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Clinical studies have indicated that high breast tumor levels of plasminogen activator inhibitor-1 (PAI-1; SERPINE1) are associated with an increased risk for metastasis, decreased patient survival, tumor angiogenesis and overall poor prognosis. Since PAI-1 is required for both the initiation of tumor-dependent angiogenesis and inhibition of capillary regression, a targeted molecular genetic approach was utilized to ablate PAI-1 synthesis in endothelial cells using antisense PAI-1 constructs and dominant-negative approaches. Such targeting provided proof-of-principle that reduced PAI-1 synthesis and inhibited capillary network formation by immortalized endothelial cells. Adaptation of this methodology to primary endothelial cells provided a means to "tag" cells with a PAI-1-GFP chimeric protein and confirmed that such engineered cells were capable of being incorporated into a hybrid capillary network in vitro. Importantly, use of a dominant-negative version of USF-1, a HLH-LZ transcription factor important in PAI-1 gene control, also attenuated PAI-1 expression in response to growth factor stimulation. Collectively, these results suggest that combinational approaches (PAI-1 antisense/dominant-negative USF-1) may form the basis for more efficient breast cancer anti-angiogenic gene therapy.

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Introduction

High tumor levels of plasminogen activator inhibitor type-1 (PAI-1; Serpine1) are consistently associated with an increased risk for metastasis, significantly decreased patient survival and an overall poor prognosis (1,2). The role of PAI-1 as a determinant in tumor progression is particularly relevant in the case of breast cancer where elevated PAI-1 expression in the primary breast carcinoma signals an aggressive angiogenic response (3-10). Tumor-initiated angiogenesis requires proteolysis of the endothelial basement membrane, migration of endothelial cells through the extracellular matrix (ECM) toward the angiogenic stimulus and continued endothelial proliferation behind the migrating front (11-13). Stimulated endothelial cell locomotion requires cycles of ECM adhesion-deadhesion and precise control of the pericellular proteolytic environment (12-14). PAI-1 functions in this process to limit plasmin generation by inhibiting the catalytic activity of urokinase plasminogen activator (uPA) (15,16) modulating, thereby, uPA-dependent ECM degradation and *in vivo* cell motility (17-19). While endothelial cell migration and capillary sprouting requires proteolysis (12,20,21), excessive protease activity prevents the coordinated assembly of endothelial cells into capillary structures highlighting the requirement for an appropriate proteolytic "balance" for a successful angiogenic response (22,23). Genetic studies *in vivo*, moreover, have implicated PAI-1 as an important regulator of this balance (24,25). Indeed, PAI-1 is expressed specifically in angiogenic "cords" and migrating endothelial cells as well as in stromal cells in direct contact with the sprouting neovessels but not in the quiescent endothelium (26-28). Most significantly, PAI-$1^{-/-}$ mice are incapable of mounting an angiogenic response either to transplanted tumors or implants of potent angiogenic growth factors (24,27,29); both tumor-associated angiogenesis and tumor invasiveness were restored by injection of PAI-1 expressing adenovirus (24,27). PAI-1 appears to promote angiogenesis specifically by inhibition of plasmin proteolysis, thus preserving an appropriate matrix scaffold for endothelial invasion as well as providing critical stability to the primitive tumor neovessels (18,24,27). Indeed, recent studies have shown that uPA-mediated plasmin generation activates MMP1 and 9 resulting in capillary regression (30). Inhibition of PAI-1 activity with neutralizing antibodies accelerates, whereas exogenous PAI-1 inhibits, capillary regression indicating that endogenous PAI-1 is the major negative regulator of this process (30). Continued PAI-1 expression by the formed capillary structures is required to maintain their stability and, in fact, to prevent regression. Our use of inducible vectors to disrupt PAI-1 synthesis, even in formed capillary structures, will be one novel approach to address the important question of whether PAI-1 targeting can have a therapeutic benefit on existing angiogenic networks. This is an important issue for the treatment of established primary tumors and their developed distant metastases.

Body of Report

Our work has focused on defining molecular controls on PAI-1 gene expression in normal and transformed cells and clarifying the role of the PAI-1 protein on cellular growth and invasive behavior. We have shown that it is possible to genetically manipulate PAI-1 synthesis in endothelial cells transfected with sense and antisense PAI-1 expression vectors (32,33). We hypothesize, based on our own preliminary and published data, as well as on work done by others (summarized in **Introduction**), that molecular targeting of PAI-1 expression can disrupt both the initial as well as the developed angiogenic response to tumor-derived stimuli. We have recently published a review article that details our in vitro data in support of this contention (Kutz and Higgins, 2004; appended). We propose that targeted attenuation of PAI-1 expression in the developing neovasculature that develops following implantation of human breast carcinoma cells into immunodeficient mice will inhibit the angiogenic response and limit subsequent tumor growth. We further suggest that human endothelial cells genetically-engineered to express inducible PAI-1 antisense transcripts may "home" to sites of active tumor-initiated angiogenesis, incorporate into the developing capillary network, and destabilize the tumor vasculature upon inducible ablation of PAI-1 synthesis. We expect that such engineered cells will ultimately serve as a therapeutic resource for inducible anti-angiogenic therapy of human breast cancer.

