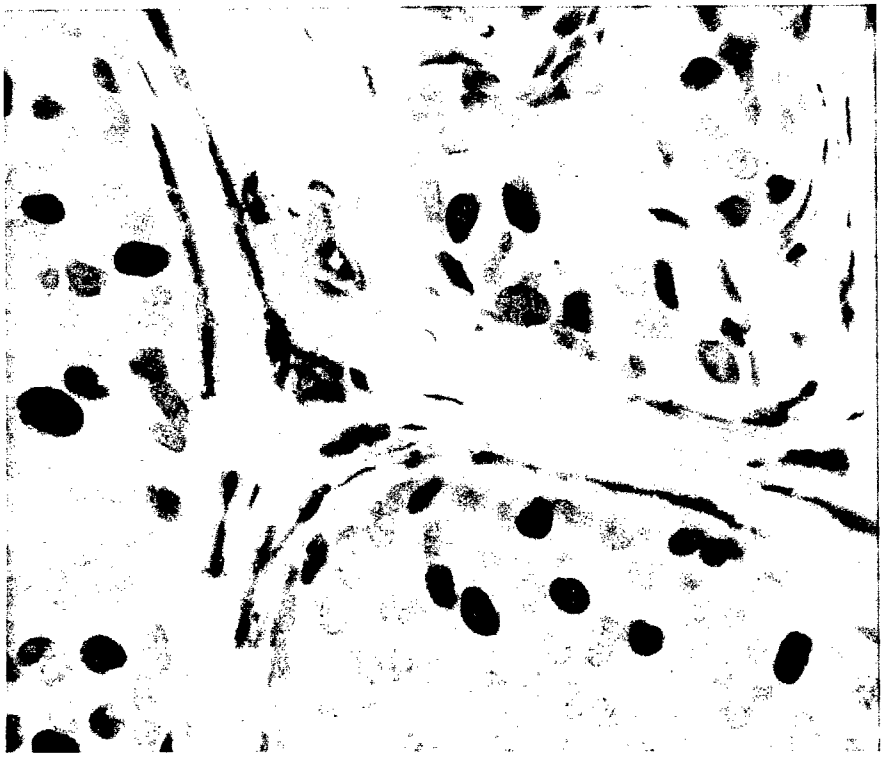

Figure 2. A tissue section of a human breast biopsy showing ductal carcinoma in situ is double labeled for proliferation (MIB-1:brown) and vessel formation (von Willebrand factor:red).

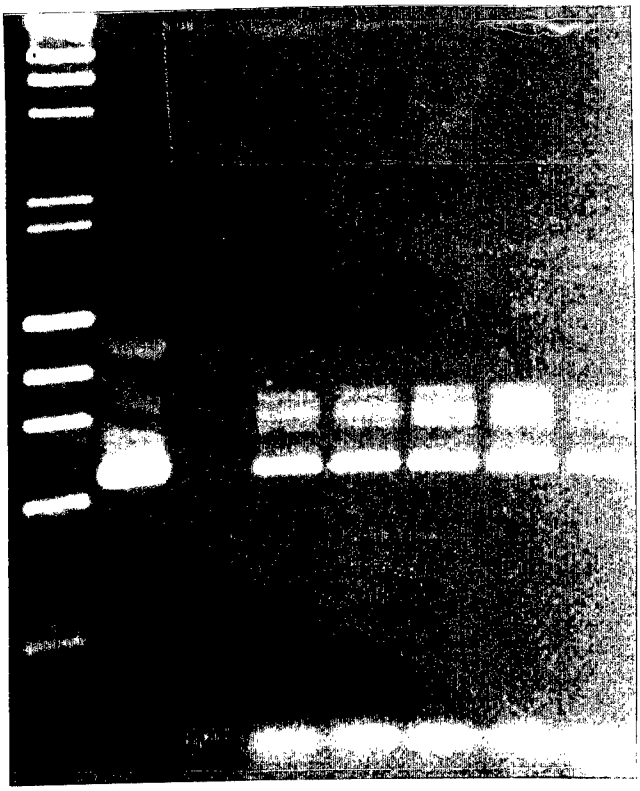

Figure 3a. RT-PCR of HUVEC RNA for VEGF; a=markers, b=actin control, d-h =individual RNA preps.

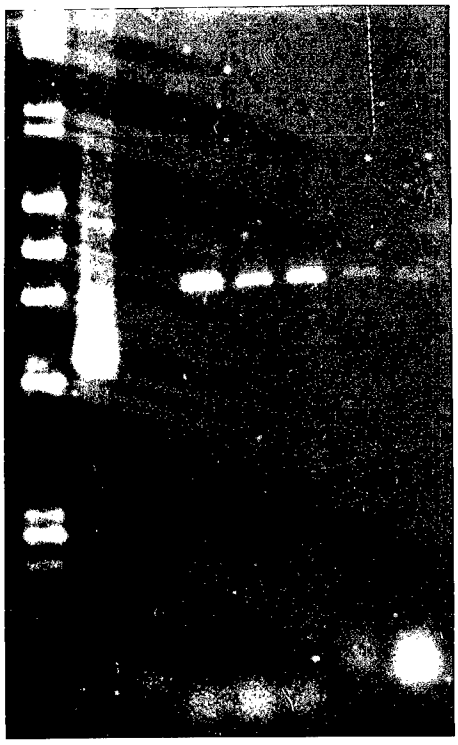

Figure 3b. RT-PCR of HUVEC RNA for KDR; a=markers, b=actin control, d-h=individual RNA preps.

