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Award Number: DAMD17-01-1-0311

TITLE: Fibrinolysis in Tumor Associated Angiogenesis

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REPORT DATE: July 2003

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Fibrinolysis in Tumor Associated Angiogenesis

5. FUNDING NUMBERS
DAMD17-01-1-0311

6. AUTHOR(S)
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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
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Angiogenesis has been shown o be necessary for tumor growth and metastasis but specific targets for antiangiogenic therapy in breast cancer have not been identified. Patient samples, a tissue assay of angiogenesis with a mammary vessel explant, and xenograft breast tumors growing in mice will be used to investigate the role(s) of the plasminogen activator/inhibitor/receptor family of proteins in breast cancer angiogenesis. In the second year, we have developed a method for identifying endothelial cells growing in culture of a mammary vessel explant that utilizes KiI-labeled acetylated LDL uptake. This method identifies the endothelial cells with a red fluorescence and enables observation of living mammary vessel cultures. Construction of retroviral vectors to infect these culture and host mouse tissues with antisense to plasminogen activator/inhibitor/receptor cDNA is underway. Also under construction viral vectors for proteins of interest coupled to green fluorescent protein. Infection of mammary vessel cultures with these viral vectors will yield information on the localization of the laveled proteins and their function in invasion of the matrix and formation of endothelial tubes. Over the next year, we will be using these vectors to study the function of the plasminogen activator/inhibitor/receptor system in the mammary vessel assay and in xenograft tumors in mice.

14. SUBJECT TERMS
Angiogenesis, endothelial, plasminogen, fibrinolysis

15. NUMBER OF PAGES
9

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	Unlimited

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Introduction

Tumors have substantial fibrin as part of their extracellular matrix and invading endothelial cells necessarily must invoke fibrinolytic activity to invade the fibrin to form new tubular structures. We proposed to investigate the role of fibrinolytic enzymes/receptors/ inhibitors in microdissected endothelial cells from xenograft breast tumors and human breast cancer, in an *in vitro* mammary vessel assay in a fibrin matrix, and in a mouse model of breast cancer angiogenesis. In the second year of the project, we have made progress in development of a mammary vessel assay of breast cancer angiogenesis by developing a method to observe these assays during culture. This method also has the potential for quantification of endothelial cells that invade the fibrin matrix. We have also made progress in the construction of sense, antisense, and green fluorescent protein (GFP) coupled vectors for proteins of interest.

Body

1. Prepare and submit manuscript with data from this application and tasks #1 and #2 from the first year.

The manuscript envisioned at the time of proposal submission was to contain data showing the differential expression of genes important in fibrinolysis in tumor-associated endothelial cells as opposed to quiescent endothelial cells. These data were initially generated from our isolation of endothelial cells from mouse xenograft tumors and mammary fat pads, followed by PCR of plasmid libraries generated from mRNA from isolated cells. That task was part of the proposal application for this grant. Confirmation of the differential expression of fibrinolytic genes in tumor-associated endothelial cells will be obtained from microdissected endothelial cells from xenograft and human tumors.

We currently have microdissection capability with both micromanipulator and laser capture microdissection. However, we have had difficulty in accurately amplifying mRNA from microdissected cells. In connection with another grant funded by the US Army Breast Cancer program (cDNA Libraries from Microdissected Cells in Pathological Specimens, DAMD17-00-1-0272), we have been developing methods for accurate and sensitive amplification of small quantities of total RNA. We are in the process of preparing our first manuscript for the microdissection project. In that manuscript, we show amplification of mRNA from 100 pg total RNA to a level of 100,000 times with preservation of representation of all but the most rarely-expressed genes. We are now in a position to apply these amplification techniques to microdissected endothelial cells and complete the data for this first paper. We foresee that we will be able to obtain these data over the next year.

2. Perform immunofluorescence for tPA, uPA, uPAR, annexin II, PAI-1, and MT1-MMP in the ring assay and analyze by confocal microscopy.

