

AD _____

AWARD NUMBER DAMD17-94-J-4173

TITLE: Stromal Components of Breast Cancer Progression

PRINCIPAL INVESTIGATOR: Sandra W. McLeskey, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20007

REPORT DATE: July 1998

19981013 039

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE July 1998	3. REPORT TYPE AND DATES COVERED Final (15 Jun 94 - 14 Jun 98)
4. TITLE AND SUBTITLE Stromal Components of Breast Cancer Progression		5. FUNDING NUMBERS DAMD17-94-J-4173
6. AUTHOR(S) Sandra W. McLeskey, Ph.D.		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Medical Center Washington, DC 20007		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES		
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE
13. ABSTRACT <i>(Maximum 200 words)</i> Angiogenesis has been shown to be a sensitive prognostic indicator for many solid tumors, including breast cancer. Since knowing the function of uniquely expressed genes could determine what is different about tumor-associated blood vessels, enabling cancer therapy to be targeted to them while sparing normal blood vessels, we proposed to study the biological process of angiogenesis in xenografts of human breast cancer into nude mice. Using tumors produced in ovariectomized mice by untransfected MCF-7 breast carcinoma cells or the same cells transfected with one of two fibroblast growth factors or vascular endothelial cell growth factor, and conditions of no treatment, estrogen or tamoxifen treatment, we have shown that edge-associated and intratumoral blood vessels in the xenograft tumors are positively correlated with favorable growth conditions. Moreover, we can distinguish rapidly growing tumors based on the tumor cells' incorporation of bromodeoxyuridine. Thus, we can study gene expression in pertinent blood vessels in rapidly growing tumors. Tumor-associated endothelial cells have been isolated from growing tumors produced in mice by these cells or from mammary fat pads, RNA extracted and used for a differential cloning technique, amplified fragment-length polymorphism. We are in the process of searching for differentially expressed genes in these RNA populations.		
14. SUBJECT TERMS Breast Cancer, Angiogenesis		15. NUMBER OF PAGES 32
		16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
20. LIMITATION OF ABSTRACT Unlimited		

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.

SUNM 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.

SUNM 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.

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

SUNM 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.

Jandra W. Meeker 7/8/98
PI - Signature Date

TABLE OF CONTENTS

1.	FRONT COVER-----	1
2.	REPORT DOCUMENTATION PAGE-----	2
3.	FOREWORD-----	3
4.	TABLE OF CONTENTS-----	4
5.	INTRODUCTION-----	5
6.	BODY-----	6
7.	CONCLUSION-----	24
8.	REFERENCES-----	26
9.	APPENDIX-----	30
	List of abbreviations-----	31
	Meeting abstracts since last report-----	32
	Publications since last report-----	32
	Manuscripts in preparation-----	32
	Personnel receiving pay from this negotiated effort-----	32

McLeskey, Sandra W.
DAMD17-94-J4173

INTRODUCTION

The overall purpose of this project is to study gene expression in endothelial cells participating in tumor-induced angiogenesis, with the aim of identifying characteristics of these cells which could serve as targets for cancer therapy. The model system used is an *in vivo* model of breast cancer which consists of wild type MCF-7 breast carcinoma cells or the same cells stably transfected with angiogenic growth factors growing as tumors in nude mice. The study includes parental MCF-7 cells transfected with *lacZ* (ML-20 and MPCX clonal cell lines), or the ML-20 cell line transfected with fibroblast growth factor 4 (FGF-4), FGF-1 (a.k.a. acidic FGF) or vascular endothelial cell growth factor (VEGF). Thus, the use of transfected cells with a common genetic background whose main difference is the transfected angiogenic growth factor enables us to look for commonalities and differences in the endothelial cells supplying the tumors produced by these cells.

Morphological and topographical studies have been conducted to determine the identity of particular blood vessels in tumors produced by the parental or transfectant cell lines which are associated with productive tumor growth, *vs.* blood vessels coincidentally associated with the tumor or blood vessels generated by the inflammatory response set up by tumor cell injection. These studies have been presented at national meetings and are the subject of a paper which is in review (1). RNA from endothelial cells isolated from growing tumors by fluorescence-activated cell sorting (FACS) has been purified from tumors produced by the parental, FGF, or VEGF transfected cells as well as more differentiated endothelial cells from mammary fat pads. This RNA has been reverse-transcribed into cDNA, which is being used for study of particular transcripts, such as receptors for FGFs or VEGF, or particular proteases associated with angiogenesis. cDNA libraries are being prepared from this RNA which will be used for a study of differential gene expression, looking for genes expressed in tumor-induced endothelial cells when compared with mammary fat pad endothelial cells.

BODY

Aim 1. As mentioned in previous reports, Aim 1 of the project, to demonstrate a positive correlation between microvessel density and tumor size, was achieved in the first year. It should be noted that the original proposal specified that endothelial cells in tumor sections would be highlighted with immunohistochemistry for von Willebrand factor, an antigen expressed by many endothelial cells. However, this antigen is not expressed as strongly on endothelial cells within the xenograft tumors as it is in the peripheral stroma. When antibodies to murine platelet-endothelial cell adhesion molecule 1 (PECAM-1) became available, we substituted this antigen for von Willebrand factor as a way of highlighting tumor associated blood vessels since PECAM-1 is more universally expressed by endothelial cells. This method, the details of which are given below, has also been used for Aims 2 and 3. This change was reported in the annual report for year 1.

Aim 2. In Aim 2, we sought to define the temporal and spatial relationships between blood vessels in tumors produced by transfected cells vs those produced by the parental cells. These studies originally only included FGF-4 transfected and parental cells. However, because FGF-4 is not expressed in human cancer, we have expanded our studies to include FGF-1 and VEGF transfected cells. These two angiogenic factors are thought to be important in many human cancers, including breast. Whereas parental MCF-7 cells require estrogen supplementation for tumorigenicity in ovariectomized nude mice, the FGF transfectants are capable of estrogen-independent growth. Additionally, in contrast to parental MCF-7 cells, the FGF transfectants are capable of growth in antiestrogen-treated mice and are reliably metastatic to lymph nodes and lungs of tumor-bearing mice (2-4). The VEGF transfected MCF-7 cells do not form tumors in ovariectomized mice, but when injected with matrix material (Matrigel, Collaborative Research, Products, Bedford, MA), form large, well-vascularized, metastatic tumors in both estrogen and tamoxifen-treated mice (5). Since all the transfectants are derived from common parents, genetic variation other than expression of the transfected angiogenic growth factor gene is minimized.

Cell lines. ML-20 cells, which have been previously described (2,3), are a clonal line of wild-type MCF-7 cells transfected with an expression vector containing the cDNA for bacterial *lacZ*. We find their *in vivo* behavior to be indistinguishable from wild-type MCF-7 cells. MKL-F cells are ML-20 cells transfected with an expression vector encoding FGF-4. This clonal cell line forms large tumors in both ovariectomized and tamoxifen-treated mice but larger tumors arise in estrogen-treated mice [(6,7) and Table 1]. MKL-4 cells are a clonal line of wild-type MCF-7 cells first transfected with an expression vector encoding FGF-4 and subsequently transfected with an expression vector encoding bacterial *lacZ*. This clonal cell line also forms tumors in ovariectomized mice under all hormonal conditions (no treatment, estrogen, or tamoxifen) but the largest tumors arise in tamoxifen-treated mice [(2,3) and Table 1]. Hence, the MKL-4 cells and MKL-F cells are both clonal cell lines derived from wild-type MCF-7 cells which contain the same two expression vectors but which are oppositely affected *in vivo* by estrogen or tamoxifen treatment (Table 1). Clone 18 cells are a clonal

line of ML-20 cells transfected with an expression vector encoding FGF-1. Clone 18 cells also form large tumors under all three treatment conditions, but the largest tumors arise in estrogen-treated mice [(4) and Table 1]. MV165-14 cells are ML-20 cells transfected with an expression vector encoding the 165 kDa form of VEGF. These cells do not form tumors in ovariectomized animals but are tumorigenic and metastatic in estrogen-treated animals or in tamoxifen-treated animals when injected with Matrigel [(5) and Table 1]. MPCX cells are ML-20 cells transfected with the expression vector for VEGF lacking the VEGF sequences. Thus, this cell line is the control transfectant line for MV165-14 (5). In our studies of microvessel density in tumors and tumor nodules, we found its behavior to be quite similar to its parent line, ML-20, and results for these two lines are pooled for these studies. The *in vivo* growth characteristics of these cell lines are summarized in Table 1.

Tumor production, harvest, and processing. Ten million cells/0.2 ml were injected into the mammary fat pad of ovariectomized nude mice as previously described (2-4). For the VEGF-transfected MV165-14 cells and their control line, MPCX, cells were mixed 1:1 with matrix material (Matrigel, Collaborative Biomedical Products, Bedford, MA) before injection. Some mice were treated with 60-day sustained release pellets containing 17β -estradiol (0.72 mg.) or tamoxifen (5 mg) (Innovative Research, Sarasota, FL). Eight hours before sacrifice and tumor harvest, 1mg bromodeoxyuridine (BrdU) was injected intraperitoneally. At indicated time points, tumors were harvested with adjacent stroma by cutting through the skin and peritoneum in a circular incision circumscribing the tumor. The tumor was fixed for 48 hours in 10% buffered formalin and subjected to X-gal staining as previously described (2-4) to delineate borders of the tumor. Stained tumors were trimmed of extraneous skin and muscle, cut in half to reveal a section from skin to muscle, fixed in 10% buffered formalin for 48 hours, followed by dehydration, paraffin embedding, and sectioning.

Immunohistochemistry for PECAM-1. Immunohistochemistry for murine PECAM-1 was accomplished using the rat monoclonal antibody Mec 13.3 (8) which was initially generously provided by Elisabetta Dejana, Rolande Berthier and Annunciata Vecchi. Subsequently, this antibody became available from Pharmingen (01951D). Following deparaffination in xylenes and rehydration in graded alcohols, slides were digested in 0.1% trypsin in phosphate-buffered saline (PBS) for 30 minutes at 37° and washed in running distilled water for 10 minutes. Endogenous peroxidases were quenched in 0.3% H₂O₂ in methanol for 30 minutes. Blocking solution (2% normal rabbit serum, 5% bovine serum albumin (Sigma #A7888) in PBS) was applied for 1 hour. Primary rat anti-murine PECAM-1 antibody was applied for 2 hours at room temperature in blocking solution at a concentration of 5 µg/ml. After PBS washes, secondary rabbit anti rat antibody coupled to biotin (Vector #BA4001) was applied for 1 hour. Slides were washed three times in PBS and ABC reagent coupled to peroxidase (Vector Elite kit #PK6001) was applied for one hour. Following three PBS washes, slides were incubated with 0.05% diaminobenzidine and 0.01% H₂O₂ for 15 minutes. Slides were then briefly counterstained with Harris acidified hematoxylin, washed,

dehydrated in graded alcohols and coverslipped from xylenes.