SP6 T7

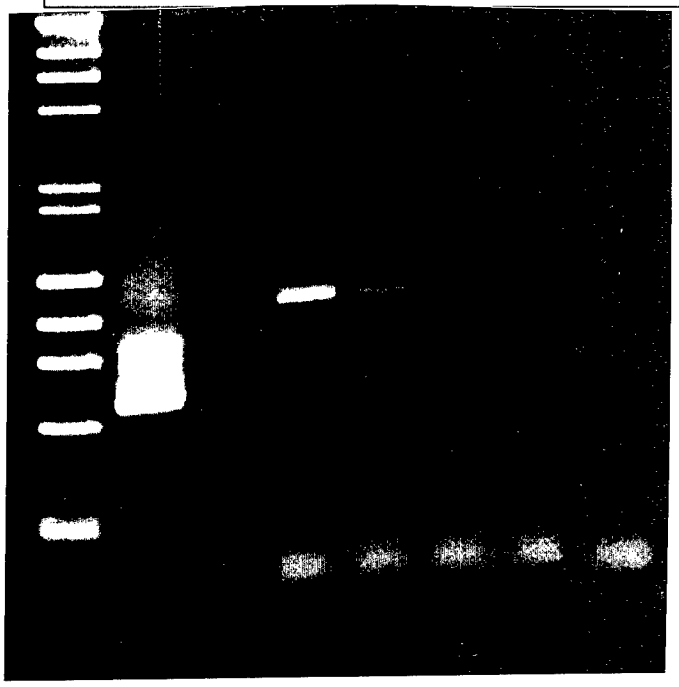

Figure 3c. RT-PCR of placental RNA for flt. a=markers, b=actin control, d-h=individual RNA preps.

Figure 4. Northern analysis of HUVEC RNA showing specific reactivity of antisense KDR probe (T7). Message size is approximately 7kb.

Table 1

PDGF: mean and st. Dev.

	epithel		myoep		endo		stroma		lymph							
Bn	0.88	0.99	1.13	0.85	0.48	0.79	0.26	0.54	0.00	0.00						
prol	0.82	0.75	1.00	0.45	0.27	0.47	0.09	0.30	0.18	0.60						
IS	0.54	1.13	0.54	0.66	0.23	0.60	0.08	0.28	0.15	0.55						
inv	0.67	0.71	0.00	0.00	0.78	0.83	0.00	0.00	0.00	0.00						

bFGF: mean and st. Dev.

	epithel		myoep		endo		stroma		WBC							
Bn	2.61	0.79	2.36	1.06	1.46	0.96	0.82	0.86	0.96	1.35						
prol	2.47	1.13	1.60	1.24	1.13	1.06	1.00	1.00	0.33	0.90						
IS	2.12	1.05	1.12	1.41	1.24	1.20	1.18	1.24	1.24	1.39						
inv	2.18	0.75	0.27	0.90	1.91	0.83	1.45	1.04	1.82	1.47						

VEGF: mean and st. Dev.

	epithel		myoep		endo		stroma		WBC							
Bn	1.89	0.94	1.00	0.82	1.63	1.01	0.37	0.60	1.32	1.49						
prol	2.10	0.74	1.10	0.99	0.70	0.67	0.40	0.84	1.20	1.32						
IS	2.22	0.97	0.56	1.13	0.78	0.97	0.22	0.44	1.33	1.58						
inv	3.13	0.64	0.00	0.00	1.75	1.04	1.00	1.31	2.25	1.04						

TGFalpha: mean and st. Dev.

			du	ct			lob	ule								
	epithel		myoep		epithel		myoep		endo		near endo		stroma		lymph	
Bn	2.90	0.99	0.40	0.97	2.82	0.98	0.55	0.69	0.40	0.70	1.64	0.67	0.00	0.00	0.18	0.63
prol	1.67	0.58	0.00	0.48	2.67	0.97	0.33	0.95	0.67	0.82	1.56	0.57	0.00	0.00	0.00	0.20
IS	0.00	0.00	2.00	0.00	2.67	0.58	2.33	2.08	0.50	1.00	2.00	0.00	0.25	0.50	0.00	0.00
inv																

Figure 5

VWF/3D9 _____ Date Read

Name: _____ Age: ____

Block Identity: ____ Date run: _____ #: ____ Sequence: ____

Days in culture: ____ BRDU Hours: ____ Design: _____

VWF+ Arteries/Veins: ____
VWF/3D9+ A/V Endo: ____
VWF/3D9+ A/V Peri: ____
VWF/3D9+ A/V Fibro: ____

VWF+ Complex Area: ____
3D9+ Complex Area: ____
VWF/3D9 + Complex Area: ____

VWF+ Capillary: ____
VWF/3D9+ Cap/Endo: ____
VWF/3D9+ Cap/Peri: ____
VWF/3D9+ Cap/Tips: ____
VWF/3D9+ Cap/Fibro: ____

Type of VWF: ____
VWF Intensity: ____
PECAM Intensity: ____

Vascular Apoptosis: ____
Vascular Preservation: ____
Matrigel Cells: ____
Matrigel 3D9+ cells: ____

