AD NUMBER

ADB246547

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Sep 98. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, 1 Jun 2001.

AD_____

GRANT NUMBER DAMD17-94-J-4271

TITLE: A Model for Breast Cancer-Induced Angiogenesis

PRINCIPAL INVESTIGATOR: Sue C. Heffelfinger, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, Ohio 45267-0562

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-94-J-4271
Contractor: University of Cincinnati
Location of Limited Rights Data (Pages): 12-22

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

8/10/99

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 - 31 Aug 98)

4. TITLE AND SUBTITLE
A Model for Breast Cancer-Induced Angiogenesis

5. FUNDING NUMBERS
DAMD17-94-J-4271

6. AUTHOR(S)
Sue C. Heffelfinger, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Cincinnati
Cincinnati, Ohio 45267-0562

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

19990820 023

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, September 1998). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

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 a complex mixture of angiogenic factors that increase in the epithelium, stroma, or infiltrating leukocytes during progression to invasive breast cancer. 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. The stroma of normal breast tissue is able to inhibit endothelial cell proliferation under some conditions. Therefore, the angiogenic potential in this tissue appears to be the result of an interplay of angiogenic stimulators and inhibitors.

14. SUBJECT TERMS
Breast Cancer
Angiogenesis, Proliferation, Carcinoma in situ

15. NUMBER OF PAGES
27

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__✓__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 9/29/98 Date

Table of Contents

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 and 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 vessels, 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 proposal had as as one of it goals the development of the organ culture system to investigate the dynamic nature of tumor-induced angiogenesis. By introducing purified angiogenic agents or cells which produce factors hypothesized to elicit angiogenesis, we proposed to study the response of capillaries and venules, as well as the response of epithelial and stromal elements within the tissue.

HYPOTHESIS/PURPOSE:

The hypothesis to be tested was 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 proposed 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) and the degree of vascularity in invasive disease is of prognostic significance (30). The signals, which cause adjacent vessels to undergo the process of angiogenesis in breast carcinoma, remain unknown. We proposed 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 proposed to define the soluble and insoluble factors that induce angiogenesis in breast cancer patients, and therefore, may be targets of therapeutic intervention. Finally, we proposed 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 were as follows:

1. Identify and quantify angiogenesis in the earliest stages of breast carcinoma, and correlate these data with clinical outcome.
2. Localize to specific cellular subsets known angiogenic factors in *in situ* carcinoma and atypical hyperproliferative lesions of the breast.
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.

The results for each specific aim and the tasks required to accomplish these aims are presented separately, below.

<u>**Specific Aim 1.** Identify and quantify angiogenesis in the earliest stages of breast carcinoma, and correlate these data with clinical outcome.</u>

Task 1: Quantify angiogenesis in in situ and early proliferative breast lesions.

Work Accomplished: All of Task 1 is complete and published (31).

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 micron sections from each specimen were de-paraffinized 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, California) at 37^{o} for 30 minutes. Next, the slides were incubated with a biotinylated goat anti-rabbit secondary antibody, followed by horse radish peroxidase-avidin, and the colorimetric reaction visualized with diaminobenzidine/H_2O_2 using copper sulfate enhancement. Negative control pre-immune rabbit serum was incorporated into each run. All slides were counterstained with hematoxylin.

Evaluation of vascularity: Histopathologic diagnoses of pre-invasive disease were ascertained for each duct or lobule by the consensus criteria (32). 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) (33). Simple hyperplasia (less than a four cell thickness per individual ductal unit) and all forms of adenosis were excluded from examination. Ductal CIS (DCIS) was subcategorized as either micropapillary, cribriform, solid, or comedo, as per consensus criteria in reference 34. 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 eighteen ducts or cross-sections of lobular alveolae within a single diagnostic category on each slide were given a vascular score and the mean 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 SH or RY. When present, invasive breast cancer was graded according to the criteria of Bloom and Richardson (35).

Results:

Table 1 is a summary of the vascular scoring system.

Table 1. Vascular Scoring Criteria

Circumference Surrounded by Vessel	Angiogenic Grade
None	0
<1/3	1
≥1/3 ⇒ <2/3	2
≥2/3 ⇒ <3/3	3
complete encircling	4

Table 2 is the summary of vascularity for each histologic subtype from this publication.

Table 2. Vascular Score for Each Histologic Type

Histology	Mean	Standard Error	Range	Number of Cases
Normal	0.187	0.0183	0.027-0.769	83
Proliferative	0.836*	0.0446	0.136-1.670	70
Carcinoma in situ	1.525*	0.0892	0.220-3.110	63

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 (36). All of these data are consistent with the concept that angiogenesis is an early phenotypic marker of cells in the process of neoplastic transformation.

Task 2: Detect c-ets-1 expression in breast tissue.