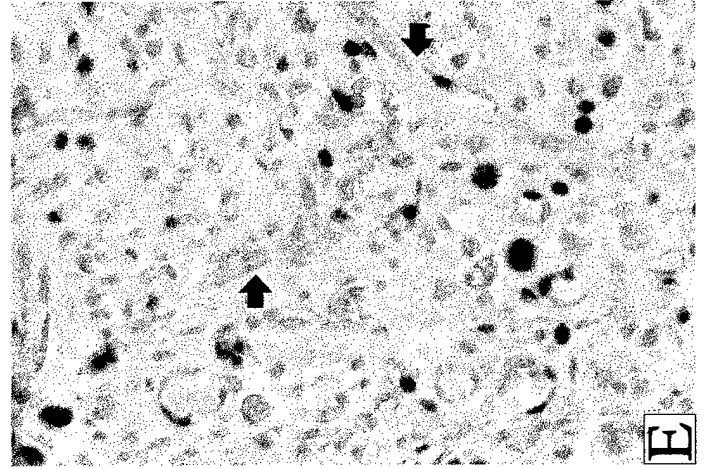
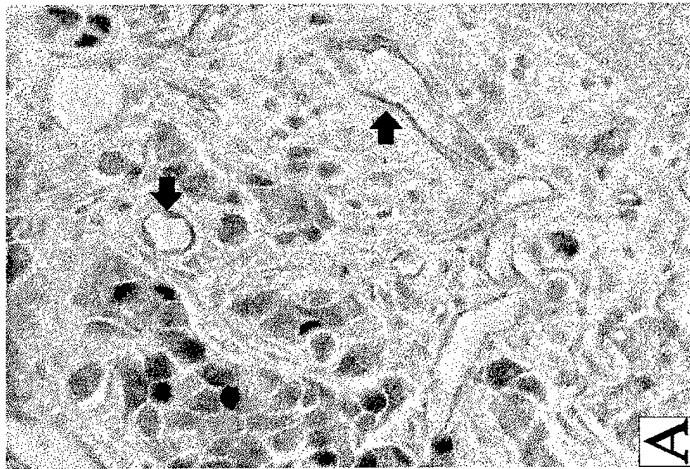
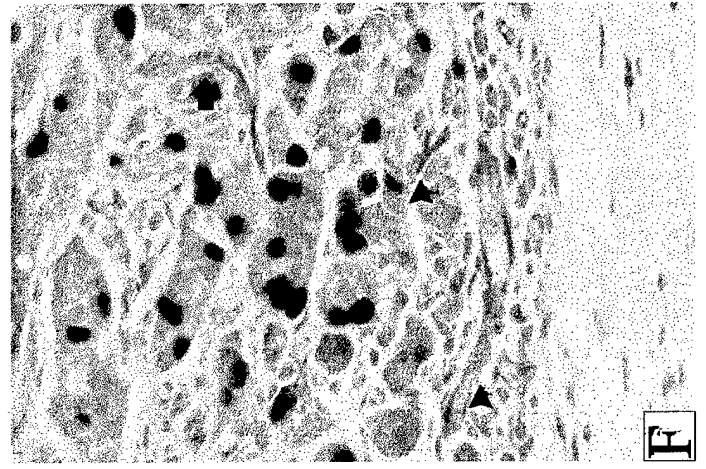
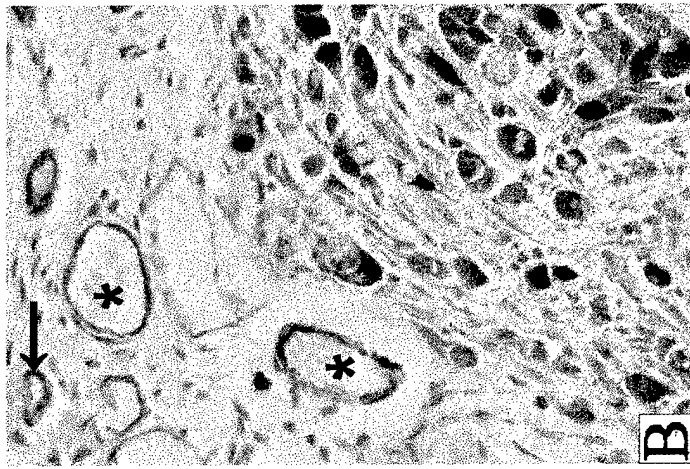
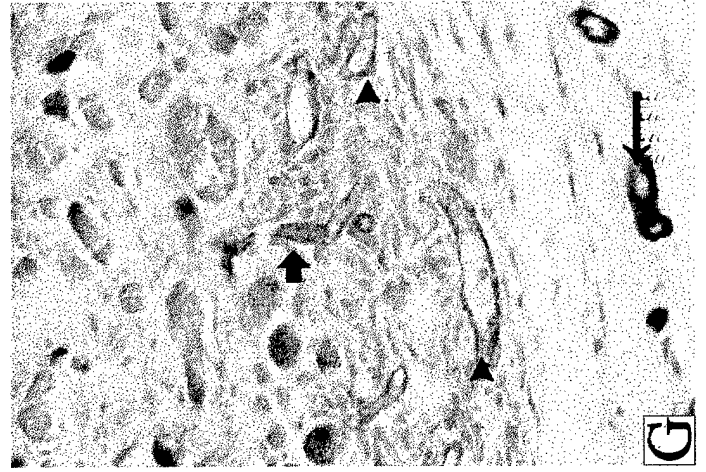
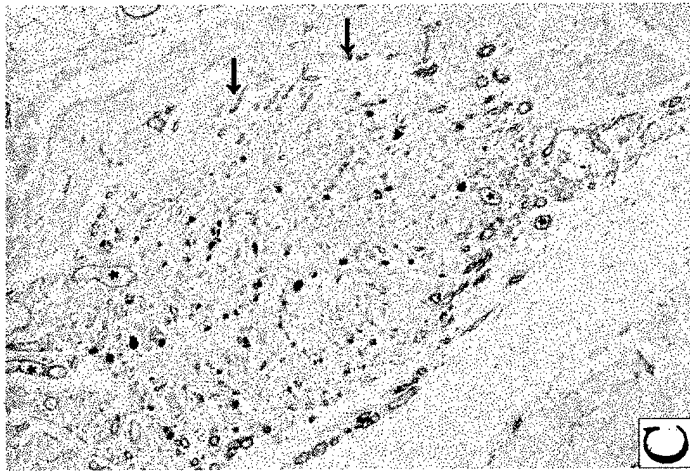
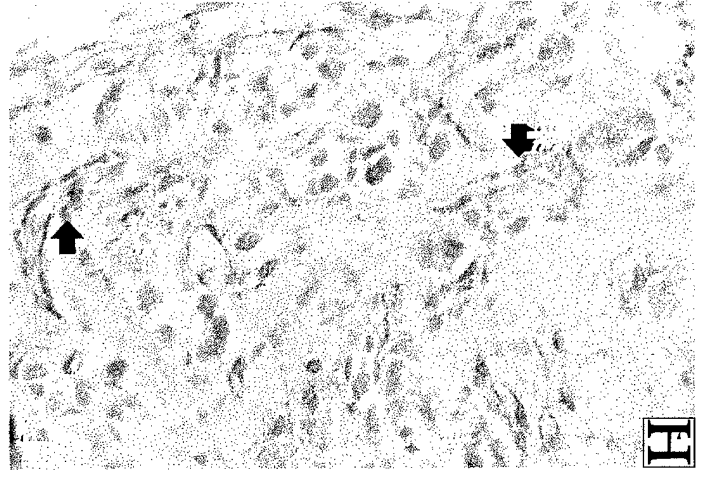
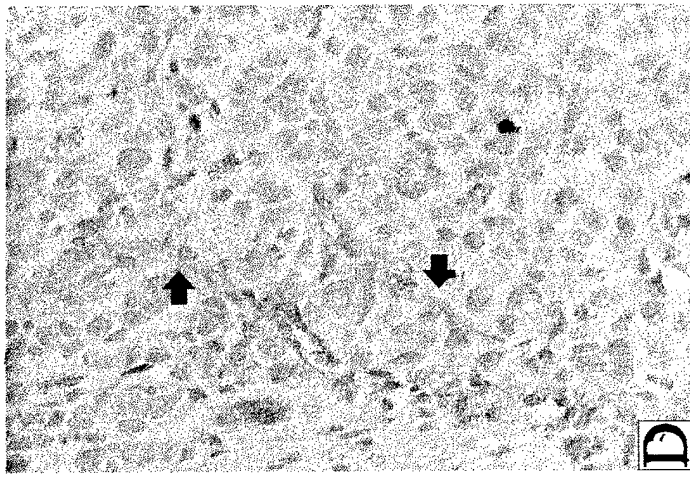
Estrogen-independent growth of transfected MCF-7 cells is characterized by increased numbers of tumor edge associated microvessels. We began these studies with the hypothesis that the phenotypic progression observed upon transfection of angiogenic factors into MCF-7 breast carcinoma cells to an estrogen independent or tamoxifen resistant phenotype could be explained, at least in part, by the angiogenic activity of the transfected

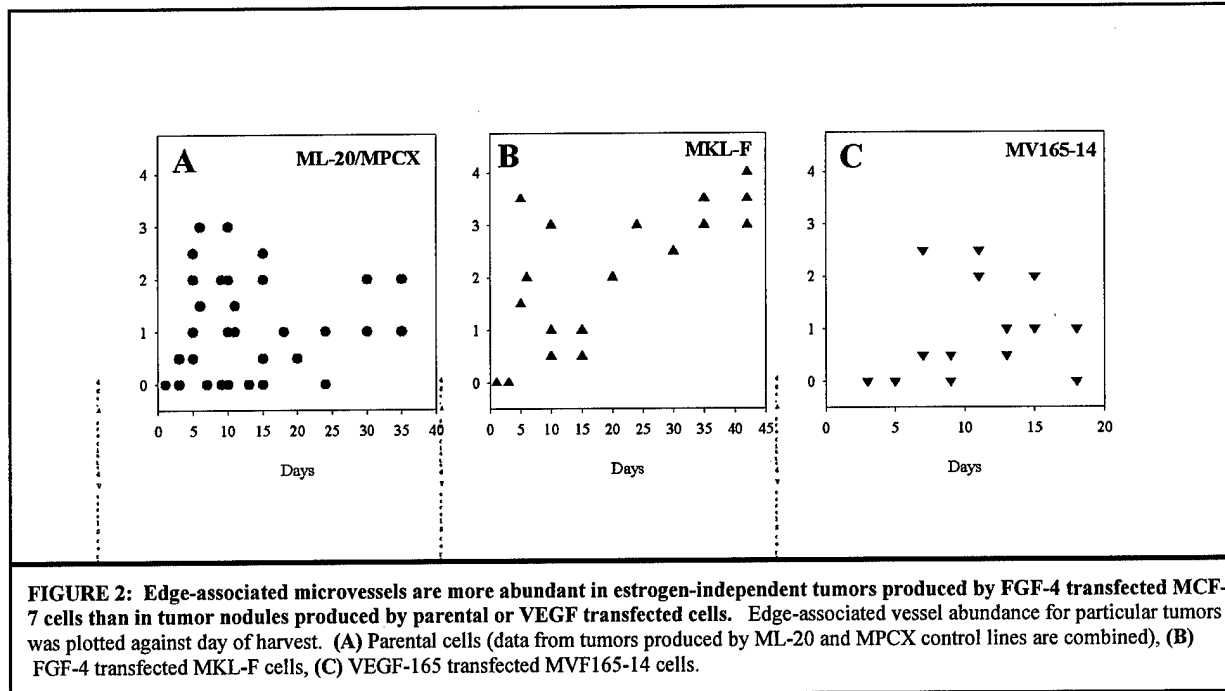
factors. To summarize this phenotypic progression, *in vivo* growth characteristics of the cell lines utilized in this study are given in Table 1. Because differences in tumorigenicity between the FGF transfectants and the parental and VEGF-transfected cells are most marked under conditions of estrogen-independent growth, we first examined characteristics of tumors in a time course from 1 day to 5-6 weeks after tumor cell injection. Under these conditions, the parental or VEGF-transfected cells form small nodules (~15 mm³ or less) which regress over about 2-3 weeks, while the FGF-transfected cells form progressively growing tumors. For these initial studies, we utilized the parental line, ML-20; the FGF-4 transfected cell line, MKL-F; the VEGF-transfected line, MV165-14; and its control line, MPCX. We were able to harvest extremely small tumor nodules produced by the parental/control cells or VEGF-transfected cells at late time points because they express bacterial *LacZ*, enabling them to be identified in the mammary fat pad by X-gal staining.