Vascular Dilation: ____
Foam cells: ____

Epi Overall: ____
Epi Preservation: ____
Epi Disorganized ____
Epi Necrosed: ____
Epi 3D9+: ____
Epi Squamous Differ: ____
Epi Squamous Branched: ____
Epi 3D9+: ____
Epi Fibrosis: ____
Epi Calcification: ____

Myo Preserved 3D9+: ____
Myo Prominence: ____

Tumor Overall: ____
Tumor Preservation: ____
Tumor Necrosed: ____
Tumor 3D9+: ____
Tumor VWF+: ____

Stroma Overall: ____
Stromal Cellularity: ____
Stromal 3D9+: ____
Stromal VWF+: ____
Stromal Density: ____

Fat Overall: ____
Fat Cellularity: ____
Fat Preservation: ____
Fat Hydrolysis: ____
Fat 3D9+: ____
Fat VWF+: ____

Outgrowth : ____
Outgrowth 3D9+: ____
Outgrowth VWF+: ____
Outgrowth VWF/3D9+: ____
Outgrowth SMA+: ____
Outgrowth Keratin+: ____

Further studies _____ Photographed: _____

Table 2.

Media
- RPMI
- M/M
- TM
- MEM

Additives
- 0.5% FCS
- 2% FCS
- 10% FCS

Growth factors
- Il-6
- Il-2
- bFGF/H2O
- bFGF/CHAPS
- ECGF
- A431 extract
- SK conditioned media
- A431 conditioned media
- heparin
- estrogen
- PMA 10ng/ml
- PMA 50ng/ml
- PMA 100ng/ml
- Matrigel
- Matrigel spiked with GF
- staurosporine
- bFGF in beads
- BSA in beads
- IMX
- forskolin
- tamoxifen citrate

Structure
- explant
- strips
- defatted strips
- chunks
- bits (1-2mm3)
- filter paper
- tissue paper
- floating
- with tumor
- with tumor explants
- with confluent fibroblasts

TABLE 3

Epithelial Cell Specific Markers

Myoepithelial	Luminal
Keratin 14	Keratin 19
α SMA	milk fat globulin
S100	casein
collagen IV	PS2
	LEA-135

Other phenotypic markers

Target	Clone	Source
Adult myosin	poly	private
α SMA	IA4	Dako
BrdU	3D9	Oncogene Science
casein	F20.14	Biogenics
collagen I	CIV-22	Dako
collagen IV	COL-94	Sigma
CD31	JC/70A	Dako
Desmin	DE-R-11	Dako
E-cadherin	6F9	Cappel
EGFR	31G7	Triton
Epithelial Membrane Antigen	Ber-EP4	Dako
estrogen receptor	NCLER-LH2	Nova Castra
Fetal myosin	poly	private
Fibronectin	568	Nova Castra
Keratin 14	LL002	Nova Castra
Keratin 19	RCK-108	Dako
Laminin	Lam-89	Sigma
Luminal epithelial antigen	LEA-135	Dako
macrophage	PG-M1	Dako
milk fat globulin	1.10.F3	Amac
PS2	poly	Nova Castra
S100	poly	Dako
Tenascin	Tn2	Dako
TGFα	213-4.4	Oncogene Science
Vimentin	3B4	Dako
von Willebrand factor	F8/86	Dako

Figure 6. Breast organ cultures were formalin-fixed and paraffin-embedded. Serial sections were prepared for immunohistochemistry. This photograph shows a double label to measure proliferation (BrdU incorporation: red) in vessels (von Willebrand factor: brown).

Figure 7. Inhibition of PKC with staurosporine induces squamous metaplasia in breast organ cultures. Here we illustrate a hematoxylin and eosin stained section of such a tissue cultured for 3 weeks in the presence of 10nM staurosporine.

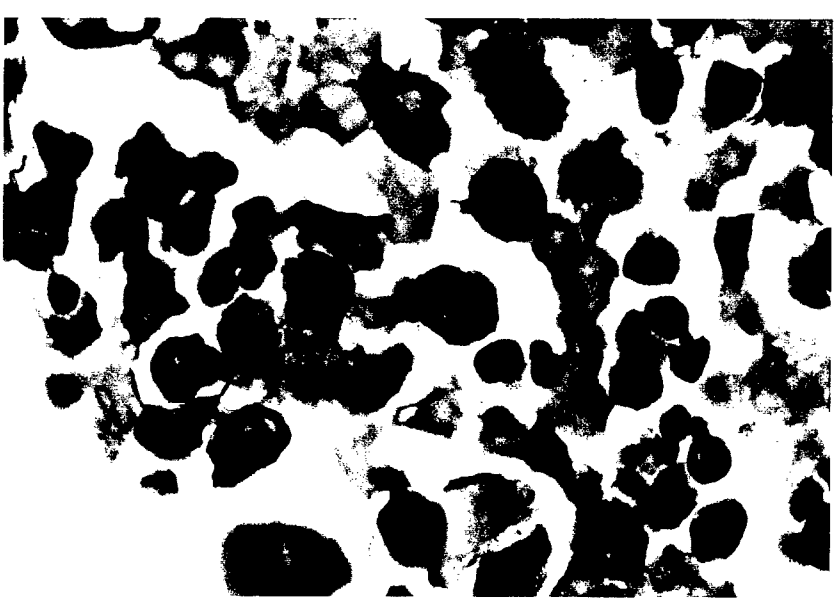

Figure 8. Normal breast epithelial cells in monolayer culture were combined to form a cell pellet for fixation and embedding. Here we show a label for ker 19 (luminal cells: red) and α-smooth muscle actin (myoepithelium: brown).