As reported last year, we were able to obtain positive immunofluorescence for PECAM-1, a membrane protein expressed by endothelial cells at relatively high levels. However, our ability to stain this protein in endothelial cells in fibrin gels has been inconsistent over the last year, in spite of our varying many of the fixation and staining conditions in a multitude of

permutations and combinations. Moreover, when we attempt to stain a less abundantly expressed protein, the results are uniformly negative. We are able to identify endothelial tubes in the mammary vessel assays by virtue of their ability to specifically take up DiI-labeled acetylated LDL. The DiI label imparts a red fluorescence to the endothelial cells (Figure 1). This method of identifying endothelial tubes in the mammary vessel assays has the advantage that it can be used for *in vivo* staining during the culture period, before fixation. In this way, the progress of the endothelial cell outgrowths can be monitored in the culture and cultures harvested for fixation at opportune times. DiI-AcLDL labeled structures can be quantified by image analysis to measure degree of outgrowth. We have concluded that DiI-AcLDL labeling is a better ay to identify endothelial cells in cultures than immunofluorescence for PECAM-1. Because the DiI-AcLDL labeling can be observed in live cultures, we can acquire images during assay culture and quantify Di-AcLDL labeled structures at different time points for the same assay.

We will be able to perform experiments blocking one or another of the fibrinolytic protein family members by including blocking antibodies in the fibrin gels and overlain medium. However, since our immunofluorescence experiments have been negative, we might not be able use this technique to localize the proteins of interest to the tips of invading endothelial cells. Because of this, we are constructing viral expression vectors in which the protein of interest is coupled to green fluorescent protein (GFP). These vectors will be used to infect the mammary vessel assays. Currently, we have on hand a vectors containing GFP-coupled uPAR and are in the process of constructing ones for MT1-MMP, tPA, and annexin II. These cDNAs are already on hand. The new vectors can also be used with ecotropic packaging to infect endothelial cells in mice for the xenograft experiments to be done in the coming year. This would obviate the need for immunofluorescence in sections of these tumors, as the proteins of interest would be labeled with GFP.

3. Finish construction of sense and antisense tPA and MT1-MMP retroviral vectors, and transfect packaging cells.

We have currently completed construction of sense and antisense viral vectors for MT1-MP and annexin II, the receptor for tPA. The tPA vectors are under construction. Packaging cells were transfected with the MT1-MMP antisense vector for last year's report, and supernatants used to infect mouse EOMA cells. Although packaging cells were transfected with high frequency, as evidenced by a GFP vector included in the transfection as a control, ecotropic supernatants were not very infective for mouse EOMA cells. However, when the packaging cells were selected with G418 (the vector confers resistance), their expression of MT1-MMP was abrogated by the antisense vector (not shown). These experiments were presented in last year's report.

4. Test retroviral vectors' infectivity and expression in the ring assay.

We produced ecotropic retrovirus expressing GFP in Phoenix-Eco packaging cells and harvested the supernatants. Mammary vessel pieces were soaked in the supernatants for 30 minutes prior to including them in the fibrin matrix. Additionally, supernatants were incorporated into the fibrin gel itself and used to overlay the cultures. Unfortunately, we were not able demonstrate that there were infected cells in the ring assay. This may have been due to low titer of the virus, although we were unable to detect even one GFP-expressing cell. One would expect that cells migrating from the explant would be mitotic, a requirement for infection by retrovirus. We have

not assessed the frequency of mitosis in the explants, but again feel it would be unusual if there were absolutely no dividing cells. Therefore, we think our failure to detect any GFP-expressing cells is due to failure of the retrovirus to infect the cells of the explant or failure of the vector to drive expression. Primary cells are notoriously difficult to transfect and endothelial cells are among the most difficult. Moreover, primary cells may not express transfected proteins to the same degree as immortal cells.

We may be able to improve our ability to obtain expression in the mammary vessel cultures by one of several possible strategies. First, we could switch to lentiviral or adenoviral vectors. These viruses are able to infect nondividing cells and could improve our infectivity. Second, we could conduct the planned experiments using immortal endothelial cell lines that could be transfected in tissue culture and then placed in fibrin matrices. We view this second solution as a last resort since it does not have the advantages of the mammary vessel assay in providing a realistic angiogenesis model. Therefore, we will continue to explore ways to induce antisense expression or expression of GFP-coupled proteins in the mammary vessel assay.

Key Research Accomplishments:

1. Development of a method to label endothelial tubes during culture of the mammary vessel assays which persists after fixation and can be used to quantify cellular outgrowths from the mammary vessels.