Table 1. *In vivo* growth characteristics of the clonal cell lines used in this study. The table summarizes results of experiments done previously with the indicated cell lines in which tumors were harvested after several months of tumor growth (2, 3, 4, 5, 6). ML-20 and MPCX, *lacZ* transfected MCF-7 cell lines, have growth characteristics typical of wild-type MCF-7 cells, *i.e.* they are estrogen-dependent for tumor growth. The transfected angiogenic factors are indicated in parentheses following the names of the clonal cell lines. Symbols are interpreted as follows: -, no tumors are produced; ±, static tumor nodules are produced; + to +++++, actively growing tumors are produced with relative sizes indicated by the number of pluses. Approximate tumor sizes are as follows: +, <30 mm³; ++, 30-150 mm³; +++, 150-350 mm³; +++++, 350-750 mm³; +++++, >750 mm³ after 2 months of tumor growth.

Cell Line(transfected factor)	Ovariectomized	Estrogen	Tamoxifen
ML-20 (a <i>LacZ</i> transfected clonal line of MCF-7)	-	+	±
MPCX (control transfection for MV165-14)	-	+++	±
MKL-4 (FGF-4)	+++	++	+++++
MKL-F (FGF-4)	+++	++++	+++
Clone 18 (FGF-1)	+++	++++	+++
MV165-14 (VEGF)	-	+++	++

(Following page) **FIGURE 1: Patterns of neovascularization after injection of parental MCF-7 and FGF-transfected MCF-7 cells into the mammary fat pads of ovariectomized nude mice.** Tumors or tumor nodules produced by injection of parental MCF-7 (A, B, and C) or FGF-4 transfected (MKL-F) (E, F, and G) cells were harvested at days 6 (A and E), 15 (B and F), and 35 (C and G), stained with X-gal (blue) to reveal tumor margins, embedded in paraffin, sectioned, and subjected to double immunohistochemistry for BrdU (red) and murine PECAM-1 (brown or reddish brown). (Since the tumors were stained with X-gal when whole, only surface tumor cells stain blue.) D and H depict VEGF-transfected cell tumors (MV165-14) harvested 18 days after tumor cell injection. Arrowheads point to edge-associated microvessels, thick arrows to intratumoral microvessels, asterisks denote ectatic stromal vessels and thin arrows indicate normal stromal vessels. Magnifications: A,B,D-H, 400X; C, 200 X.





At 1-10 days after tumor cell injection, neovessels were present in stroma surrounding and infiltrating the tumor cells in tumor nodules produced by all cell lines (examples of day 6 in Figure 1, A and 1E). At later time points, however, in tumors produced by the FGF transfectants, small microvessels were closely associated with the tumor periphery and extended into the tumor (examples of day 15 and 35 in Figure 1, F and G) while in tumor nodules produced by parental cell injection, blood vessels remained at the periphery of the nodule, in some cases separated from the edge of the nodule by an avascular space (examples of day 15 and 35 in Figure 1, B and C). Moreover, some of the vessels in parental cell nodules gradually assumed a dilated, ectatic appearance with irregular contours (Figure 1, B and C). At very late time points after tumor cell injection, tumors produced by the FGF transfectants continued to exhibit intratumoral microvessels as well as large numbers of tumor edge-associated microvessels (Figure 1G). Vessels in parental cell nodules at these time points were almost exclusively peripheral to the tumor (Figure 1C).

Because it seemed that regressing tumor nodules produced by the parental cells were not lacking in blood vessels, but instead exhibited blood vessels with different characteristics when compared to those of the progressively growing tumors produced by the FGF transfectants, we further characterized these sections with regard to microvessel topography and morphology in tumor sections produced by the parental and transfected cells. Sections were rated by a blinded observer for abundance of four distinct morphologies of microvessels noted in the tumor sections on a rating system of 0-4, with 4 being the highest and 0 denoting absence of the parameter. These parameters

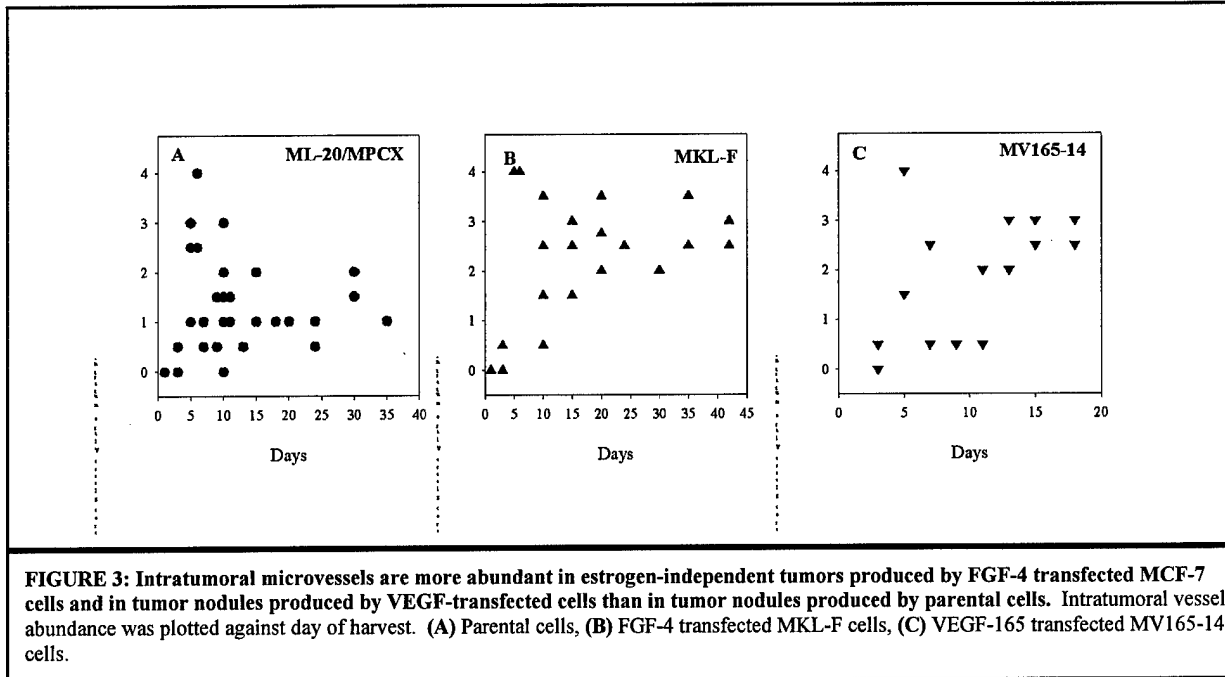


FIGURE 3: Intratumoral microvessels are more abundant in estrogen-independent tumors produced by FGF-4 transfected MCF-7 cells and in tumor nodules produced by VEGF-transfected cells than in tumor nodules produced by parental cells. Intratumoral vessel abundance was plotted against day of harvest. (A) Parental cells, (B) FGF-4 transfected MKL-F cells, (C) VEGF-165 transfected MV165-14 cells.

were (1) tumor edge-associated microvessels (Figure 1, F and G), (2) intratumoral microvessels (Figure 1, A, E, F, and G), (3) ectatic (dilated) stromal microvessels (Figure 1, B and C), and (4) normal stromal microvessels (Figure 1, B, C, and G).

When tumors or tumor nodules produced in ovariectomized mice by parental/control or transfected cells were compared over the entire time course with respect to abundance of these four vessel types, it was found that edge-associated microvessels were more abundant in the progressively growing tumors produced by the FGF transfected cells (Figure 2B and not shown) than in tumor nodules produced by the parental or VEGF-transfected cells (Figure 2, A and C). Statistical analysis of scores for edge-associated microvessels at time points 5 days or later after tumor cell injection revealed that scores for tumors produced by the tumorigenic FGF-4 transfected MKL-F cell line were significantly higher than those for tumor nodules produced by the ML-20 and MPCX control lines ($p < 0.05$) while edge-associated microvessel scores for tumor nodules produced by the nontumorigenic VEGF-transfected cell line, MV164-14 were not different from control. Thus, edge-associated microvessels were significantly more abundant in tumors produced by tumorigenic MKL-F cells in ovariectomized nude mice than in tumor nodules produced by cell lines which are nontumorigenic under these conditions.

Intratumoral microvessels also appeared more abundant in tumors produced by the FGF transfected cells when compared with the parental cells (Figure 3) in ovariectomized mice.

Statistical analysis of intratumoral vessels scores at time points 5 days or later after tumor cell injection again revealed that tumors produced by the FGF-4 transfected cell line (MKL-F) had significantly higher intratumoral vessel scores than the parental/control lines (ML-20 and MPCX) ($p < 0.05$). The intratumoral vessel scores of tumor nodules produced by the VEGF-transfected line, MV165-14, appeared higher than control but this apparent difference did not achieve statistical significance. Since the VEGF transfectants do not form tumors under these conditions, significant intratumoral blood vessel abundance was correlated with estrogen-independent growth.

No significant differences were observed between tumors produced by FGF or VEGF transfected cells and parental cells in densities of ectatic or normal stromal microvessels (data not shown). Taken together, these data suggest that edge-associated and intratumoral microvessels may be important in promoting estrogen-independent tumor growth in ovariectomized nude mice.

Hormonal treatments which affect tumor growth also produce different topography and morphology of tumor-induced blood vessels. We extended our study to include tumors produced from injection of FGF- or VEGF-transfected or parental/control cells into ovariectomized mice treated with sustained-release pellets of 17- β estradiol or tamoxifen. To differentiate effects which were hormonally produced *versus* those produced by tumor growth, we additionally utilized a clonal line of FGF-4 transfected MCF-7 cells (MKL-4) which is growth-inhibited by estrogen treatment of tumor-bearing mice and growth-stimulated by tamoxifen treatment of similar mice [(2) and Table 1].