Table 4.

48 hour incorporation of 3H-thymidine into human umbilical vein endothelial cell monolayers. Elvax beads are made with carrier [bovine serum albumin (BSA)] or growth factor [basic fibroblast growth factor (bFGF)]. Reported is mean +/- one standard deviation of quadruplicate samples.

Condition	Counts per minute
No beads (media alone)	2343+/-270
BSA beads	2867+/-399
bFGF beads	5667+/-1104

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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
HUVECS	human umbilical vein 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 Numenclature 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

Vascularity of Proliferative Breast Disease and Carcinoma *in Situ* Correlates with Histological Features[1]

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ABSTRACT

The level of vascularity within an invasive breast carcinoma is a predictor of metastatic potential and survival. However, little is known about the vascular potential and prognostic value of angiogenesis in preinvasive breast pathology. Women with proliferative breast disease or carcinoma *in situ* are at increased risk of developing invasive breast cancer. This relative risk increases in correlation with defined histopathological features. We asked whether these early proliferative lesions and carcinoma *in situ* were capable of inducing a vascular supply. Vascularity in preinvasive archival paraffin-embedded breast tissue from 90 patients was quantified by immunohistochemical identification of vessels using anti-von Willebrand factor. Vascular scores were analyzed with respect to histopathological diagnosis, age at diagnosis, and presence of coincident invasive disease. These data indicate that: (*a*) the vascularity of histopathologically normal epithelium is greater in breasts containing invasive disease than in breasts lacking invasive disease; (*b*) simple proliferative breast disease induces a vascular supply greater than that of normal breast epithelium; and (*c*) vascularity increases in proportion to epithelial lesion progression and relative risk of invasion. These studies indicate that the vascularity of preinvasive breast pathology may be a clinically useful predictor of invasive breast cancer.

INTRODUCTION

Breast tumorigenesis can be characterized by a succession of histologically defined "precursor states" in which there is epithelial growth (1). These include epithelial hyperplasias (PBD)[3] and CIS. Women diagnosed with these forms of pathology are at increased risk of having invasive disease, especially if there is a family history of breast cancer (2–5).

All tumors require an adequate blood supply to grow beyond a size of 2–3 mm^3 (6), and quantification of tumor-induced vascularity is of prognostic value. This was first demonstrated for breast cancer by Weidner *et al.* (7), who showed that the number of vessels in areas of intense vascularity correlates with metastatic potential and patient survival. These observations have now been confirmed by others (8–15). Since the ability of tumor cells to induce a vascular supply occurs early in the process of cell transformation (16), it seemed reasonable that preinvasive breast lesions may also induce a vascular supply. Indeed, in 1975, *in vivo* assays by Gimbrone *et al.* (17) showed that murine preinvasive breast tissues contain the ability to induce angiogenesis in rabbit irises. Since that time, others have documented that the vascular supply in preinvasive breast disease is greater than in normal breast (18–20). Furthermore, one study indicated that vascular density in the tissue of fibrocystic change was a predictor of progression to invasive disease (20). The studies reported here examine the vascularity of preinvasive breast disease to test the hypothesis that vascularity increases with progression toward invasive disease.

Quantification of vascularity in invasive tumors is not a determination of "new vessels" but a measure of local vessel density (21). In tumors, the local vessel density is focally greater than in normal tissue, and therefore by implication, new vessels must have been produced or migrated into the tumor by tissue remodeling (22). A similar approach has been taken by several groups to quantify vessels in preinvasive breast disease (19–20, 23, 24). We chose a different approach for our studies for two reasons: (*a*) the distribution of vessels in breast stroma is very heterogeneous and difficult to relate to individual ducts or lobules by position, especially in breasts with marked epithelial proliferation; and (*b*) ducts and lobules in close proximity can display more than one pathological diagnosis. Since capillaries rarely touch the basement membrane of normal breast epithelium, we used this feature to identify and quantify "new" vessels by restricting our assessment to vessels that touched the basement membrane of epithelium defined as either normal, proliferative, or *in situ* carcinoma. Here we report that compared with normal breast tissue, vascularity is increased in preinvasive breast pathology, that the degree of vascularity correlates with histological features that are predictive of invasion, and that histologically normal breast tissue from cancerous breasts is more vascular than similar tissue in normal breasts.

MATERIALS AND METHODS

Immunohistochemistry. Specimens were routinely processed by the surgical pathology service of each hospital submitting tissues. Tissue processing included formalin fixation and paraffin embedding. Four-μm sections from each specimen were deparaffinized with xylenes and hydrated through graded alcohol. Vessels were stained using the Ventana automated immunohistochemistry stainer 320ES. Briefly, sections were pretreated with trypsin and then incubated with polyclonal anti-von Willebrand factor 1:3000 (Dako Corp, Carpinteria, CA) at 37°C for 30 min. Next, the slides were incubated with a bioti-

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[3] The abbreviations used are: PBD, proliferative breast disease; CIS, carcinoma *in situ*; DCIS, ductal CIS; LCIS, lobular CIS.