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. We repeated the studies at very high stringency and found that smooth muscle cells of arterioles highly express this transcription factor. Furthermore, there was little to no expression in small vessel endothelium, even in tissue that was highly angiogenic. This past year there was a report by Ito et al (37) reporting c-ets-1 oligonucleotides for in situ hybridization. Based on these studies we synthesized the following oligos:

Antisense: GCCCAGCTTCATCACAGAGTCCTATCAGAC
Sense: GTCTGATAGGACTCTGTGATGAAGCTGGGC

Methods:

Transcription: RNA antisense and sense probes were transcribed from the linearized cDNA that had been cloned into Promega's pGEM 3Z vector. The probes were labeled with S^{35} using Promega's Riboprobe Sp6 - T7 System and alkaline degraded to approximately 100 to 200 bp as recommended by Cox *et al.* They were dissolved in hybridization solution (50% formamide; 0.3 M NaCl; 20 mM Tris, pH 8.0; 5 mM EDTA; 10 mM $NaPO_4$, pH 8.0; 10% dextran sulfate; 1 X Denhardt's; 0.5 mg/ml yeast tRNA) at 2 ng/ul/kb length of cloned fragment (or 1 x 10^5 cpm/ul.)

Hybridization: Rehydrate by putting slides through graded ethanols. Digest with proteinase K at 200 μg/ml for formalin-fixed paraffin embedded tissue. Incubate at 37° for 15 min in a humid chamber. Post-fix in 4% paraformaldehyde in PBS for 20 min. Acetylate with 0.25% acetic anhydride in triethanolamine. Dehydrate. Denature probe at 80° for 2 min. Cool on ice. Place probe on the slide. Incubate overnight at a temperature appropriate for G-C content of the probe (~50°) in a hymid chamber.

Washing: Remove slides from box immerse in 5 X SSC, 10 mM DTT at the hybridization temp for ~30 min for coverslips to fall off.

Wash slides for low stringency in:

50% formamide, 5 X SSC, 20 mM DTT	50°	60 min
0.5 M NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA	RT	10
0.5 M NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA	37°	10
0.5 M NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA	37°	10
20 μg/ml RNase in0.5 M NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA	37°	30
0.5 M NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA	37°	15
2 X SSC	RT	15
2 X SSC	RT	15
0.1 X SSC	RT	15
0.1 X SSC	RT	15

Dehydrate sections. Place X-ray film over slides in an exposure cassette. Expose overnight at room temp.

Autoradiography: In the darkroom, prepare: Kodak NTB-2 nuclear track emulsion. Dip each experimental slide. Dry. Transfer slides to slide boxes containing a sachet of desiccant . Seal with electrical tape and place at 4° in a light-tight box for the desired period of time. Develop. Counterstain the sections with nuclear fast red.

Conclusion: Using these probes we found essentially the same results as with the riboprobe analyses. Finally, 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 (38) 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.

Additional Work: In the past couple years we have continued to search for markers of angiogenic endothelium, particularly markers which could be used in archival tissue. The markers we have explored include the Vascular Endothelial Growth Factor (VEGF) receptor, KDR, and the integrins, $\alpha v\beta 3$ and $\alpha v\beta 5$. Angiogenic endothelium has increased expression of the VEGF receptor, KDR (39). 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. Thus far we have seen uniform hybridization of vessels around all histologic subtypes of carcinoma in situ and less consistently around atypical hyperplasia and proliferative breast disease. In most archival specimens, the level of KDR mRNA is low, necessitating isotopic labeling and emulsion exposures on the order of three months. Because of a rare, unexplained high background in vessels with the sense probe, we recently cloned the KDR sequence from bases 1531-2413 into pbluescript and are repeating a few of the analyses to confirm the specificity of these results. Despite the fact the these experiments are not quite finished, we believe that it is fair to report that KDR is upregulated in vessels surrounding all types of carcinoma in situ. This is consistent with the recent data of Brown et al. (40). Furthermore, KDR is expressed in vessels immediately adjacent to proliferative breast disease indicating that, at least in some cases, VEGF may have a role in increasing the vascularity of these types of lesions.

A second feature of angiogenic endothelium 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 (41). We contacted Dr. Cheresh to get his antibodies, as well as those that are commercially available. However, in our hands and in his, these antibodies do not work in formalin fixed, paraffin embedded tissue. In the frozen specimens of invasive breast cancers we have examined, the expression of these integrins in endothelium is clearly found in many but not all venules and capillaries. Therefore, these reagents do not seem particularly well suited to define processes in which the level of angiogenesis is low.

<u>**Specific Aim 2.** Localize to specific cellular subsets known angiogenic factors in *in situ* carcinoma and atypical hyperproliferative lesions of the breast</u>.

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.

Work Accomplished: VEGF and its receptors, KDR and flt, Transforming Growth Factor alpha (TGFα), and Epidermal Growth Factor Receptor (EGFR) were cloned into PGEM-3Z vector for riboprobe production.

Task 4: Examination of breast tissue for angiogenic growth factors.

Work Accomplished: 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), Platelet Derived Growth Factor (PDGF), and Platelet Derived Endothelial Cell Growth Factor (PD-ECGF), which is also known as thymidine phosphorylase (TP). A list of reagents is provided in Table 3.

Table 3. Reagents

Antibody Specificity	Manufacturer	Clone	Dilution	Pretreat.
TGFα	Oncogene Res.	213-4.4	1/25	Trypsin
EGFR	Zymed	31G7	1/15	Trypsin
VEGF	Biogenix	poly	1/10	MW*
IGF I	R & D Antibodies	poly	1/400	Trypsin
IGF II	R & D Antibodies	poly	1/400	Trypsin
bFGF	Oncogene Res.	poly	1/50	Trypsin
TGFβ	Santa Cruz	poly	1/30	MW
PDGF-B	Santa Cruz	poly	1/125	Trypsin
PD-ECGF	Lab Vision	P-GF.44C	1/200	MW
MMP2	Oncogene Res.	42-4D11	1/120	MW
MMP9	Oncogene Res.	56-2A4	1/20	MW

* = antigen retrieval by microwaving in citrate buffer.