Findings in this study confirmed correlation of increased edge-associated microvessels with hormonal conditions which promote tumorigenicity or increased tumor size. Tumors produced by FGF-transfected cells grown under conditions of estrogen treatment exhibited increased abundance of edge-associated microvessels when compared with parental cell tumors (Table 2). This effect was statistically significant for two of the three FGF-transfected cell lines ($p < 0.05$). These two lines (MKL-F and Clone 18) produce larger tumors under conditions of estrogen treatment than the control lines (Table 1). Tumors produced by VEGF-transfected cells with estrogen treatment had comparable scores for edge-associated microvessels compared to the parental cell tumors (Table 2), which also correlates with their comparable eventual tumor size compared to the control line (Table 1). Parental cell tumor nodules produced under conditions of tamoxifen treatment had a significantly reduced abundance of edge-associated microvessels when compared to estrogen-treated parental cell tumors ($p = 0.045$, Table 2). This was not true of tumors produced in tamoxifen-treated animals by the FGF or VEGF transfectants, which exhibit tamoxifen-resistant growth (Table 2). Moreover, the edge-associated microvessel abundance in tumors produced by the same two transfected lines (MKL-F and Clone 18) was significantly greater in tamoxifen-treated animals than that in tumors produced by control cells ($p < 0.05$). These data again imply that edge-associated vessels contribute positively to tumor growth under conditions of estrogen or tamoxifen treatment.

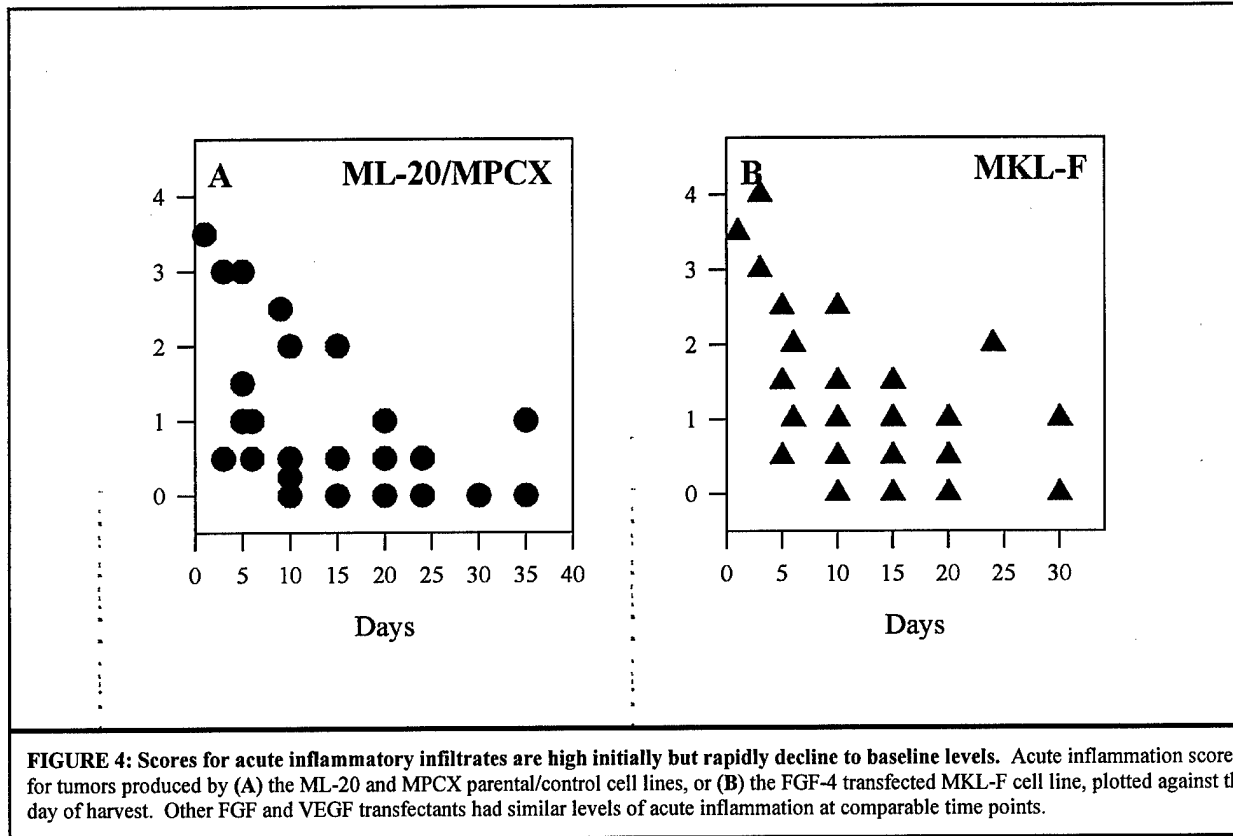
Table 2. Abundance of microvessel types in tumors produced in estrogen or tamoxifen treated mice by parental or transfected MCF-7 cells. Microvessel score averages \pm the standard error of the mean are given for the various microvessel types from tumor sections harvested from mice treated with estrogen or tamoxifen pellets from time points 5 days or later after tumor cell injection. Asterisks indicate scores significantly different from the parental cell scores for a particular treatment. $^{\circ}$ indicates scores of tumors in tamoxifen treated animals receiving control cells differed significantly from those receiving estrogen treatment.

Vessel Type	Treatment	Parental/ Control	MKL-F	MKL-4	Clone 18	MV165-14
Edge Associated	Estradiol	0.84 \pm 0.18	2.60 \pm 0.24 *	1.25 \pm 0.35	2.42 \pm 0.44 *	0.71 \pm 0.14
	Tamoxifen	0.36 \pm 0.08 $^{\circ}$	2.30 \pm 0.33 *	1.17 \pm 0.42	1.92 \pm 0.41 *	1.14 \pm 0.29
Intratumoral	Estradiol	1.23 \pm 0.19	2.65 \pm 0.66	2.07 \pm 0.50	2.00 \pm 0.49	1.86 \pm 0.30
	Tamoxifen	0.83 \pm 0.13	3.05 \pm 0.41 *	2.17 \pm 0.42 *	2.07 \pm 0.54 *	2.61 \pm 0.30 *
Ectatic Stromal	Estradiol	1.09 \pm 0.18	1.40 \pm 0.53	1.14 \pm 0.39	1.71 \pm 0.51	1.29 \pm 0.19
	Tamoxifen	1.45 \pm 0.25	1.90 \pm 0.60	0.83 \pm 0.31	1.86 \pm 0.29	1.25 \pm 0.21
Normal Stromal	Estradiol	1.75 \pm 0.19	2.40 \pm 0.51	1.96 \pm 0.50	2.71 \pm 0.43	1.68 \pm 0.25
	Tamoxifen	1.89 \pm 0.25	2.70 \pm 0.37	1.96 \pm 0.57	2.21 \pm 0.42	2.14 \pm 0.27

Intratumoral vessel abundance was not significantly increased in the tumors produced by the FGF-transfected and VEGF-transfected cells when compared with the parental cells with estrogen treatment (Table 2), a condition in which all lines are tumorigenic. Although there was a slightly decreased abundance of intratumoral vessels in tamoxifen-treated parental cell tumor nodules, this effect did not reach statistical significance. However, intratumoral microvessel abundance in tumors produced by all the transfected cell lines was significantly increased when compared with control/parental cell tumor nodules under conditions of tamoxifen treatment ($p < 0.05$, Table 2). This was particularly evident in the VEGF-transfected cells (Figure 1, D and H). Thus, these data and data on intratumoral vessels under estrogen-free conditions suggest that intratumoral vessels may have an important role in promoting growth under conditions where the parental cells are not tumorigenic, especially in the case of tamoxifen-resistant growth.

There were no differences in abundance of ectatic stromal or normal stromal vessels between different cell lines or treatment conditions. These data combined with the data from tumors produced in ovariectomized animals imply that these vessel types do not influence tumor growth.

Sustained angiogenesis produced by tumor cell injection is not due to a prolonged inflammatory response. Although breast tumor xenografts in nude mice are a commonly used tumor model, injection of tumor cells into the mammary fat pad does not mimic early stages of



growth of a spontaneously arising breast tumor. We were therefore concerned that the early angiogenic response might be due to inflammation as a result of the tumor cell injection and therefore would not be representative of spontaneous tumor angiogenesis. Similarly to the microvessel abundance scoring, inflammation was scored on a rating system of 0-4, with 4 being the highest and 0 denoting absence of the inflammation. Inflammatory infiltrates were evaluated from hematoxylin and eosin stained sections and scored as acute (primarily neutrophilic) or chronic (primarily lymphocytic), in addition to being given a numerical rating. We examined inflammatory infiltrates over the entire time course, above, and found that while in a few tumors high levels of acute, primarily neutrophilic, inflammation were initially present, after fewer than 10 days, this rapidly declined to baseline levels (Figure 4). Chronic, primarily lymphocytic, inflammatory infiltrates remained constant throughout the time course (not shown). There was no difference in the degree of acute or chronic inflammatory reaction produced by different cell lines (Figure 4 and not shown). Moreover, injection of the medium used as a vehicle for the tumor cell injections did not produce neovascularization or inflammation when the injection sites were harvested 10 days later (data not shown). In addition, immunohistochemistry of tumor sections showed negligible expression of the inflammatory adhesion molecule, ICAM-1, in tumor-associated blood vessels

under all treatment conditions in spite of robust expression in several adjacent lymph nodes harvested coincidentally with the tumor (data not shown). In some tumors produced by all transfected cells, but more frequently in ones produced by VEGF-transfected cells, there was sporadic expression of ICAM-1 in blood vessels in adjacent tissues not intimately associated with the tumor, such as overlying skin (data not shown). Since VEGF has been shown to increase ICAM-1 expression (9), and FGF-2 (basic FGF) can either upregulate or downregulate ICAM-1 in cultured endothelial cells (9,10) this finding may be due to an effect of diffusion of the transfected FGF or VEGF away from the tumor into surrounding tissues and actually argues for an ICAM-1 suppressing mechanism within the tumor. In any case, we conclude that sustained tumor-associated angiogenesis occurring after the initial period in xenografts produced by parental or transfected MCF-7 cells is not due to inflammation.

Angiogenesis produced by FGF-transfected cells is not due to increased expression of VEGF. Recently it has been reported that transfection of mammary epithelial cells or MCF-7 cells with FGF-4 induces expression of VEGF (11,12). Since the validity of our model depends on our being able to ascribe angiogenic effects of the transfections to the transfected factors, we examined RNA from the parental and FGF-transfected cells for mRNA encoding VEGF.