Table 1 ●●●

Vascularity of ductal or alveolar epithelium was determined on slides stained by immunohistochemistry for von Willebrand factor. The proportion of basement membrane touched by vessels for each epithelial unit was scored according to this formula. Up to 18 ducts or cross-sections of lobular alveolae were examined per slide per diagnostic category and averaged. Therefore, each slide will have a mean vascular score for each diagnostic category identified.

Circumference surrounded by vessel	Angiogenic grade
None	0
<1/3	1
≥1/3 → <2/3	2
≥2/3 → <3/3	3
Complete encircling	4

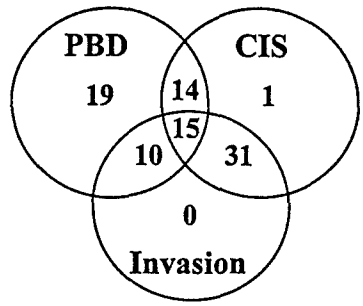

Fig. 1 Venn diagram showing distribution of cases containing PBD, CIS, and invasive breast cancer. Numbers indicate numbers of cases per diagnostic category.

nylated goat antirabbit secondary antibody, followed by horseradish peroxidase-avidin, and the colorimetric reaction was visualized with diaminobenzidine/H_2O_2 using copper sulfate enhancement. Negative control preimmune rabbit serum was incorporated into each run. All slides were counterstained with hematoxylin.

Evaluation of Vascularity. Slides were examined independently by R. Y. and S. H. Histopathological diagnoses of preinvasive disease were ascertained for each duct or lobule by the consensus criteria (25). Categories of epithelium included the following: normal, proliferative (including florid ductal hyperplasia and lobular hyperplasia), atypical hyperplasia (ductal and lobular), and carcinoma *in situ* (ductal and lobular; Ref. 26). Simple hyperplasia (less than a four-cell thickness per individual ductal unit) and all forms of adenosis were excluded from examination. DCIS was subcategorized as either micropapillary, cribriform, solid, or comedo, as per consensus criteria in Ref. 27, and by two recently suggested criteria for the classification of DCIS (28, 29). The classification system by Silverstein *et al.* (28) is dependent upon nuclear grade and the presence of necrosis. The classification system by Holland *et al.* (29) depends upon epithelial polarity and nuclear size and cytological features. Only the positively stained vessels in direct contact with either ductal or lobular basement membrane were considered. Each duct or cross-section of a lobular unit was graded as shown in Table 1. Up to 18 ducts or cross-sections of lobular alveolae within a single diagnostic category on each slide were given a vascular score, and the mean was determined for the final vascular score. Therefore, a unique vascular score was determined for each diagnostic category found on a slide. The level of vascularity around ducts or lobules containing epithelial proliferation or *in situ* carcinoma was scored independently by two pathologists. Normal epithelium was scored by S. H. or R. Y. When present, invasive breast cancer was graded according to the criteria of Bloom and Richardson (30).

Clinical Characteristics. Specimens from 90 patients were examined: 34% premenopausal (ages 32–50) and 65% postmenopausal (ages 51–81). Follow-up data were available for a mean of 4.7 years after diagnosis (range, 1–16 years). Cases were accessioned from the surgical pathology case files at the University of Cincinnati Hospital and from surrounding regional hospitals. Specimens included mastectomies, excisional biopsies, and reduction mammoplasties. Fig. 1 illustrates the relationship of PBD, CIS, and invasive disease among these cases. As shown in this Venn diagram, 58 (64%) patients had at least one form of PBD, with or without atypia, 61 (68%) individuals had some form of CIS, and 56 (62%) patients had invasive carcinoma. All but one case of invasion was coincident with the preinvasive pathology.

Statistics. All statistics were performed using SigmaStat (Jandel Scientific, San Rafael, CA). Among histological subtypes, means for each patient were compared by ANOVA on ranks and multiple comparison testing by Dunn's method. Variation between pathologists and comparison of multiple slides from one specimen were tested by Wilcoxin sign ranks (31).

RESULTS

Interobserver differences were evaluated for each diagnostic category on 136 slides. Ninety-one % showed no significant differences between the two pathologists ($P > 0.05$). The discrepant comparisons were equally distributed between proliferative and *in situ* lesions. No significant differences were found between different slides from the same patient.

Fig. 2 shows an example of normal, proliferative, and atypical proliferative epithelium, respectively. The proliferative epithelium partially fills the duct with swirls of cells that are irregular in size and shape (Fig. 2*b*). Fig. 2*c* shows proliferative epithelium with atypia in which some of the cells have an increased nuclear:cytoplasmic ratio with prominent nucleoli (*arrow*) and focal gland formation similar to cribriform DCIS (*).