Methods: 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. In all cases the intensity of staining was graded 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).

Results: Table 4 lists for each growth factor the mean and standard deviation of staining intensity for the epithelium and percent positive cases in each diagnostic category. **Table 5** is a similar set of data for the stroma. **Table 6** shows the significant data for leukocyte staining of these growth factors. * = a statistically significant increase relative to normal epithelium ($p < 0.05$).

Table 4. Epithelial Staining Intensity and Percent Positive Cases

		VEGF	bFGF	TGFβ	TGFα	EGFR	IGF1	IGF2	PDGF	PDECGF
Normal	Mean	2.203	2.788	1.28	2.52	0.56	0.85	1.89	1.05	0.78
	S.D.	1.033	0.803	0.53	1.01	0.92	0.87	1.17	0.86	1.02
	% pos.	96	100	97	97	33	59	83	74	49
Prolif.	Mean	2.326	2.915	**1.84***	2.43	**1.60***	1.13	2.15	1.37	1.25
	S.D.	0.715	0.747	0.87	0.83	1.17	1.00	1.22	0.97	0.96
	% pos.	100	89	100	98	**77***	69	85	83	**67***
Atypia	Mean	2.667	2.727	1.70	2.73	1.29	0.69	1.85	1.58	1.17
	S.D.	0.500	0.786	0.48	0.90	0.95	0.75	1.34	1.16	1.17
	% pos.	100	100	100	100	86	nd	nd	83	67
In Situ	Mean	**2.745***	2.582	**2.15***	2.17	0.79	**1.34***	**2.42***	1.24	**1.82***
	S.D.	0.793	1.031	0.86	1.10	1.10	1.11	1.42	1.09	1.22
	% pos.	100	93	100	89	44	72	78	69	**85***
Invasive	Mean	**3.192***	2.966	**2.73***	2.48	0.36	1.21	**2.61***	1.07	**1.48***
	S.D.	0.634	0.680	0.96	1.16	0.95	1.29	1.26	1.09	1.22
	% pos.	100	100	94	93	16	61	89	57	**75***

Table 5. Stromal Staining Intensity and Percent Positive Cases

		VEGF	bFGF	TGFα	EGFR	IGF1	IGF2	PDGF	PDECGF
Normal	Mean	0.554	0.774	0.31	0.56	0.46	0.48	0.18	0.01
	S.D.	0.665	0.869	0.47	0.82	0.69	0.67	0.56	0.11
	% pos.	96	55	32	40	36	39	14	1
Prolif.	Mean	0.51	1.085	0.35	0.43	0.44	0.58	0.13	0.02
	S.D.	0.631	0.996	0.55	0.74	0.65	0.85	0.34	0.14
	% pos.	43	66	32	29	35	40	13	2
Atypia	Mean	0.556	0.909	0.46	0.73	0.23	0.31	0.17	0
	S.D.	0.527	0.302	0.66	0.65	0.83	0.85	0.39	0
	% pos.	56	91	38	64			17	0
In Situ	Mean	0.872	1.200	0.51	0.36	0.55	0.64	0.07	0.04
	S.D.	0.875	0.951	0.65	0.62	0.75	0.91	0.26	0.19
	% pos.	60	**73***	44	30	42	45	9	4
Invasive	Mean	0.846	**1.448***	0.32	0.68	0.68	0.89	0.07	0.15
	S.D.	0.967	1.213	0.63	0.99	0.90	1.07	0.26	0.53
	% pos.	54	76	24	45	46	54	7	7

Table 6. Leukocyte Staining Intensity and Percent Positive Cases

		PD-ECGF	bFGF
Normal	Mean	0.94	1.19
	S.D.	0.1.37	1.46
	% pos.	35	42
Prolif.	Mean	1.60	1.13
	S.D.	1.56	1.48
	% pos.	56	40
Atypia	Mean	1.83	0.91
	S.D.	1.60	1.30
	% pos.	67	36
In Situ	Mean	**2.61***	1.509
	S.D.	1.50	1.502
	% pos.	**82***	55
Invasive	Mean	**3.22***	**2.276***
	S.D.	1.50	1.509
	% pos.	**89***	**72***

A brief summary of the highlights from these data are as follows:

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 immunoreactivity is present in normal breast epithelium and proliferative breast disease. Furthermore, VEGF immunoreactivity shows a statistically significant increase during progression at the level of carcinoma in situ and invasive disease. Stromal VEGF also tends to increase with progression, but due to large variations in intensity among cases, this increase is not statistically significant.

3. Epithelial bFGF is uniformly present throughout disease progression in nearly 100% of cases and does not show an increase with invasion. However, stromal and leukocyte bFGF immunoreactivity is statistically greater in invasive disease relative to normal epithelium. This increase is not due to increases in staining intensity but rather due to a statistically significant increase in the number of cases with either stromal or leukocyte staining.

4. TGFβ shows the earliest increase in epithelial staining, with 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.

5. Staining of epithelial or stromal TGFα did not change during progression, but the staining of leukocytes was significantly higher at the level of IS and invasion, relative to normal tissue. On the other hand, EGFR shows an early statistically significant increase in PBD, gradually decreasing in CIS and invasion. Stromal EGFR is fairly constant throughout progression.