Parental or transfected cells were grown to 60% confluence in media containing 5% fetal bovine serum. RNA was harvested using Triazol (Life Technologies, Gaithersburg, MD) and ribosomal bands were determined to be intact by agarose gel electrophoresis followed by ethidium bromide staining. The riboprobe vector for human GAPDH has been previously described (2). The riboprobe for human VEGF produces a 432 bp probe which contains plasmid sequences and a 389 bp fragment

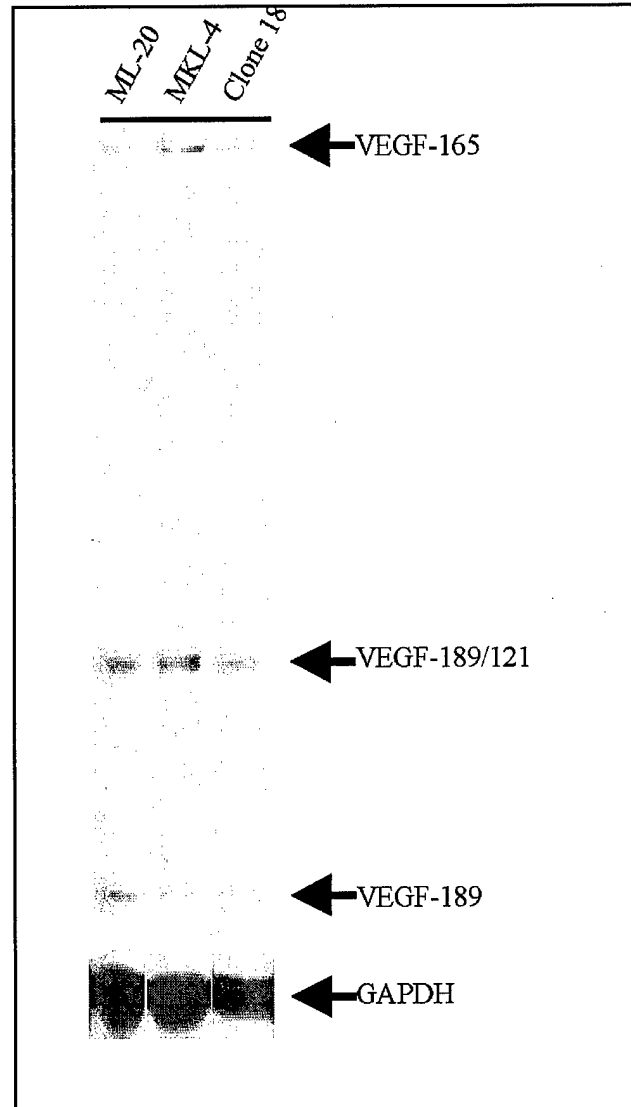


FIGURE 5: Expression of VEGF mRNA is not elevated in FGF-transfected cells. RNase protection of 30 μ g RNA from the indicated cell lines shows that the parental ML-20 cells and the two FGF transfectants have similar levels of mRNA for all isoforms of VEGF detected. Isoforms detected by the riboprobe are as indicated.

identical to partial sequence of the mRNA which gives rise to the 165 amino acid form of VEGF. When hybridized to VEGF-165 mRNA, a 389 bp protected fragment is produced. When hybridized to mRNA from VEGF-189 or -121, which share mRNA sequences with VEGF-165, a 216 bp fragment is protected. VEGF-189 transcripts also protect a 173 bp fragment. Thirty μ g total RNA was subjected to RNase protection as previously described (2) using 32 P-labeled antisense riboprobes for human VEGF and human GAPDH followed by denaturing polyacrylamide gel electrophoresis and autoradiography.

RNase protection assays revealed that neither transfection with FGF-4 nor FGF-1 induced expression of any of the detected isoforms of VEGF (Figure 5). These data, in addition to the different *in vivo* phenotypes of the FGF-transfected and VEGF-transfected cell lines (Table 1), argue that the angiogenic effects of FGF transfection in these cell lines are not due to increased expression of VEGF.

Identification of an *in situ* predictor of future tumor growth in tumor sections from transfected and parental MCF-7 cells. Our data, above, validate the importance of particular microvessels in producing active tumor growth. As mentioned, our goal is to study gene expression in these microvessels at early time points in tumor growth when we believe angiogenesis would be most active. From the time course experiments depicted in Figure 2 and Figure 4 and from our previous work (2,4,5,7,13), this would seem to be in the time range of 10-20 days after tumor cell injection, after the acute inflammatory response has subsided, but just before or concurrent with a substantial increase in tumor growth rate. Thus, we know *which vessels* to study and *when* to study them. However, tumor cell injection of any cell line under any conditions produces a range of tumor sizes or no tumor at all. Therefore, in order to correlate endothelial expression of particular genes with active tumor growth, it is important to know whether an individual early tumor is actively growing, before differences in tumor size become apparent. In other words, we want to know *which tumors* to study. Since tumor growth is thought to be due to the algebraic sum of cell proliferation and cell death, we asked whether measurements which estimate either of these parameters could identify actively growing tumors *versus* static or regressing ones. Since the individual cell lines have different responses to hormonal treatment *in vivo* (Table 1), we were able to utilize conditions of estrogen or tamoxifen treatment of tumor-bearing animals to answer this question.

For these studies, we utilized the FGF-4 transfected cell line (MKL-4) which produces tumors which are stimulated by tamoxifen treatment of tumor-bearing mice, the FGF-1 transfected cell line (Clone 18), and the VEGF transfected cell line (MV165-14), as well as the ML-20/MPCX parental/control lines (Table 1). Our hypothesis was that if proliferation, as measured by BrdU incorporation (14), were a good *in situ* predictor for future tumor growth, the pattern of the mean BrdU labeling indices for tumors produced by a given cell line under conditions of estrogen or tamoxifen treatment would have the same relationship as the known growth pattern (measured at much later time points) for that cell line as influenced by estrogen or tamoxifen (Table 1). Conversely, since terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) labeling is one

measurement of cell death, if it were a good *in situ* predictor of future tumor growth, the mean TUNEL labeling index for a given cell line under conditions of estrogen or tamoxifen treatment would have the *opposite* pattern to the growth pattern produced by estrogen or tamoxifen treatment for that cell line. We were *not* interested in explaining growth characteristics of tumors produced by the different cell lines as the algebraic sums of proliferation and cell death. This would be a very difficult task because of the substantial differences in time sensitivity of the two assays, the probability that TUNEL, which is only one of several *in situ* measurements of apoptosis, identifies only a subset of apoptotic cells and may also stain necrotic cells (15-17), and the possibility of entry of apoptotic cells into S-phase (18,19), which might produce positive staining for both BrdU incorporation and TUNEL in some cells.

Representative tumor sections from tumors produced by FGF or VEGF transfected cells or parental cells in estrogen or tamoxifen treated mice harvested between 10 and 20 days after tumor cell injection were stained for BrdU incorporation or TUNEL labeling. Immunohistochemistry for BrdU incorporation followed the same protocol as for PECAM-1 except that before the trypsin digestion, slides were placed in 2N HCl for 30 minutes, and washed thoroughly in deionized water. The primary antibody was a rat monoclonal anti-BrdU antibody (Accurate Antibodies #MAS250b) used at a dilution of 1:32 in blocking solution and incubated for 1 hour at room temperature. Following incubation with the same secondary antibody as for PECAM-1, slides were incubated with an ABC reagent coupled to alkaline phosphatase (Vector AK5000). Slides were then incubated with the Vector red alkaline phosphatase substrate (Vector SK-5100) according to the manufacturer's directions. For double PECAM-1 and BrdU immunohistochemistry, PECAM-1 staining utilizing the peroxidase ABC kit and diaminobenzidine was performed first. Following the wash step after diaminobenzidine disclosure, slides were left overnight in PBS and begun the next morning at the HCl step of the BrdU protocol. TUNEL staining was performed using the TACS 2 TdT kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. Labeling indices for each section were determined by image analysis. BrdU incorporation and TUNEL staining in tumor cells was analyzed utilizing image analysis which counted numbers of positively stained nuclei in a given area (in square microns). Tumor cell density was determined by manually counting numbers of tumor cells in a given area and dividing by the area. Labeling indices for BrdU incorporation and TUNEL staining were then derived by converting areas to numbers of cells. Sections from mammary fat pads of mice injected with media alone and harvested after 10 days were used to evaluate the effect of vehicle injections.

For tumors produced by the ML-20/MPCX parental or control cell lines, the BrdU labeling index pattern correlated well with the tumor growth pattern, and the TUNEL labeling index pattern was weakly inverse to the growth pattern (Figure 6). Thus, for these cell lines, BrdU labeling index is the best *in situ* predictor of future tumor growth. For the FGF transfected MKL-4 cell line, the BrdU labeling index pattern correlated with the tumor growth pattern and the TUNEL labeling index was opposite to the growth pattern, so that either a high BrdU labeling index or low TUNEL labeling index could serve as an *in situ* predictor of future tumor growth for this cell line (Figure 6). For the

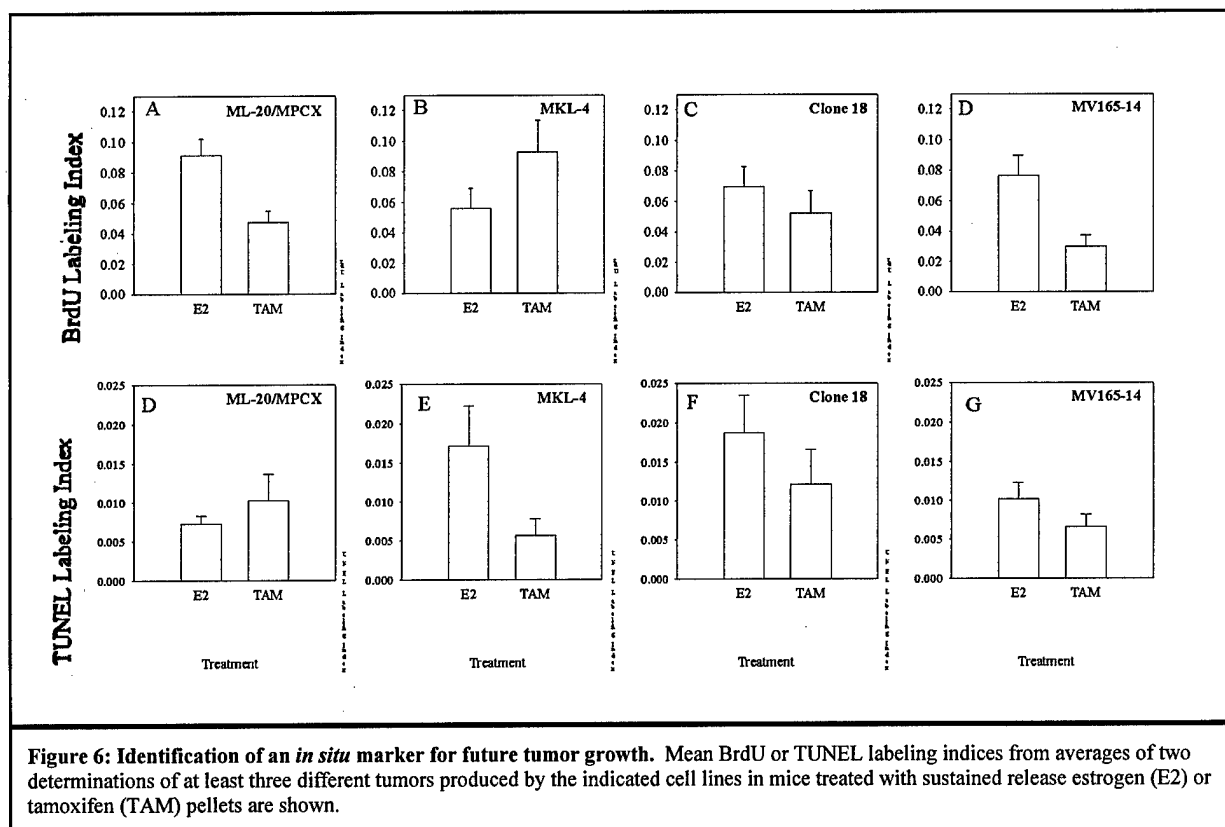


Figure 6: Identification of an *in situ* marker for future tumor growth. Mean BrdU or TUNEL labeling indices from averages of two determinations of at least three different tumors produced by the indicated cell lines in mice treated with sustained release estrogen (E2) or tamoxifen (TAM) pellets are shown.

clone 18 and MV165-14 cell lines, BrdU is a good predictor, but TUNEL is not, since the BrdU patterns correlate with the growth patterns, but the TUNEL patterns are not inverse. Thus, for the parental/control and all the transfected cells, BrdU incorporation is the best *in situ* predictor of tumor growth since it most consistently correlated with growth patterns of the transfected cell lines under conditions of estrogen and tamoxifen treatment. Moreover, rapidly growing tumors produced by individual cell lines can be identified as those whose BrdU labeling indices exceed means of the growth-stimulated tumors in these experiments, and slowly growing tumors can be identified as those whose BrdU labeling indices are lower than means of the growth-inhibited tumors in these experiments. These data will therefore assist us in assessing the effects of expression of particular genes on individual tumors as our studies of gene expression progress.