An example of *in situ* carcinoma that is highly vascular is illustrated in Fig. 3. Immunological staining of vessels (Fig. 3, *brown*) demonstrates the extent of vascularity along the basement membrane of a duct filled with *in situ* carcinoma. Because the sum of all the vessels around the duct would cover greater than two-thirds of the circumference, this duct would have a vascular score of "3" (see Table 1). In some cases, numerous vessels were prominent in the adjacent periductal connective tissue; however, these were not considered part of the vascular score. The vascular score of normal epithelium, PBD, and CIS is summarized in Table 2. This table indicates the number of cases in which each diagnostic category was identified, the mean vascular score for each diagnostic category, the S.E., and the range of vascular means found for each diagnostic category overall of the slides examined. These data show that PBD has an increased level of vascularity relative to normal epithelium

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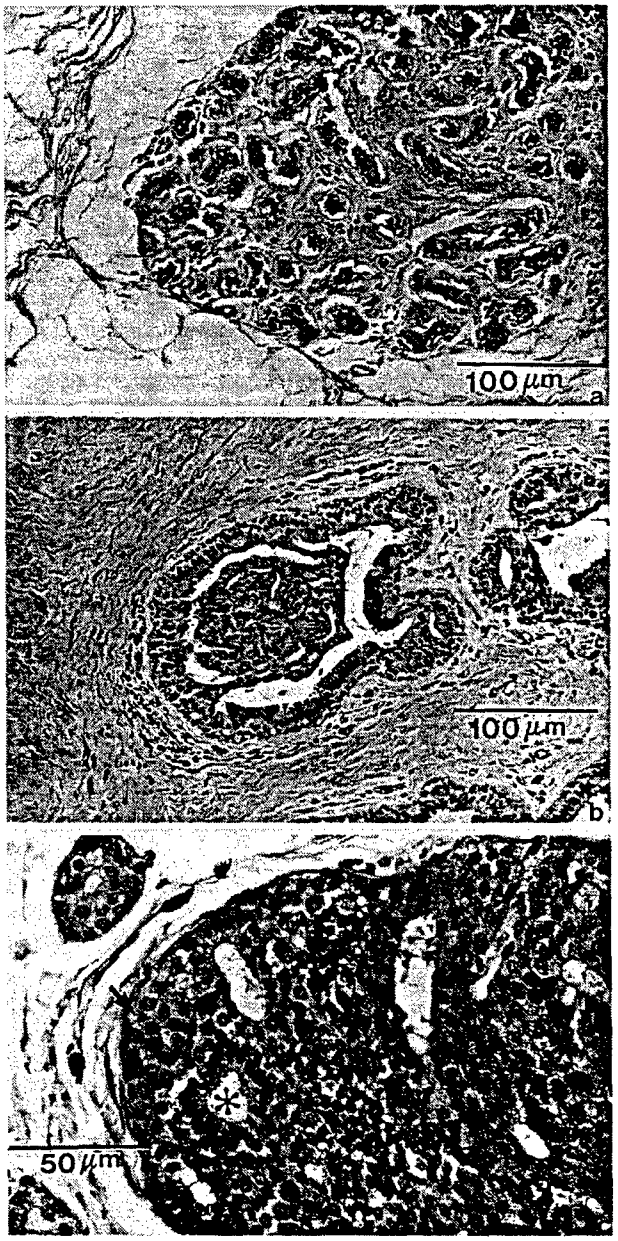

Fig. 2 H&E-stained sections of: *a*, normal breast epithelium; *b*, PBD; and *c*, proliferative breast disease with atypia. Diagnostic criteria were as described in Ref. 27. Note that the epithelium in *c* has features of both simple proliferative epithelium and carcinoma *in situ*. In particular, there are foci of cells with cytological atypia including an increased nuclear: cytoplasmic ratio and prominent nucleoli (*arrow*) and lumen formation with cribriform-like palisading of the cells (*).

(0.836 *versus* 0.187), with $P < 0.0001$. Similarly, CIS is more vascular than PBD (1.525 *versus* 0.836), with a $P < 0.0001$. Analysis of vascularity among histological subtypes of PBD and CIS was difficult because of the small number of cases in some of the categories, particularly LCIS and micropapillary and comedo DCIS. However, the levels of vascularity differed sufficiently that even using a nonparametric ANOVA on ranks we found a statistically significant difference among some groups

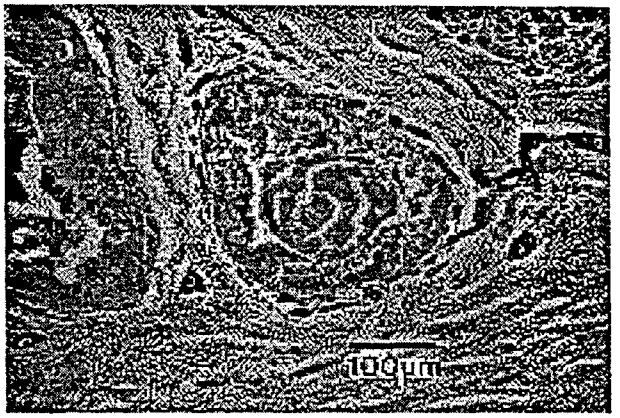

Fig. 3 Histological section of DCIS stained by immunohistochemistry for von Willebrand factor. Note that the basement membrane of the duct is touched by numerous vessels. According to the criteria in Table 1, this duct would have a vascular score of 3 and > two-thirds circumference but not completely surrounded.

Table 2 Summary of pooled data on vascularity for normal breast epithelium, PBD including proliferative breast disease with and without atypia, and CIS, both ductal and lobular

Ninety patients were included in this study. The number of cases per category shown here illustrates that many tissues contained epithelium that fell into more than one diagnostic category. The mean vascular score is derived from the mean of all slides on which each diagnostic category was identified. The range of vascular scores is the lowest and highest mean for each diagnostic category found on any individual slide.