6. IGF1 shows a marked increase in mean epithelial immunoreactivity during progression, both in quantity and number of positive cases, at the level of in situ disease. Generally, about 50-60% of cases are positive at all levels of progression. On the other hand, IGFII is positive in the epithelium in 80-90% of cases, with only a slight increase in staining intensity at the level of CIS or invasion. Others have indicated that IGFII replaces IGFI immunoreactivity in the stroma during progression (42). Our data show that IGFI staining is constant during

progression but that the intensity of IGFII staining in the stroma increases begining as early as PBD. Also, IGFII staining intensity increases beginning at the level of PBD within leukocytes, indicating that both stromal cells and infiltrating leukocytes may be important in regulating vascularity of these tissues.

7. PDGF, on the other hand, showed no statistically significant increase in either the level of staining or % positive cass with progression in the epithelium, endothelium, stroma, or leukocytes. The epithelium was generally positive in 70-80% of cases whereas the stroma was positive in only 10-15% of cases.

8. Because of the recent emphasis in the literature on the angiogenic activity of PD-ECGF/TP, we included this growth factor in this study within the last year. PD-ECGF/TP increases in the epithelium with progression being statistically greater in in situ and invasive disease relative to normal epithelium, not due to an increase in stain intensity, but due to an increase in the percent of positive cases. In addition, leukocyte staining also increases in in situ carcinoma and invasion reflecting both an increase in leukocyte number in these later forms of progression and a true increase in staining intensity within the leukocytes. These data suggest again that the inflammatory component of tumors may be important in regulating angiogenesis.

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 7** 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 ($p<0.05$).

Table 7. 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

Conclusion: From these data we conclude the following: 1. No one growth factor obviously controls angiogenesis during progression in the majority of cases. Correlation coefficients were calculated for each growth factor with progression versus vascular score for the tissue at each stage of progression. In no case was a clear correlation demonstrated. Furthermore, there was no correlation with the presence of subsequent invasion. 2. Angiogenic growth factors are plentiful even in normal epithelium. 3. At the level of PBD, epithelial TGFβ increases as does the stromal and leukocyte staining intensity for IGFII. Beginnning at this level, endothelial cells close to the epithelium began to exhibit changes in that increased TGFα staining was identified relative to vessels at a distance. 4. In situ disease shows increases in epithelial VEGF staining intensity, whereas IGFI and PD-ECGF/TP are more commonly found in the epithelium beginning at this level of progression (increased percent positive cases). PD-ECGF/TP leukocyte staining intensity and number of cases also increased beginning with CIS. 5. Finally, invasive disease is associated with the presence of intense staining for many of these angiogenic factors in a large proportion of cases. In particular, epithelial, stromal, and leukocyte staining for bFGF and VEGF are prominent. PD-ECGF/TP staining of the leukocytes in most cases of invasive disease is also impressive.

Task 5: Examination of breast tissue for expression of metalloproteinases and their inhibitors.

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.

Results: The results of these studies are shown in **Table 8**. 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 8** 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 and have not been analyzed.

Table 8. Matrix Metalloproteinase Immunoreactivity

		MMP2	MMP9
Normal	Mean	0.30	0.03
	S.D.	0.67	0.82
	% pos.	22	19
Prolif.	Mean	0.52	1.40
	S.D.	0.87	0.82
	% pos.	36	24
Atypia	Mean	0.75	0.50
	S.D.	1.04	0.76
	% pos.	50	38
In Situ	Mean	**1.09***	**0.66***
	S.D.	0.97	1.00
	% pos.	69	44
Invasive	Mean	**1.42***	**1.00***
	S.D.	0.64	0.85
	% pos.	92	73

* = $p<0.05$ relative to normal epithelium.

Specific Aim 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 that relies on the ability of endothelial cells to produce tubular structures in ECM gels (43). This model has allowed the identification of numerous cell-specific events that occur during angiogenesis. Nonetheless, it is a poor model for elucidating the events that 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).

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 was used to look at these questions. To utilize this model to its fullest potential, we proposed 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.

Task 6: Develop culture conditions in which explant cultures give reliable angiogenesis.

Work Accomplished: We have performed and analyzed 77 organ culture experiments. The form used to analyze these experiments is included. As indicated in reference 44, 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, 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 (44).

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 that has multiple cell types.

Methods: 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 (concentrations listed below each data table to follow) are added along with fresh media every other day or are included in a diffusion vector (gelfoam or Elvax beads). BrdU (100μM) and Fldu (10μM) are added 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.

Results: 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 9** 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. 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. Being an extremely complex material, we did not analyze the serum effect further, but continued to keep the level at 2% for our additional experiments. Less than 2% FCS does not permit extended epithelial or endothelial cell viability (data not shown).

Table 9. 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.

Task 7: Isolate VEGF from HL60 cells.

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.

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

Work Accomplished: This task was the main focus of our activity in years 3 and 4. As noted above, we switched serum concentrations and repeated some of the earlier experiments. 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 77 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, ECGF, PDGF, cell culture conditioned media containing VEGF, PMA, staurosporine, calphostin C, forskolin, IMX, tamoxifen citrate, and co-culture with isolated breast fibroblasts. 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.