Since BrdU can serve as an *in situ* predictor of tumor growth for the transfected cell lines, and edge-associated or intratumoral microvessel abundance seemed to be increased in tumors with increased growth potential (above) we asked whether BrdU labeling were correlated with the abundance of edge-associated or intratumoral microvessels in individual tumor sections harvested between 10 and 20 days after tumor cell injection. Statistical analysis such as this is difficult for

several reasons. First, since both variables are the result of measurement (both are dependent variables), there is associated error present in both variables. Second, there must be a good spread of data across a range of values so that linear regression can be performed adequately. Third, the data must be normally distributed with a constant variance. To increase the validity of our analysis in this part of the study, we enlisted a second observer who performed ratings of microvessel abundance on the same sections as were evaluated by the first observer. Additionally, since we seek correlations for six individual cell lines and are thus conducting six linear regression analyses, we must divide the criterion for significance, a p-value of less than 0.05, by 6 to rule out the possibility that one of our regressions will achieve a $p < 0.05$ by chance. Therefore, our criterion for significance in this analysis is a p-value of less than 0.008. For tumor sections produced by the ML-20 or MPCX control cells, all but one of the sections examined had average edge-associated vessel scores of less than 2.5. Thus, these data did not meet the second criterion above and we would not consider a linear regression analysis to be valid (data not shown). For tumors produced by one FGF-4 transfected cell line (MKL-4), average edge-associated vessel scores were highly correlated with BrdU labeling indices in the tumor sections analyzed ($p=0.0045$), and intratumoral vessel density showed a weak correlation ($p=0.07$) (data not shown). For the other transfected cell lines, there was no demonstrable correlation between BrdU labeling index and edge-associated or intratumoral vessel scores. Thus, these data provide additional evidence, at least in one cell line, that increased tumor growth as identified by BrdU incorporation, is associated with increased abundance of edge-associated or intratumoral vessels.

The data described here for Aim #2 form the content of a paper "Tumor growth of FGF or VEGF transfected MCF-7 breast carcinoma cells correlates with density of specific microvessels independent of the transfected angiogenic factor" which is in press in *The American Journal of Pathology* (1).

In this aim, we were going to identify immature blood vessels by their expression of *ets-1*, a transcription factor reported to be induced by FGFs and VEGF (20) in newly angiogenic endothelial cells. Unfortunately, possibly due to technical difficulties, we have been unable to replicate published results of others (21) in mouse embryos using *in situ* hybridization. In addition, in spite of multiple attempts using many different methods, we have not been able to definitely show staining of tumor-associated endothelial cells with polyclonal rabbit antibodies directed toward murine ETS-1. In searching the literature, most of the data implicating *ets-1* expression in angiogenesis is from cultured endothelial cells, not from tumor sections. We have abandoned this effort for the present since it appears that technical constraints and unavailability of effective reagents make it unfeasible at this time.

Aim 3. In Aim 3, we were to investigate gene expression in tumor-derived endothelial cells. This aim was the most speculative, as we had to develop a method to separate endothelial cells from the rest of the cells in the tumor. We felt that we could not use methods which involved culturing

the cells, as gene expression is known to change dramatically in tissue culture (22-25). We were able to develop a one-step separation of endothelial cells utilizing flow cytometry (FACS) and three different monoclonal antibodies to murine PECAM-1. This method was detailed in the last annual report. Briefly, tumors produced by the various cell lines or mammary fat pads were finely chopped, digested with collagenase and DNase I, reacted with fluorochrome labeled anti-PECAM-1 antibodies and subjected to FACS. A murine hemangioma cell line [EOMA (26)] which expresses PECAM-1 was used as a positive control to set the fluorescence levels for tumor-associated endothelial cell selection. RNA was extracted from the selected cells using the RNeasy kit (Qiagen, Santa Clarita, CA). This RNA was demonstrated by reverse transcription followed by the polymerase chain reaction (RT-PCR) to contain transcripts for PECAM-1 and murine GAPD. Many batches of RNA so produced did not contain transcripts for human keratinocyte growth factor receptor (KGFR), implying that the level of tumor cell contamination of the selected cells was very low. In other batches of RNA, RT-PCR revealed a faint human KGFR band, implying a somewhat higher level of tumor cell contamination (These data were reported in last year's report). However, the degree of purity which can be obtained in a one-step procedure such as this is probably limited, and we have obviously achieved a very high degree of enrichment of endothelial cells by this method. Hence we deemed this RNA suitable for differential cloning techniques to show gene expression in tumor derived endothelial cells.

The quantity of RNA obtained from endothelial cells in tumors produced by the various cell lines varied but was on the order of a few micrograms. Mammary fat pad endothelial cells were very few in number and RNA obtained from mammary fat pads of 40 mice was not able to be quantitated by spectrophotometry, although we were able to show PECAM and GAPDH transcripts by RT-PCR. Because of these small amounts, the RNA cannot be used to analyze gene expression by conventional techniques. We therefore utilized it in a PCR-based method of synthesizing larger quantities of cDNA. The method we chose was the SMART PCR cDNA synthesis system (Clontech Laboratories, Palo Alto, CA). In this method, the RNA is reverse transcribed utilizing a poly-dT primer, which selects poly-adenylated mRNA and a reverse transcriptase (RT) enzyme (Superscript II, Life Technologies, Gaithersburg, MD) which is capable of template-switching. The poly-dT primer contains a manufactured sequence at its 5' end, which is then incorporated into the 5' end of the first strand cDNA. The reverse transcriptase (RT) synthesizes the particular cDNA species and then appends multiple "Cs" to the 3' end of the first strand cDNA. A manufactured oligonucleotide present in the reaction contains 5' "Gs" which hybridize to the 3' "Cs" on the cDNA. The RT will then switch to the template of the manufactured oligonucleotide, appending a known sequence at the 3' end of the first strand cDNA. Thus, first strand cDNA is produced with manufactured sequences at each end. This first strand cDNA can then be used as template for a PCR reaction with the 5' and 3' manufactured sequences as primers. Because the manufactured sequences and PCR conditions are optimized, it is thought that the PCR reaction will not amplify particular cDNA species preferentially. We have produced cDNA by this method from RNA from all the tumor cell lines (grown in tissue culture), the RNA from the tumor and mammary fat pad endothelial cells isolated

by FACS, and murine EOMA and NIH 3T3 cell lines grown in tissue culture. We are subjecting these cDNAs to a differential cloning procedure known as amplified fragment length polymorphism (AFLP) (27). This method is similar to differential display PCR, which was the method proposed in the original proposal.

The method of AFLP is somewhat more suitable for utilization with small amounts of cDNA than differential display PCR, which requires somewhat larger amounts of RNA. Moreover, it is less likely to produce artifactual bands through false priming than is differential display PCR. Briefly, each candidate cDNA is digested with two restriction enzymes, TaqI (a frequent cutter) and AseI (a rare cutter). Adapters which have a manufactured sequence followed by AseI or TaqI sticky ends are then ligated to the cDNA fragments. The cDNA fragments at this point have manufactured sequences at each end and are preamplified with primers complementary to these manufactured sequences. At this point, a small portion of the amplified cDNA fragments are further amplified with primers which contain the manufactured sequence and two internal bases which are varied through all possible combinations. The primers are used two at a time (one each corresponding to each end of the cDNA) for high-stringency PCR to amplify those particular cDNA species which contain the sequences of the chosen internal bases at their 3' or 5' ends. Since these reactions are done under high stringency conditions with primers which have an

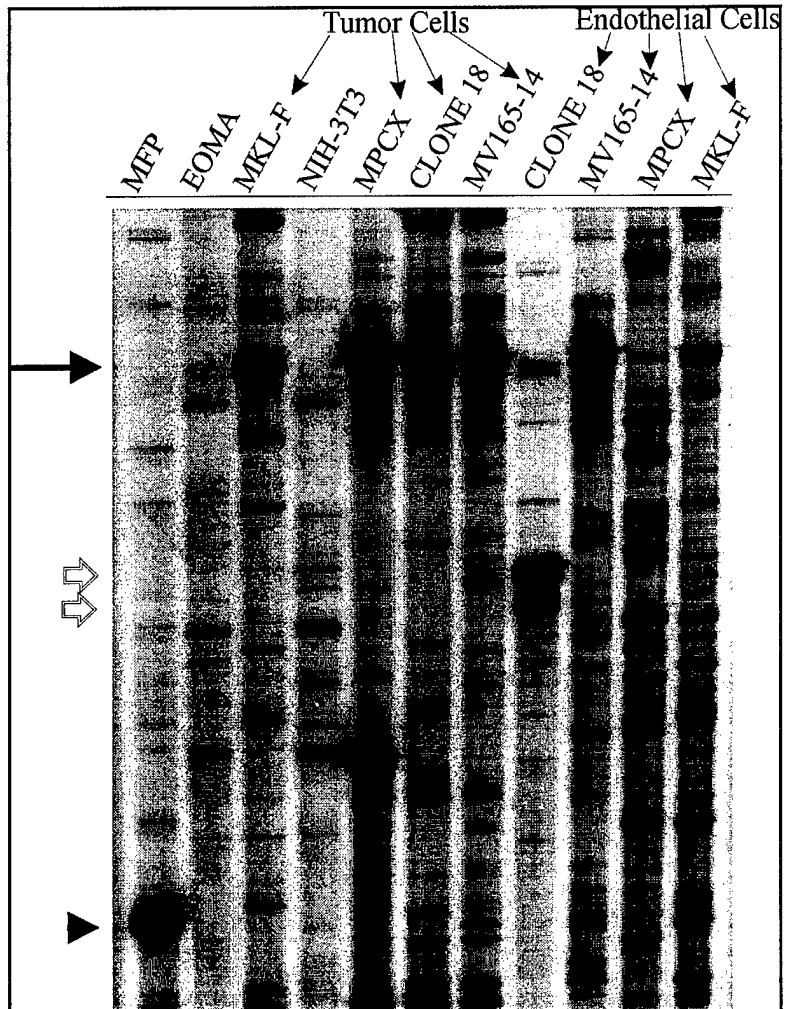


Figure 7. Autoradiogram of AFLP gel. Bands result from mRNAs present in RNA isolated from cells as indicated. MFP, mammary fat pad endothelial cells; EOMA, a murine hemangioma cell line, MKL-F (FGF-4 transfected) tumor cells grown in culture; NIH-3T3, murine fibroblasts; MPCX (parental/control) tumor cells grown in culture; Clone 18 (FGF-1 transfected) tumor cells grown in culture; MV165-14 (VEGF transfected tumor cells grown in culture; Clone 18, endothelial cells isolated from a tumor produced by Clone 18 cells; MV165-14, endothelial cells isolated from a tumor produced by MV165-14 cells; MPCX, endothelial cells isolated from a tumor produced by MPCX cells; MKL-F, endothelial cells isolated from a tumor produced by MKL-F cells.