Histology	Mean	SE	Range	No. of cases
Normal	0.187	0.0183	0.027–0.769	83
Proliferative	0.836[a]	0.0446	0.136–1.670	70
CIS	1.525[a]	0.0892	0.220–3.110	63

[a] $P < 0.0001$ compared with normal.

with a $P < 0.0001$ (Table 3). All forms of PBD and CIS differed from normal epithelium, confirming the previous pooled analyses. Among these subtypes, levels of vascularity fell into four groups. In order of increasing vascularity, these were: normal epithelium; all proliferative breast disease, LCIS, and micropapillary DCIS; cribriform and solid DCIS; and comedo DCIS. Detailed analysis indicated that further separation of groups would be statistically significant, but the power to discern these differences was insufficient in this preliminary study. In particular, there was a separation of PBD from a group containing atypical PBD/LCIS/micropapillary DCIS and a separation of cribriform from solid DCIS.

By this classification system (27), subtypes of DCIS were related to the Bloom and Richardson grade of invasive ductal cancer (30). Grades of invasion were associated with DCIS as follows: cribriform, 1.818 ± 0.603; solid, 2.286 ± 0.644; and comedo, 2.500 ± 0.577. These data show a trend for less vascular forms of DCIS to develop into low-grade invasive carcinoma, whereas the highly vascular comedo carcinoma tends to develop into high-grade carcinoma. More cases need to be analyzed to acquire enough power for statistical analysis.

Several new classification systems for DCIS have recently

Table 3 Data shown in Table 2 displayed according to diagnostic subtype

Diagnostic categories include normal epithelium; PBD; proliferation with atypia; micropapillary, cribriform, solid, and comedo DCIS; and lobular CIS. Note the marked difference between vascularity in PBD without atypia and normal breast, indicating that vascularity is increased very early in the progression to invasive disease. In addition, note that comedo DCIS is markedly more vascular than any other form of CIS.

Histology	Mean	SE	No. of Cases
Normal	0.187	0.0183	83
Proliferative	0.814[a]	0.0502	50
Atypia	0.889[a]	0.0940	20
Micropapillary	0.962[a]	0.1455	9
Cribriform	1.418[a]	0.1670	15
Solid	1.616[a]	0.1345	28
Comedo	2.216[a]	0.2582	7
Lobular	1.341[a]	0.2014	4

[a] $P < 0.0001$ compared with normal.

Table 4 Vascularity *versus* the classification system for DCIS

Comparison of vascular score *versus* DCIS classified according to two recently proposed classification systems. The DCIS on each slide was classified according to the proposed criteria of Silverstein *et al.* (28) listed as either Group 1, 2, or 3, or by the criteria of Holland *et al.* (29), listed as either Class 1, 2, or 3. The mean vascularity ± SD were calculated for each diagnostic category. The mean vascularity showed no statistically significant association within either classification system.

Classification	Mean Vascularity ± SD
Group 1[a]	1.483 ± 0.723
Group 2[a]	1.165 ± 0.603
Group 3[a]	1.695 ± 0.748
Class 1[b]	1.349 ± 0.578
Class 2[b]	1.473 ± 0.711
Class 3[b]	1.699 ± 0.818

[a] According to the proposed criteria of Silverstein *et al.* (28).
[b] According to the criteria of Holland *et al.* (29).

Table 5 Mean vascularity *versus* menopausal status

Premenopausal women were defined as patients ≤50 years old at the time of diagnosis. Postmenopausal women were >50 years old. Thirty-one patients were premenopausal, and 59 were postmenopausal. Differences in the vascular score for each diagnostic category were not statistically different.

Age	Normal	Proliferative	In situ
≤50 years old	0.202	0.842	1.445
>50 years old	0.180	0.803	1.600

Table 6 Vascular score *versus* coincident invasion

The vascular score was calculated for each diagnostic category and segregated according to whether the patient had coincident invasive disease or had no evidence of invasion. The vascularity of normal epithelium was significantly more vascular when derived from a breast with invasive disease ($P = 0.006$). None of the other comparisons reached statistical significance. Numbers given are the mean vascular score ± SD.

Pathology	Normal	Proliferative	In situ
No invasion	0.127 ± 0.134[a]	0.805 ± 0.301	1.366 ± 0.574
With invasion	0.221 ± 0.176[a]	0.830 ± 0.416	1.596 ± 0.756

[a] $P = 0.006$.

been proposed (28, 29). To determine whether these classification systems also correlated with vascular potential, each case of DCIS was reclassified according to these proposed criteria. Table 4 shows the results of these studies. In brief, vascularity among these DCIS subtypes could not be distinguished by statistical analysis. However, the vascularity tended to increase with increasing grade in the classification system proposed by Holland *et al.* (29).

Because breast carcinoma in pre- and postmenopausal women has unique characteristics (32), we examined the vascularity of these preinvasive lesions with respect to age at diagnosis and coincident invasive carcinoma. The mean vascularity of normal epithelium and PBD in premenopausal women (≤50 years old) was greater than in postmenopausal women (<50 years old; 0.202 *versus* 0.180, and 0.842 *versus* 0.803), although the difference was not statistically significant (Table 5). However, the vascularity of CIS showed the opposite relationship. As shown in Table 6, vascularity in PBD or CIS showed no correlation with coincident invasion, regardless of the histological grade. However, the vascularity of normal epithelium was greater when associated with invasive disease (0.221 *versus* 0.127; $P = 0.006$).