Results: Summary data for experiments with growth factors are presented in **Tables 10-19.** Data from other agents are published (44). Shown are the mean and standard deviation of proliferation (BrdU labeling) for endothelial cells (endo), pericytes adjacent to microvessels (peri), pericytes which appear to be oriented perpendicular to the vessel, as if moving away (or toward) the vessel (tips), and stromal cell immediately adjacent but not touching the vessel (fibro). Concentrations of each agent are listed below each table.

VEGF (**Table 10**): 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.

Table 10. 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

VEGF = 50 μg/ml. 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.

Basic FGF (**Table 11**): 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.

Table 11. 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

BFGF = 10 ng/ml. * = statistically significant increase in fibro proliferation with bFGF ($p<0.001$). Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2 each, 10% and 2% serum.

TGFα (**Table 12**): These experiments were done in 2% serum only. TGFα had no effect on the proliferation of any cell type.

Table 12. 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

TGFα = 20 ng/ml. Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2

EGF (**Table 13**): 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.

Table 13. 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

EGF = 10 ng/ml. Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2.

PDGF (**Table 14**): These experiments were done in 2% serum only. PDGF had no effect on the proliferation of any cell type.

Table 14. Organ Cultures with PDGF.

		PDGF				Control			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
2% FCS	Mean	1	0.3	0.1	0	1.82	0.5	0.2	0.06
	S.D.	1.13	0.5	0.3	0	1.24	0.6	0.4	0.24

PDGF-B = 2.5 ng/ml. Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 3.

TGFβ (**Table 15**): We tested TGFβ at two different concentrations, as shown. As indicated in **Table 9**, serum is inhibitory to endothelial cell proliferation. This effect of serum is often attributed to TGFβ. Indeed at the higher concentration, TGFβ-treated cultures showed a significant loss of endothelial cell proliferation.

Table 15. Organ Cultures with TGFβ.

	$TGF\beta^a$				$TGF\beta^b$				Cont			
	Endo	Peri	Tips	Fibro	Endo	Peri	Tips	Fibro	Endo	Peri.	Tips	Fibro
Mean	1.76	1.0	0.5	0.06	**0.43***	0.9	0	0	1.82	0.5	0.2	0.06
S.D.	1.2	0.9	0.6	0.24	0.53	0.4	0	0	1.24	0.6	0.4	0.24

$TGF\beta^a$ = 50 pg/ml. $TGF\beta^b$ = 1 ng/ml. Cont = no added growth factor. * = statistically significant decrease in endothelial proliferation vs control (p=0.015). Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 4.

Protease (**Table 16**): Our data on human tissues indicates that metalloproteinases are present. However, due to the length of these experiments (one month) and the large amount of media required, it was not reasonable to use metalloproteinases from private sources in this assay. However, plasmin is known to activate metalloproteinases, as well as TGFβ. Since this was available commercially in sufficient quantities to perform the assay, we tested plasmin in this system. These data are summarized in **Table 16**. Plasmin had essentially no effect on vascular proliferation.

Table 16. Organ Cultures with Plasmin.

		Plasmin				Control			
		Endo.	Peri.	Tips	Fibro.	Endo.	Peri.	Tips	Fibro.
2% FCS	Mean	2.0	0.7	0.3	0.08	1.5	0.6	0.3	0
	S.D.	1.41	0.8	0.7	0.29	1.1	0.9	0.9	0

Plasmin = 1U/ml. Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 2. There was no statistical difference in any form of vascular proliferation with plasmin.

Directed growth factors (**Table 17**): 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 17**, the beads containing bFGF had the same cell proliferation as beads containing bovine serum albumin, a protein control. As a positive control, these beads were first tested for their ability to induce mitogenesis in isolated endothelial cells. They performed as well as purified bFGF (data not shown).

Table 17. 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.

While these organ culture 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 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 less endothelial cell proliferation in fibrous tissue, when other cell types proliferate there. Second, our analyses are based on the means of all experiments, whereas some wells of tissue are far more angiogenic than others. Third, proliferation is just one aspect of angiogenesis.

Fibroblast Co-culture (**Table 18**): In order to test the potential effect of normal breast fibroblasts on endothelial cell proliferation in this system, we performed the organ cultures in the presence of confluent, isolated normal breast fibroblasts. Replicate fibroblast cultures were plated on the bottom of each culture dish, such that during one week of co-culture, each set of cells would be confluent but not stressed. Organ cultures were placed in transwells above the monolayer. As shown in **Table 18**, at 10% serum normal breast fibroblasts had an inhibitory effect on endothelial cell proliferation. Note however that at 2% serum, this inhibitory effect is lost. Potentially the inhibitory effect at high serum could be due to activation of TGFβ by proteases produced by the fibroblasts. We have not formally tested this hypothesis.

Table 18. 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
2% FCS	Mean	2.06	0.9	0.7	0.06	1.78	1.1	0.6	0.11
	S.D.	1.06	0.9	0.9	0.24	1.22	1.2	0.9	0.32

Endo. = endothelium; Peri. = pericyte; Tips = pericytes pointed away from the vessels; Fibro. = stromal cells adjacent to vessels. N = 3 for 10% FCS and 4 for 2% FCS.