McLeskey, Sandra W.
DAMD17-94-J4173

exact match to the manufactured sequence and the two internal bases, there is little false priming. There are 256 possible primer combinations at this degree of degeneracy. Radiolabeled nucleotides are incorporated into the PCR reaction and the products are electrophoresed on a denaturing 6% polyacrylamide (sequencing) gel. The amplified fragments are identified via autoradiography. Typically an RNA population will produce about 30 bands per primer set (27).

We have produced cDNA fragments with the AFLP method and have currently used two of the degenerate primers to look for bands which are expressed in RNA populations extracted from the tumor-derived endothelial cells but are not present in mammary fat pad endothelial cells. As additional negative controls, we have also produced cDNA fragments from RNA extracted from each of the parental or transfected cell lines, and from EOMA and NIH-3T3 murine cell lines in tissue culture. The last two controls are meant to exclude genes expressed solely as a result of proliferation. Figure 7 is an example of an autoradiogram of an AFLP gel which shows bands representing expressed mRNAs from the various controls and the tumor derived endothelial cells. The band identified by the thick arrow represents a mRNA expressed by all tumor cell lines. Since this band is also present in lanes produced by mRNA from the isolated endothelial cells, they may also express that particular mRNA. Alternatively, the band may be present in those lanes as a result of tumor cell contamination of the FACS-selected cells. At any rate, we would not pursue this band as a differentially expressed gene even though it is not present in the lane from mammary fat pad endothelial cells. This band illustrates how we can control for the small amount of tumor cell contamination present in our isolated endothelial cells by rejecting from consideration bands also present in lanes from tumor cell RNA. This autoradiogram does not show a band common to all tumor-derived endothelial cells but absent from mammary fat pad endothelial cells and tumor cells. It does show a band which is very strong in the mammary fat pad endothelial cells and absent/strongly downregulated in tumor-derived endothelial cells (arrowhead). In addition, it shows a band which is very strongly present in endothelial cells derived from FGF-1 transfected cell tumors which is absent in mammary fat pad endothelial cells and absent or weakly present in the other tumor-derived endothelial cell lanes. A second lower molecular weight band meets the same criteria (open arrows).

At present, our goal is to identify a band present in lanes from in tumor-derived endothelial cells but absent in mammary fat pad endothelial cells and also absent in the lanes from the tumor cells themselves. Ideally, this band would also be absent from NIH-3T3 cell and EOMA cell lanes, indicating it is not a marker for proliferation in general. If during our screen of 16 possible combinations of the 4 forward and 4 backward primers we have obtained, we do not identify such a band, we may pursue some of the interesting bands as identified above. We will, however, obtain additional primers and continue our search for a band which meets our most stringent criteria for candidate bands.

Candidate bands will be excised from the gel, reamplified with the same primers and re-

McLeskey, Sandra W.
DAMD17-94-J4173

electrophoresed to supply large amounts of the particular band. This reamplified band will be excised from the gel and ligated into the pGEM-T vector (Promega, Madison, WI) followed transformation of bacteria. Purified plasmids will be dotted on duplicate nitrocellulose membranes and differential expression will be confirmed by probing the membranes with library probes from the tumor-derived endothelial cell RNA and from the tumor cells themselves. Description of the method for obtaining library probes is given below. If differential expression is confirmed in this test, sequence data will be obtained, sequence data bases will be screened and the identity of the transcript will be obtained. Although a large portion of the mouse genome has been sequenced to date and many expressed sequence tags have also been identified in mice, it is possible that our candidate sequence will be unknown. In that case, we will search human sequence data bases, translate all possible reading frames and search protein databases for clues as to the identity of the transcript. We would also screen RNA from various mouse tissues, both by Northern blot and by *in situ* hybridization or immunohistochemistry (if a suitable antibody is available), to see whether the gene were expressed exclusively in tumor endothelial cells or in other endothelial or nonendothelial cells. Particular attention would be paid to tissues of the female reproductive tract and of the immune system. In addition, tissues from healing wounds and models of inflammation would be examined.

Efforts are also currently underway to prepare cDNA libraries from RNA extracted from the tumor-derived endothelial cells. In making a library from such small amounts of RNA, there are serious problems concerning amplification of the RNA while preserving the relative abundance of various transcripts in the library. Because our AFLP method is heavily based on PCR, first in preparing the cDNA by the SMART cDNA system, and second in performing the AFLP procedure, we wanted to prepare our library in a way that does not involve PCR. The preparation of amplified RNA is such a technique. In preparing amplified RNA, one first performs reverse transcription on candidate RNA utilizing a poly-dT primer with a T7 RNA polymerase promoter appended to its 5' end. The same RT enzyme (Superscript II, Life Technologies, Gaithersburg, MD) is used as for SMART cDNA synthesis and the SMART oligonucleotide is included in the reaction mixture so that the SMART manufactured sequence is appended to the 3' end of the first strand cDNA. Second strand cDNA synthesis utilizes the 3' SMART primer, so that the resultant double-stranded cDNA has the SMART sequence at one end and a T7 promoter sequence at the other end. Amplified RNA is then produced by T7 RNA polymerase. We have obtained approximately 1500-fold amplification of RNA at this step. At this point, a second round of cDNA synthesis is done, using the manufactured sequence to prime the RT reaction and the poly-dT-T7 promoter sequence to prime the second strand. This newly synthesized cDNA is used to produce second-round amplified RNA via T7 RNA polymerase. In this step, we have obtained an additional 1500-fold amplification with sizes of aRNA in excess of 7.5 kb. The amplified RNA will then be used as template for double-stranded cDNA as for the second round cDNA synthesis except that Not I and Xho I restriction sites will be appended to the SMART and poly-dT oligonucleotides. This will give us cDNA with an approximately 2 million-fold abundance when compared to the original RNA (28-30) The newly

McLeskey, Sandra W.
DAMD17-94-J4173

synthesized cDNA will be doubly digested with Not I and Xho I and ligated into a similarly digested cloning vector (LambdaZap II, Stratagene, La Jolla, CA). This technique has the advantage over PCR in that the amplifications produced are for the most part arithmetic rather than exponential, and do not depend on primer/template characteristics so that the product is not as likely to contain preferentially amplified species. Therefore, we think the libraries generated by this technique will contain cDNA species which are representative of the original mRNA populations. In addition, since the LambdaZap II vector has a Bluescript plasmid embedded in it with T3 and T7 RNA polymerase promoters, RNA library probes can be generated with ease and used to probe the dot blots, above.

Conclusion

Scientific conclusions to date:

1. Edge-associated and intratumoral microvessel density in tumors produced by parental, FGF-transfected or VEGF-transfected MCF-7 cells correlate with tumor growth characteristics of the transfectants whereas other vessel types do not. Such correlations are in place irrespective of the identity of the transfected angiogenic factor.
2. BrdU incorporation is the best *in situ* predictor of future tumor growth in tumors produced by parental and transfected cell lines.
3. RNA purified from endothelial cells isolated from growing tumors by flow cytometry can serve as a substrate for differential cloning techniques despite small amounts of tumor cell contamination provided the right controls are included.
 - A. AFLP is an effective way to display differential gene expression in this model.
 - B. AFLP identifies genes differentially expressed by mammary fat pad endothelial cells or by endothelial cells isolated from particular tumor types.
 - C. To date, AFLP has not identified genes differentially expressed by all tumor-derived endothelial cells but not expressed by mammary fat pad endothelial cells. However, only two primer pairs have been analyzed to date, so future primer pairs may reveal such genes.
4. The technique of amplified RNA coupled with template switching can produce large amounts of high molecular weight RNA which can be used to produce cDNA libraries.

Discussion:

With recent new information concerning the effect of angiogenesis inhibitors on tumor

McLeskey, Sandra W.
DAMD17-94-J4173

growth in animal models (31-34), it has become clear that targeting tumor vasculature may be an effective way of treating many types of cancer. That being the case, it is even more important to investigate the biology of the *process* of tumor-induced angiogenesis than when this proposal was written in 1993. It is only through an understanding of this process that effective antiangiogenesis therapies can be developed which will target tumor vasculature specifically. However, study of human endothelial cells is very difficult since it is very clear that cultured endothelial cells do not represent the situation *in vivo* and isolation of endothelial cells from human tissues is logistically quite difficult, even using the isolation protocol we have developed (which requires extremely fresh tissue and approximately 10 hours to complete). Moreover, genetic diversity among humans makes comparisons between individuals extremely difficult. However, with the techniques we have developed in the course of this project, we believe we will be able to extend our studies to endothelial cells in human tumors. We have been able to produce amplified RNA from one endothelial cell microdissected from a paraffin-embedded section which was had been subjected to immunohistochemistry for PECAM-1 to highlight endothelial cells. The amplified RNA obtained was of high molecular weight (unpublished). The slide used for this preliminary experiment was one of our mouse xenograft slides, but there is no reason this could not be done on a paraffin embedded section of a human breast tumor. In the future, in addition to exploring any differentially expressed genes we identify from the mouse xenografts, we will be engaged in molecular cloning techniques to analyze gene expression in microdissected endothelial cells from human tumors. We feel we are in the forefront of the field in this regard and hope to serve as a resource for others who wish to develop similar research programs.

This project was subject to a long start-up time because of the developmental aspects of the techniques used. We did not complete all the tasks outlined in the "Statement of Work". However, this contract is not for tasks accomplished, it is for the *conduct of research*. It is characteristic of research that nothing ever comes out the way you think it will, that things rarely work the first time you try them, and that new and exciting findings are often completely unanticipated and are therefore not found in a "Statement of Work." We feel that the last four years have been very productive and thank the Department of Defense for supporting us during that time.