DISCUSSION

The angiogenic potential of preinvasive breast disease of murine origin was first shown over two decades ago (17–18). Since that time, several groups have quantified the vascularity of PBD or DCIS. Ottinetti and Sapino (19) examined vascular number and size within a 100-μm perimeter of normal epithelium, PBD, and DCIS. These studies showed an increased mean vascular size in PBD and DCIS relative to normal epithelium but no increase in vessel number. Using the same approach, Guinebretiere *et al.* (20) examined the vascularity in fibrocystic disease and showed an increased relative risk for developing subsequent invasive breast cancer. Other methods were used by Fregene *et al.* (23) and Guidi *et al.* (24). Fregene *et al.* (23) quantified the number of vessels per ×400 field in the most intensely vascular regions near benign breast epithelium of both proliferative and nonproliferative categories. Although this is similar to the methods used in invasive cancers (reviewed in Ref. 33), when applied to small intraductal pathology, it is more difficult to assess how much stroma to include relative to the lesion size. These studies showed no difference in vascularity between proliferative and nonproliferative benign pathology but a significant increase in the vascularity of PBD in postmenopausal women *versus* premenopausal women (23). Finally, Guidi *et al.* (24) used both a 1–3+ estimate of vascularity and quantitative counts of microvessels within 500 μm of DCIS (24). These data showed that vascularity is greater in comedo than non-comedo DCIS, proportional to nuclear grade, and correlated with Her2/neu expression. All of these methodologies examined vessel number or size within a predetermined field size or within the connective tissue 100–500 μm from the

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epithelial basement membrane. Because of the tremendous heterogeneity of vascular density in the connective tissue of benign breast epithelium, the influence of fibroplasia (24), and the inability to discriminate induced from preexistent vessels, we developed a method based on quantifying vessels in a location where vessels rarely exist, along the epithelial basement membrane.

As explained in Table 1, the vascularity scoring system is based upon the proportion of an individual duct or lobular cross-section that is surrounded by vessels. The 0–4+ grading system was readily reproduced by two pathologists and showed a clear distinction in vascularity among normal epithelium, PBD, and CIS. This is contrary to the results of Ottinetti and Sapino (19) and Fregene *et al.* (23), who showed no correlation between vascular number and histological features, but consistent with Guidi *et al.* (24), who demonstrated a difference in vascularity between comedo and non-comedo DCIS. Furthermore, our vascular scores correlated well with the previously defined relative risk of invasion for each histopathological subtype (4). Since two new classification systems for DCIS have been proposed recently, we evaluated vascularity for each proposed DCIS subtype (Table 4; Refs. 28 and 29). Vascularity was not statistically different among DCIS classes using either system. However, the system proposed by Holland *et al.* (29) showed a trend of increased vascularity with increasing grade of DCIS.

Previous studies on vascularity of DCIS described two patterns of vascularization: an increase in stromal vessels; and "cuffing" of the effected ducts (24). We also saw heterogeneity in stromal vascularity, which as described above, has been measured by others using multiple different methodologies. Guidi *et al.* (24) showed cuffing in 38% of DCIS (24), whereas Weidner *et al.* (7) reported cuffing in 23% of DCIS. Using the criteria of Guidi *et al.* (24), cuffing was found in 30% of DCIS in our study.

In 1982, using a rabbit iris model system, Jensen *et al.* (34) demonstrated that normal breast epithelium is more angiogenic when derived from a cancerous breast than a noncancerous breast. Our data on normal epithelium is consistent with this study (Table 6). However, this difference was not found with disease progression to PBD or CIS.

Fregene *et al.* (23) showed a statistically significant increase in vascularity in PBD of postmenopausal patients, whereas in invasive tumors, vascularity is significantly higher in premenopausal women (35). Our data showed no statistical difference in vascularity of normal, PBD, or CIS *versus* age (Table 5).

One study has shown that vascularity of fibrocystic disease is predictive of subsequent invasion (20). This study examined 48 women with fibrocystic disease, one-half of which developed invasive disease. Although our study demonstrates a correlation between vascularity and recognized histopathological features that are predictive of invasion, this study cannot test this hypothesis because most of the invasive disease in our patient population was concurrent with the preinvasive disease. Furthermore, the follow-up time interval for patients with benign diagnoses (2.14 years) was insufficient for such an analysis.

The relation of vascularity to noninvasive breast epithelium has two clinical implications: (*a*) if our data and the work of Guinebretiere *et al.* (20) are confirmed by others, vascularity could become a powerful tool to discriminate who is at high risk for invasive disease. Discrimination of vessels that are undergoing angiogenesis from nascent vessels will potentially make the characterization of vessels in preinvasive disease an even better marker for subsequent invasion. Factors such as endothelial cell tissue factor, specific integrins and other adhesion molecules, and transcription factors are all potential markers for the angiogenic phenotype (36–39); and (*b*) inhibition of vascularization of these premalignant lesions may inhibit progression to invasion. It is well understood that tumor growth is vessel dependent, and numerous antiangiogenic agents have been tested *in vitro* and *in vivo* (reviewed in Ref. 33). However, studies have yet to show that preinvasive progression to malignancy is vessel dependent. If proven, antiangiogenic agents may provide a new class of tumor-preventive therapy.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

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1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
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