Angiogenic Inhibitor (**Table 19**): Finally, the archival tissue studies indicate that while numerous angiogenic factors are present in normal tissue, they are not sufficient to drive angiogenesis. Indeed in our organ culture experiments, we see a similar result in that we are not able to overcome inhibitory effects of the adjacent tissue with exogenous angiogenic factors. Since in most cases we tend to see maximal endothelial proliferation in 2% serum without exogenous growth factors, we decided to test this proliferation against the potent angiogenic inhibitor, TNP-470 (TAP Holdings, Inc.) (45). This compound is known to inhibit the proliferation of isolated endothelial cells. As shown in **Table 19**, this potent angiogenic inhibitor had no effect on endothelial proliferation in the organ culture system. Therefore, the regulation of endothelial proliferation in this model system may be very different from angiogenesis, in vivo.

Table 19. Organ Cultures with TNP-470.

		10 pg/ml	100 pg/ml	1 ng/ml	1 μg/ml	Control
2% FCS	Mean	1	1	1.5	2	1
	S.D.	1.2	1	0.71	1.41	0.71

Mean and standard deviation are for endothelial proliferation at each concentration of TNP-470. N = 2.

Organ Culture Conclusion: Using this organ culture system, we have been unable to demonstrate regulation of endothelial cell proliferation or sprouting using a variety of potent angiogenic agents. Furthermore, the angiogenic inhibitor, TNP-470, does not appear to have the expected inhibitory effect. Therefore, we believe that this organ culture system is not appropriate for testing novel angiogenic agents or inhibitors.

New Approach: Within the past year we have developed a more "traditional" angiogenic assay in the laboratory in which human umbilical vein endothelial cells (HUVEC) are cultured in three-dimensional collagen gels. Although in unrelated studies (testing PKC isozyme activity in endothelial shape changes) we used Matrigel as a model for in vitro vessel formation, we found that tubules form too efficiently in the absence of an exogenous stimulus for testing angiogenic factors from tissue. Therefore, we spent a large part of this year developing the collagen gel system in which we can obtain quantitative data by morphometry. This system responds well to angiogenic agents such as bFGF and VEGF, and we have begun to characterize the angiogenic effects of breast epithelium in this system.

Method: Assays are performed in 48 well plates. 30,000 HUVEC (Clonetics) are plated on 175 μl of gelled type I collagen (Bectin Dickinson) in defined media (EBM, Clonetics). Two hours later, the cells are overlayed with another 175 μl of collagen. As soon as the collagen is solid, media is placed on the sandwich. If the test reagent is conditioned media, it is mixed 1:1 with the HUVEC basal media. If tissue is tested, the minced tissue or isolated epithelial organoids are placed directly on the upper layer of collagen. Seven hours later 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma) is added to a final concentration of 1mg/ml.

Live cells take up this vital dye and form an insoluble formazan crystal, which stains the vessels blue. Tiff files are collected from each well nine hours after the addition of media. Each condition is run in triplicate wells. Mean vascular areas and lengths are calculated for each well. 50 ng/ml bFGF is run as a positive control in each assay. **Table 20** shows an example of how bFGF and VEGF function in this assay system.

Table 20. Three-dimensional Collagen Gel Assay

	Media Control	bFGF	VEGF
Mean	12.07	34.79	28.82
S.D.	1.22	2.64	3.94

Mean (mm) and standard deviation of tubule length using basal media, 50 ng/ml bFGF, and 10 ng/ml VEGF.

We know that conditioned media from tumors and isolated human normal breast organoids can be angiogenic in this assay. We are currently comparing the data from this in vitro assay with the vascularity determined in the fixed surgical specimens from the same patients. To date we have cultured about 50 specimens, both from mastectomies and reduction mammoplasties. **Table 21** shows the results of plating passage 2 tumor epithelium in defined media in a transwell over this sandwich assay.

Table 21. Three-dimensional Collagen Gel Assay with Tumor Epithelium

	Media Control	Tumor Epithelium
Mean	16.8	23.71
S.D.	0.99	3.86

Mean vascular area (mm^2) of HUVEC co-cultured with defined media versus tumor epithelium at passage 2.

Task 9: Examine tumor epithelium and tumor stroma for angiogenic factors.

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: As described above, the organ culture assay is insufficient to test the angiogenic characteristics of breast tissue. Therefore, we are continuing these studies using the three-dimensional collagen gel assay system, described above. Within the past year we have also worked out conditions for successful isolation and culture of normal and tumor breast fibroblasts and epithelium using a defined media (46). With this assay system and culture techniques in hand, over the next year (during our one year extension) we shall test the following hypotheses:

Hypothesis 1. Fibroblasts derived from normal breast tissue can inhibit the activity of angiogenic stimulators such as bFGF.

Hypothesis 2. Fibroblasts derived from tumors do not inhibit angiogenesis.

Hypothesis 3. Epithelium from either normal or tumor specimens is angiogenic when isolated from its stromal elements.

Conclusions:

The vascularity of breast tissue increases continuously with progression to invasive disease. The largest increase is at the transition from atypical hyperplasia to carcinoma in situ. However, even normal breast tissue is more vascular in women who have or will soon have invasive carcinoma, indicating that vascularity may be a marker of genetic or epigenetic changes within the tissue. This increase in angiogenesis is regulated by an array of angiogenic factors that are present in the normal breast, but show some increases with progression within the epithelium, stroma, and invading leukocytes. Since the increases, although statistically significant, are not great, it is likely that loss of inhibitors may also be important. What those inhibitors may be is not yet known. Normal breast tissue in vitro is extremely resistant to exogenous angiogenic agents. However, isolation of endothelial cells from the adjacent matrix and substitution of that matrix with collagen gels allows rapid stimulation of vessel formation, indicating that the matrix of breast tissue is generally inhibitory to vessel formation. We are currently using isolated fibroblasts and epithelial organoids from normal and malignant breast tissue to discover the cell types responsible for this inhibitory phenotype.