McLeskey, Sandra W.
DAMD17-94-J4173

References

1. McLeskey, S.W., Tobias, C.A., Vezza, P.R., Filie, A.C., Kern, F.G., and Hanfelt, J. Tumor growth of FGF or VEGF transfected MCF-7 breast carcinoma cells correlates with density of specific microvessels independent of the transfected angiogenic factor. *Am. J. Pathol.* 1998.
2. McLeskey, S.W., Kurebayashi, J., Honig, S.F., Zwiebel, J., Lippman, M.E., Dickson, R.B., and Kern, F.G. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. *Cancer Res.* 53: 2168-2177, 1993.
3. Kurebayashi, J., McLeskey, S.W., Johnson, M.D., Lippman, M.E., Dickson, R.B., and Kern, F.G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and *LacZ*. *Cancer Res.* 53: 2178-2187, 1993.
4. Zhang, L., Kharbanda, S., Chen, D., Bullocks, J., Miller, D.L., Ding, I.Y.F., Hanfelt, J., McLeskey, S.W., and Kern, F.G. MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized metastatic tumors in ovariectomized or tamoxifen-treated nude mice. *Oncogene*, 15: 2093-2108, 1997.
5. Bullocks, J., Zhang, L., Ding, I.Y.F., McLeskey, S.W., Tobias, C.A., Miller, D.L., and Kern, F.G. Overexpression of vascular endothelial cell growth factor (VEGF) in MCF-7 breast carcinoma cells facilitates growth in tamoxifen-treated nude mice and tumor cell dissemination. *Proc. Am. Assoc. Cancer Res.* 38: 35211997.
6. McLeskey, S.W., Zhang, L., Kharbanda, S., Kurebayashi, J., Lippman, M.E., Dickson, R.B., and Kern, F.G. Fibroblast growth factor overexpressing breast carcinoma cells as models of angiogenesis and metastasis. *Breast Cancer Res. Treat.* 39: 103-117, 1996.
7. McLeskey, S.W., Zhang, L., El-Ashry, D., Trock, B.J., Lopez, C.A., Kharbanda, S., Tobias, C.A., Lorant, L.A., Hannum, R.S., Dickson, R.B., and Kern, F.G. Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen, ICI 182,780, and two aromatase inhibitors. *Clin. Cancer Res.* 4: 697-711, 1998.
8. Vecchi, A., Garlanda, C., Lampugnani, M.G., Resnati, M., Matteucci, C., Stoppacciaro, A., Schnurch, H., Risau, W., Ruco, L., Mantovani, A., and Dejana, E. Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. *Eur. J. Cell Biol.* 63: 247-254, 1994.

McLeskey, Sandra W.
DAMD17-94-J4173

9. Melder, R.J., Koenig, G.C., Witwer, B.P., Safabakhsh, N., Munn, L.L., and Jain, R.K. During angiogenesis, vascular endothelial growth factor and basic fibroblast growth factor regulate natural killer cell adhesion to tumor endothelium. *Nature Medicine*, 2: 992-997, 1996.
10. Griffioen, A.W., Damen, C.A., Martinotti, S., Blijham, G.H., and Groenewegen, G. Endothelial intercellular adhesion molecule-1 expression is suppressed in human malignancies: the role of angiogenic factors. *Cancer Res.* 56: 1111-1117, 1996.
11. Deroanne, C.F., Hajitou, A., Calberg-Bacq, C.-M., Nusgens, B.V., and Lapière, C.M. Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. *Cancer Res.* 57: 5590-5597, 1997.
12. Hajitou, A., Deroanne, C.F., Noël, A., Colette, J., Nusgens, B.V., Foidart, J.M., and Calberg-Bacq, C.-M. FGF-3 increases tumorigenicity in MCF7 breast cancer cells but FGF-4 effect is more pronounced and associated with VEGF upregulation. *Proc. Am. Assoc. Cancer Res.* 39: 341998.
13. McLeskey, S.W., Zhang, L., Trock, B.J., Kharbanda, S., Liu, Y., Gottardis, M.M., Lippman, M.E., and Kern, F.G. Effects of AGM-1470 and pentosan polysulphate on tumorigenicity and metastasis of FGF-transfected MCF-7 cells. *Br. J. Cancer*, 73: 1053-1062, 1996.
14. Weidner, N., Moore, D.H., II, Ljung, B.-M., Waldman, F.M., Goodson, W.H.I., Mayall, B., Chew, K., and Smith, H.S. Correlation of bromodeoxyuridine (BRDU) labeling of breast carcinoma cells with mitotic figure content and tumor grade. *Am. J. Surg. Path.* 17: 987-994, 1993.
15. Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.P., Toyka, K.V., and Lassmann, H. Differentiation between cellular apoptosis and necrosis by the combined use of *in situ* tailing and nick translation techniques. *Lab. Invest.* 71: 219-225, 1994.
16. Mundle, S.D. and Raza, A. The two *in situ* techniques do not differentiate between apoptosis and necrosis but rather reveal distinct patterns of DNA fragmentation in apoptosis. *Lab. Invest.* 72: 611-612, 1995.
17. Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.P., Toyka, K.V., and Lassmann, H. The two *in situ* techniques do not differentiate between apoptosis and necrosis but rather reveal distinct patterns of DNA fragmentation in apoptosis --- reply. *Lab. Invest.* 72: 612-613, 1995.

McLeskey, Sandra W.
DAMD17-94-J4173

18. Mundle, S., Iftikhar, A., Shetty, V., Dameron, S., Wright-Quinones, V., Marcus, B., Loew, J., Gregory, S., and Raza, A. Novel *in situ* double labeling for simultaneous detection of proliferation and apoptosis. *J. Histochem. Cytochem.* 42: 1533-1537, 1994.
19. Raza, A., Mundle, S., Iftikhar, A., Gregory, S., Marcus, B., Khan, Z., Alvi, S., Shetty, V., Dameron, S., Wright, V., Adler, S., Loew, J.M., Shott, S., Ali, S.N., and Preisler, H. Simultaneous assessment of cell kinetics and programmed cell death in bone marrow biopsies of myelodysplastics reveals extensive apoptosis as the probable basis for ineffective hematopoiesis. *Am. J. Hemat.* 48: 143-154, 1995.
20. Iwasaka, C., Tanaka, K., Abe, M., and Sato, Y. Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. *J. Cell. Physiol.* 169: 522-531, 1996.
21. Maroulakou, I.G., Papas, T.S., and Green, J.E. Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. *Oncogene*, 9: 1551-1565, 1994.
22. Speir, E., Sasse, J., Shrivastav, S., and Casscells, W. Culture-induced increase in acidic and basic fibroblast growth factor activities and their association with the nuclei of vascular endothelial and smooth muscle cells. *J. Cell. Physiol.* 147: 362-373, 1991.
23. Auerbach, R., Auerbach, W., and Polakowski, I. Assays for angiogenesis: A review. *Pharmac. Ther.* 51: 1-11, 1991.
24. Liaw, L. and Schwartz, S.M. Comparison of gene expression in bovine aortic endothelium *in vivo* versus *in vitro*. *Arteriosclerosis & Thrombosis*, 13: 985-993, 1993.
25. Craig, L.E., Spelman, J.P., Strandberg, J.D., and Zink, C.M. Endothelial cells from diverse tissues exhibit differences in growth and morphology. *Microvasc. Res.* 55: 65-76, 1998.
26. Obeso, J., Weber, J., and Auerbach, R. A hemangioendothelioma-derived cell line: Its use as a model for the study of endothelial cell biology. *Lab. Invest.* 63: 259-269, 1990.
27. Bachem, C.W.B., van der Hoeven, R.S., de Bruijn, S.M., Vreugdenhil, D., Zabeau, M., and Visser, R.G.F. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *Plant J.* 9: 745-753, 1996.
28. Eberwine, J. Amplification of mRNA populations using aRNA generated from immobilized oligo-dT-T7 primed cDNA. *Biotechniques*, 20: 584-586, 1996.

McLeskey, Sandra W.
DAMD17-94-J4173

29. Eberwine, J., Yeh, H., Miyashiro, K., Cao, Y., Nair, S., Finnell, R., Zettle, M., and Coleman, P. Analysis of gene expression in single live neurons. *Proc. Natl. Acad. Sci. U. S. A.* 89: 3010-3014, 1992.
30. Eberwine, J., Crino, P., and Dichter, M. Single cell mRNA amplification: Basic science and clinical implications. *Neuroscientist*, 1: 200-211, 1995.
31. O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H., and Folkman, J. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell*, 79: 315-328, 1994.
32. Cao, Y., O'Reilly, M.S., Marshall, B., Flynn, E., Ji, R.-W., and Folkman, J. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. *J. Clin. Invest.* 101: 1055-1063, 1998.
33. Boehm, T., Folkman, J., Browder, T., and O'Reilly, M.S. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature*, 390: 404-407, 1997.
34. Arap, W., Pasqualini, R., and Ruoslahti, E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science*, 377-380, 1998.

201501
McLeskey, Sandra W.
DAMD17-94-J4173

APPENDIX

LIST OF ABBREVIATIONS AND ACRONYMS

AFLP	Amplified fragment-length polymorphisms
a.k.a.	Also known as
BrdU	Bromodeoxyuridine, a thymidine analog
EOMA	A mouse hemangioma cell line
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
FGF-1	Fibroblast growth factor 1, a.k.a. acidic FGF
FGF-1, clone 18	A clonal cell line of ML-20 cells (below) transfected with FGF-1
FGF-4	Fibroblast growth factor 4, a.k.a. Kaposi FGF, <i>hst-1</i>
FGFR	FGF receptor
KGFR	Keratinocyte growth factor receptor
<i>lacZ</i>	A bacterial gene encoding β -galactosidase
MCF-7	An estrogen receptor positive breast carcinoma cell line
MKL-F	A clonal cell line of ML-20 cells cotransfected with FGF-4 and <i>lacZ</i>
MKL-4	A clonal cell line of MCF-7 cells cotransfected with FGF-4 and <i>lacZ</i>
ML-20	A clonal cell line of MCF-7 cells transfected with <i>lacZ</i>
NIH 3T3	A cell line of immortalized mouse fibroblasts
PECAM	Platelet-endothelial cell adhesion molecule
RT-PCR	Reverse transcription followed by the polymerase chain reaction
TUNEL	Terminal deoxynucleotidyl transferase - mediated dUTP-biotin nick end labeling
VEGF	Vascular endothelial cell growth factor

McLeskey, Sandra W.
DAMD17-94-J4173

Meeting abstracts since the last report:

Tobias, C.A., Vezza, P.R., O'Brien, J.A., Filie, A., and McLeskey, S.W. Development of a model of tumor-induced angiogenesis. "Era of Hope", The Department of Defense Breast Cancer Research Program Meeting, Washington, DC., October 31-November 4, 1997.

Vezza, P.R., Filie, A.F., Tobias, C.A., and McLeskey, S.W. A model of tumor-induced angiogenesis. 89th Annual Meeting of the American Association for Cancer Research, New Orleans, LA, March 28-April 1, 1998

Publications since the last report:

McLeskey, S.W., Zhang, L., El-Ashry, D., Trock, B. J., Lopez, C.A., Kharbanda, S., Tobias, C. A., Lorant, L.A., Hannum, R.S., Dickson, R. B. and Kern, F.G. Tamoxifen-resistant FGF-transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen, ICI 182,780, and two aromatase inhibitors. 1998, *Clinical Cancer Res.*, **4**, 697-711.

McLeskey, S. W., Tobias, C. A., Vezza, P. R., Filie, A. F., Kern, F.G., and Hanfelt, J. Tumor growth of FGF or VEGF transfected MCF-7 breast carcinoma cells correlates with density of specific microvessels independent of the transfected angiogenic factor. 1998, *Am. J. Pathol.*, in press.

Manuscripts in preparation:

McLeskey, S.W. and Lippman, M.E. The Biology of tumor-induced angiogenesis --- unanswered questions. 1998, *Cancer Res.*, manuscript in preparation.

Personnel receiving pay from this negotiated effort:

Sandra W. McLeskey, PhD