2. Construction of a retroviral vector encoding one of the proteins of interest coupled to GFP that can be used to infect the mammary vessel assays and mouse tissues in xenograft experiments.

3. Construction of retroviral vectors with sense and antisense coding sequences for MT1-MMP.

4. Preliminary tests of retroviral vectors' infectivity in the mammary vessel assay.

Reportable Outcomes:

McLeskey, S.W., Watson, P., and Passaniti, A. Ex Vivo Angiogenesis Assay Using a Mammary Vessel Explant, 94th Annual Meeting of the American Association for Cancer Research, Washington, DC, July, 11-14, 2002

Conclusions: The past year has seen progress in vector construction and identification of endothelial cells in the mammary vessel assays during culture. Difficulties have been encountered in immunofluorescence of mammary vessel cultures in fibrin matrices and the alternative strategy of GFP-coupled proteins has been developed. Difficulties in infection of the mammary vessel with retroviruses are probably due to a combination of low viral titer and rare dividing cells in the explants. Use of alternative viral systems will be attempted during the next year, as well as transfection of endothelial cell lines in culture followed by embedding of the transfected cells in a fibrin matrix.

These studies have increasing importance because of recent reports of the success of an antibody-based antiangiogenic therapy in patients with metastatic colon cancer[1]. The antibody in these studies was directed against VEGF-A, a very important angiogenic factor. However, previous to this trial, there were multiple trials of multiple antiangiogenic therapies in multiple types of cancer that produced disappointing results. The current successful trial is proof that this approach may be a viable form of therapy in cancer, but it does not explain the failure of similar approaches in other trials. Therefore, although the current success can serve to spur us on, the challenge remains to identify viable antiangiogenic targets in breast and other cancers. Thus, the work we are doing with the mammary vessel assay has become even more important with the success of the current antiangiogenic trial.

References:

1. N. H. Fernando and H. I. Hurwitz, Inhibition of vascular endothelial growth factor in the treatment of colorectal cancer *Semin.Oncol.* 30, 39-50 (2003).

Appendices:

1. Abstract presented to the American Association for Cancer Research, July, 2003.
2. Figure 1.

Mammary vessel angiogenesis *ex vivo*

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Breast carcinomas recruit mammary gland vessels that enhance tumor growth and metastasis. To establish a more physiological model of breast cancer angiogenesis, we adapted the fibrin-based aortic ring assay to mammary vessels. In this assay, a segment of mammary blood vessel is placed in a fibrin matrix that stimulates the formation of tube-like structures composed of invasive endothelial cells. These vessels can be quantitated and evaluated by immunofluorescence using antibodies reactive to molecules of interest. Young female mice were treated with subcutaneous estrogen pellets to stimulate mammary gland development. Two weeks following pellet implantation, the central vessel (the epigastric vein) of the inguinal mammary fat pad is harvested and cut into short segments. Chambered coverslips were prepared by thrombin polymerization of a small layer of a fibrinogen/growth medium mixture in the bottom of the chambers. Pieces of the mammary vessel were placed in chambered coverslips on top of the fibrin clot and another layer of fibrin was added to the top of the vessel pieces. After approximately one week, cells migrated from the explant into the matrix, some forming branched, tube-like structures. A subset of these tubular structures are composed of endothelial cells, which can be identified by immunofluorescence for PECAM-1, von Willebrand factor, and DiI-labeled acetylated LDL uptake. The chambered coverslip enables analysis by confocal microscopy, with 3-dimensional reconstruction of the endothelial network. Image analysis on the DiI-AcLDL labeled branching structures was used to quantitate the number and length of tubes and the number of branch points. Comparison of angiogenic factors, such as bovine pituitary extract, FGF-2, and/or VEGF, on the incidence of sprouts was done. Time courses and dose response curves were analyzed. Development of this *ex vivo* angiogenesis assay specific to breast endothelium in a matrix relevant to the carcinoma environment will enable elucidation of the role of molecules in breast tumor angiogenesis and will have applicability for many molecular families and matrix materials.