References:

1. ACS (American Cancer Society), Cancer Facts and Figures. Atlanta, GA, American Cancer Society, 1993.
2. Harris JR, Lippman Me, Veronsesi U, et al: Breast cacner (Part1). N Engl J Med 327:319-328, 1992.
3. Miller BA, Baines CJ, To T, et al: Canadian National Breast Screening Study: Breast cancer detection and death rates among women aged 50-59 years. Can Med Assoc J 147:1477-1488, 1992.
4. Miller BA, Ferer EF, Handey BF: Recent incidence trends for breast cancer in women and relevance of early detection: An update. CA Cancer J Clin 43:27-41, 1993.
5. Kelsey JL, Gammon MD: Epidemiology of breast cancer. Epidemiol Rev 12:228-240, 1993.
6. Marshall E: Search for a killer: focus shifts from fat to hormones in a special report on breast cancer. Science 259:618-621, 1993.
7. Fielding LP, Fenoglio-Preiser CM, Freedman L: Prediction of outcome: The future of prognostic markers. Cancer 70:2367-2377, 1992.
8. Folkman, J., Klagsbrun, M. Angiogenic Factors. Science. 235:442-447, 1987.
9. Folkman, J. What Is the Evidence that Tumors are Angiogenesis Dependent? J. Natl. Cancer Inst. 82:4-6, 1990.
10. Auerbach, R. Angiogenesis-inducing factors: A review. Lymphokines 4:69-88, 1981.
11. Sato R, Rifkin DB: Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. J Cell Biol 109:309-315, 1989.
12. Folkman, J., Haudenschild, C. Angiogenesis *in vitro.* Nature (Lond.) 288:551-556, 1980.
13. Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Sotrel A, Manseau EJ, Van De Water L, Senger DR. Distribution of vascular permeability facotr (Vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. J Exp Med 174:1275-1278, 1991.
14. Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NPH, Risau W, Ullrich A. High affinity VEGF binding and developmental expression suggest FLK-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72:835-846, 1993.
15. Senger DR, Perruzzi CA, Feder J, Dvorak HF. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res 4a6:5629-5632, 1986.

16. Shibuya M, Yamaguchi S, Yamane A, Ideda T, Tojo A, Matsushime H, Sato M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 5:519-524, 1990.
17. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB. Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 6:1677-1683, 1991.
18. De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989-991, 1992.
19. Senger DR, Connolly DT, Van De Water L, Feder J, Dvorak HF. Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. Cancer Res 50:1774-1778, 1990.
20. Charnock-Jones DS, Sharkey AM, Rajput-Williams J, Busch D, Schofield JP, Fountain SA, Boocock CA, Smith SK. Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. Biol Reprod 48:1120-1128, 1993.
21. McCormick BA, Zetter BR. Adhesive interactions in angiogenesis and metastasis. Pharmac Ther. 53:239-260, 1992.
22. Folkman, J. Tumor angiogenesis. Adv. Cancer Res. 43:175-203, 1985.
23. Bouck, N. Tumor Angiogenesis: The role of Oncogenes and Tumor Suppressor Genes. Cancer Cells 2:179-185, 1990.
24. Montesano R, Orci L. Tumor-Promoting Phorbol Esters Induce Angiogenesis In Vitro. Cell 42:469-477, 1985.
25. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 64:327-336, 1991.
26. Rifkin DB, Moscatelli D. Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol. 109:1-6, 1989.
27. Risau W. Angiogenic growth factors. Prog Frowth Factor Res 2:71-79, 1990.
28. Grotendorst GR, Seppa HEJ, Kleinman HK, Martin GR. Attachment of smooth muscle cells to collagen and their migration toward platelet-derived growth factor. Proc Natl Acad Sci USA 78:3669-3672, 1981.
29. Sporn M, Roberts AB, Wakefield LM, Assoian RK. Transforming Growth Factor-β: Biological function and chemical structure. Science 233:532-534, 1986.
30. Weidner, N., Semple, J.P., Welch, W.R., Folkman, J. (1991) Tumor Angiogenesis and Metastasis - Correlation in Invasive Breast Carcinoma. New Eng. J. Med. 324: 1-8.
31. Heffelfinger SC, Yassin R, Miller MA, Lower E. Vascularity of proliferative breast disease and carcinoma in situ correlates with histologic features. Clinical Cancer Res, 2:1873-1878, 1996.
32. Hutter RVP. Consensus meeting: is "fibrocystic disease" of the breast precancerous? Arch Pathol Lab Med 110:171-173, 1985.
33. Jensen RA, DuPont WD, Page DL. Diagnostic criteria and cancer risk of proliferative breast lesion. J Cell Biochem. 17G59-64, 1993.
34. Page D, Anderson T: Diagnostic histopathology of the breast. Churchill Livingstone, UK, 1987, p157.
35. Bloom HJG, Richardson WW. Histologic grading and prognosis in breast cancer. Br J Cancer 11:359-377, 1957.
36. Jensen HM, Chen I, DeVault MR, Lewis AE. Angiogenesis induced by "normal" human breast tissue: a probable marker for precancer. Science 218:293-294, 1982.
37. Ito T, Nakayama T, Ito M, Naito S, Kanematsu T, Sekine I. Expression of the ets-1 proto-oncogene in human pancreatic cancinoma. Modern Pathol. 11:209-215, 1998.
38. Nakayama T, Ito M, Ohtsuru A, Naito S, Nakashima M, Fagin JA, Yamashita S, Sekine I. Expression of Ets-1 proto-oncogene in human gastric cancer: correlation with tumor invasion. Am J Pathol. 149: 1931-1939, 1996.
39. Senger DR, Van De Water L, Brown LF, Nagy JA, Yeo KT, Yeo TK, Berse B, Jackman RW, Dvorak AM, Dvorak HF. Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Metastasis Rev. 12: 303-324, 1993.
40. Brown LF, Berse B, Jackman RW, Tognazzi K, Giudi AJ, Dvorak HF, Senger DR, Connolly JL, Schnitt SJ. Expression of vascular permeabiltiy factor (Vascular Endothelial Growth Factor) and its receptors in breast cancer. Human Pathol. 26:86-91, 1995.

41. Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA. Definition of two angiogenic pathways by distinct alpha v integrins. Science 270:1500-1502, 1995.
42. Giani C, Cullen KJ, Campani D, Rasmussen A. IGF-II mRNA and protein are expressed in the stroma of invasive breast cancers: an in situ hybridization and immunohistochemistry study. Breast Cancer Res Treat. 41:43-50, 1996.
43. Heffelfinger SC, Hawkins HH, Barrish J, Taylor L, Darlington GJ. SK Hep-1: A Human Cell Line of Endothelial Origin. In Vitro Cell Devel Biol 28A:136-142, 1992.
44. Heffelfinger SC, Miller MA, Gear RB, DeVoe G. Staurosporine-induced versus spontaneous squamous metaplasia in pre- and post-menopausal breast tissue. J Cell Physiol 176: 245-254, 1998.
45. Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. Nature (Lond.) 348:555-557, 1990.
46. Petersen OW, Ronnov-Jessen L, Bissell MJ. The microenvironment of the breast: three-dimensional models to study the roles of the stroma and the extracellular matrix in function and dysfunction. The Breast J. 1: 22-35, 1995.

APPENDIX 1

Personnel receiving pay during the four-year period:
Sue C. Heffelfinger, PI
Robin Gear, technician
Tom Miller, graduate student
Valerie Rogers, graduate student

APPENDIX 2

Manuscripts funded by this proposal:
Heffelfinger SC, Yassin R, Miller MA, Lower E. Vascularity of proliferative breast disease and carcinoma in situ correlates with histologic features. Clinical Cancer Res, 2:1873-1878, 1996.
Heffelfinger SC, Miller MA, Gear RB, DeVoe G. Staurosporine-induced versus spontaneous squamous metaplasia in pre- and post-menopausal breast tissue. J Cell Physiol 176: 245-254, 1998.
Several additional manuscripts are currently in review or in preparation.

APPENDIX 3

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
HUVECS	human umbilical vein endothelial cells
IGF I and II	Insulin-like growth factors
Il-1	Interleukin 1
Il-6	Interleukin 6
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
PD-ECGF	Platelet-derived endothelial cell growth factor
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
TP	Thymidine phosphorylase
TPA	Tissue plasminogen activator
UC	University of Cincinnati
UPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor

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

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

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

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

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

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be changed to "Approved for public release; distribution unlimited"

Grant Number	Accession Document Number
DAMD17-94-J-4147	ADB221256
DAMD17-93-C-3098	ADB231640
DAMD17-94-J-4203	ADB221482
DAMD17-94-J-4245	ADB219584
DAMD17-94-J-4245	ADB233368
DAMD17-94-J-4191	ADB259074
DAMD17-94-J-4191	ADB248915
DAMD17-94-J-4191	ADB235877
DAMD17-94-J-4191	ADB222463
DAMD17-94-J-4271	ADB219183
DAMD17-94-J-4271	ADB233330
DAMD17-94-J-4271	ADB246547
DAMD17-94-J-4271	ADB258564
DAMD17-94-J-4251	ADB225344
DAMD17-94-J-4251	ADB234439
DAMD17-94-J-4251	ADB248851
DAMD17-94-J-4251	ADB259028
DAMD17-94-J-4499	ADB221883
DAMD17-94-J-4499	ADB233109
DAMD17-94-J-4499	ADB247447
DAMD17-94-J-4499	ADB258779
DAMD17-94-J-4437	ADB258772
DAMD17-94-J-4437	ADB249591
DAMD17-94-J-4437	ADB233377
DAMD17-94-J-4437	ADB221789
DAMD17-96-1-6092	ADB231798
DAMD17-96-1-6092	ADB239339
DAMD17-96-1-6092	ADB253632
DAMD17-96-1-6092	ADB261420
DAMD17-95-C-5078	ADB232058
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
DAMD17-94-J-4433	ADB236087
DAMD17-94-J-4433	ADB254499
DAMD17-94-J-4413	ADB232293
DAMD17-94-J-4413	ADB240900

Encl