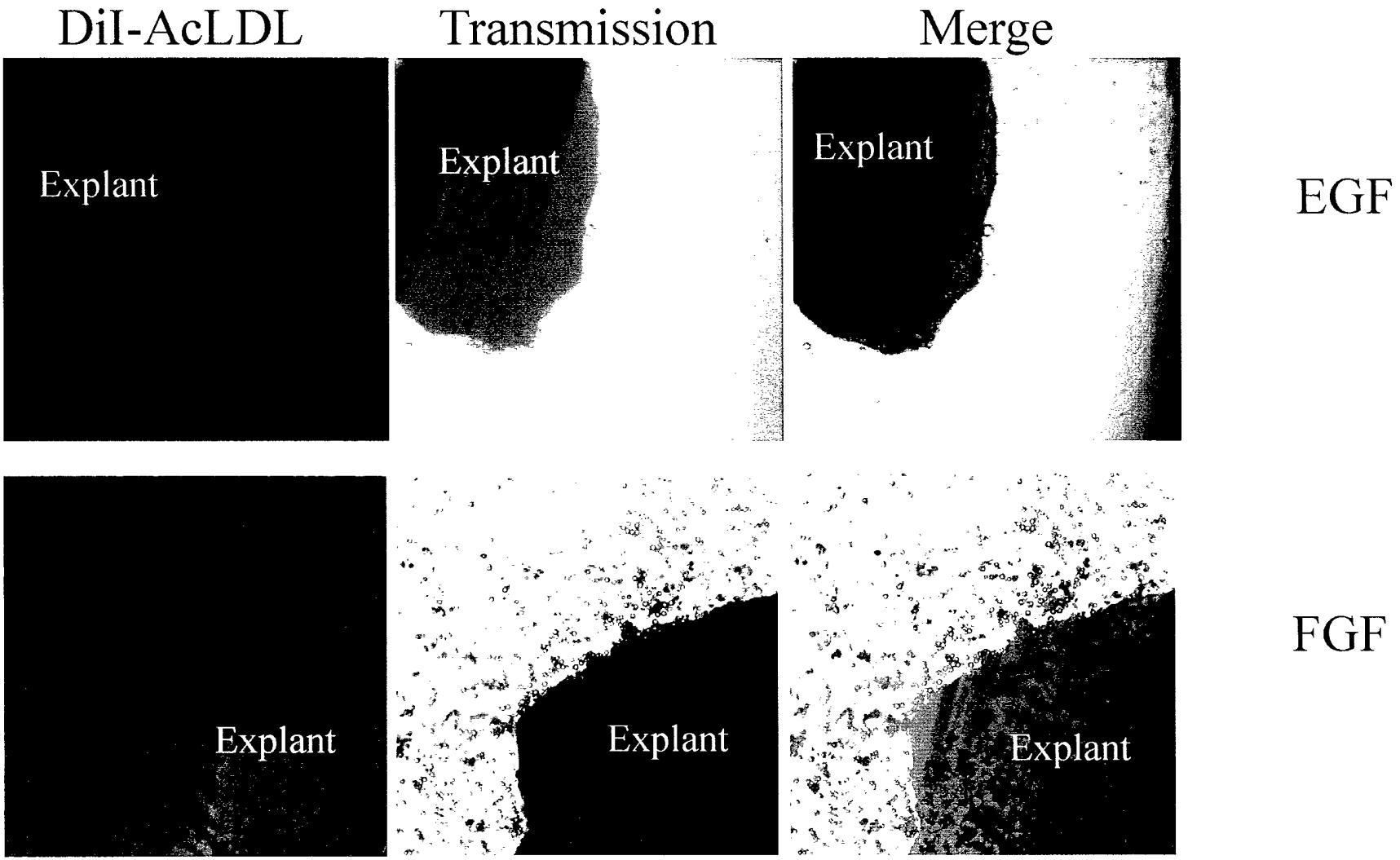

Figure 1. Live imaging of DiI-AcLDL uptake in mammary vessel implants growing in fibrin matrix. Mammary vessel explants were placed in fibrin matrix made with medium 199(E) supplemented with 10% fetal bovine serum and EGF (500 pg/ml) or FGF (25 ng/ml). For both treatments, multiple cells exit the explant and invade the fibrin. However, only a subset of the cells are positive for DiI-AcLDL uptake (red cells in 2 panels on the left and in merged images). The positive cells are much more numerous with FGF treatment, and also form linear structures.