To achieve these aims, the goals in Task 2 in the originally proposed **Statement of Work** were addressed in year 02 of this study.

Task 2. To assess the ability of infused genetically-engineered human endothelial cells, inducible for expression of PAI-1 antisense transcripts, to incorporate into the developing human breast tumor vasculature and disrupt the supporting capillary network.

a. Establish that infused green fluorescent protein-"tagged" human endothelial cells are incorporated into the developing angiogenic network.

b. Assess the ability of induced PAI-1 antisense transcript expression in vasculature-incorporated human endothelial cells to disrupt tumor-associated capillary vessels and inhibit tumor growth.

Based on the data summarized in the **Introduction**, our working hypothesis is that genetically-induced temporal changes in the expression of PAI-1 may influence endothelial cell migration, capillary formation and/or capillary network stability. Effort in year 02 of this study was devoted to confirmation that the genetic constructs (PAI-1 antisense expression vectors) developed would, in fact, result in attenuated PAI-1 synthesis when transfected into human

endothelial cells (both primary cultures of human endothelial cells as well as the established HMEC-1 line of human microvessel endothelial cells). Transfection studies established that our selected rat PAI-1 mRNA coding sequence, when cloned in antisense orientation into CMV promoter-driven constructs (i.e., in the Rc/CMV expression vector backbone), effectively attenuated PAI-1 synthesis in both mouse (MS1) and rat (T2) endothelial cells (detailed in year 01 Progress Report). We have established (in immortalized rat cells) that genetically-targeted down-regulation of PAI-1 synthesis can inhibit in vitro tubulogenesis likely through the combined action of "scaffold" unstability and vessel regression (described in appended paper by Kutz and Higgins, 2004). Rc/CMV plasmid vectors that drive expression of a full-length PAI-1 cDNA insert, cloned in antisense (IAP) orientations, were constructed to be under control of a CMV promoter. To assess the success of Rc/CMVIAP driven down-regulation of PAI-1 synthesis and matrix accumulation, saponin-extracts of ^{35}S-methionine-labeled cells were separated by gel electrophoresis and proteins visualized by fluorography. One derivative (4HH) did not express detectable PAI-1 protein nor accumulate PAI-1 in the matrix. Wild-type T2 cells formed highly-branched and anastomizing capillary networks when suspended in a complex support matrix consisting of a 3:1 misture of Vitrogen-Matrigel. Many of these tubular processes had clearly evident lumens. Extensive sprout formation was evident at the tips of T2 branches, moreover, indicative of both invasive and differentiated compartments. PAI-1$^{-/-}$ 4HH cells, in contrast, failed to construct stable tubular structures and extensively degraded the gel matrix. Thus, at least within the setting of immortalized rodent endothelial lines, our targeting strategy (i.e., delivery of constitutively-expressing PAI-1 antisense constructs) has the expected outcome on gene expression and the tubulogenic phenotype.

To address specifically the issue that genetically-engineered human endothelial cells are capable of incorporation into a developing angiogenic network, the pEGFP-1 vector in which the GFP insert was expressed as a fusion protein with PAI-1 and driven by PAI-1 promoter sequences was used (Kutz and Higgins, 2004; appended). Human microvessel endothelial cells (HMEC-1) were transfected with pEGFP-1 and the ability of these cells to incorporate into a tubulogenic network confirmed by co-culture with T2 cells on Matrigel-coated surfaces. Once proof-of-principle was established, primary cultures of mouse endothelial cells were prepared to confirm that "normal" (i.e., non-immortalized) cells could be similarly "tagged" and incorporate into hybrid vessels (a paper describing both preparation of engineered endothelial cells and their ability to produce chimeric capillary networks is in preparation and will be forwarded to USAMRMC upon acceptance for publication).

We are currently testing tet-inducible PAI-1 antisense constructs in both human (HMEC-1) and primary mouse endothelial cells to determine if expression induction will produce network instability results similar to those induced by the constitutively-expressed Rc/CMVIAP vector in immortalized cells (described in Kutz and Higgins, 1004; appended).

We have also determined that a major transcriptional regulator of PAI-1 expression is the transcription factor USF-1. When expressed as a dominant-negative construct (USF-1A), this factor markedly suppresses PAI-1 transcription. We are currently investigating the possibility that PAI-1 antisense and USF-1 expression vectors will be therapeutically useful as a combinational anti-angiogenic strategy.

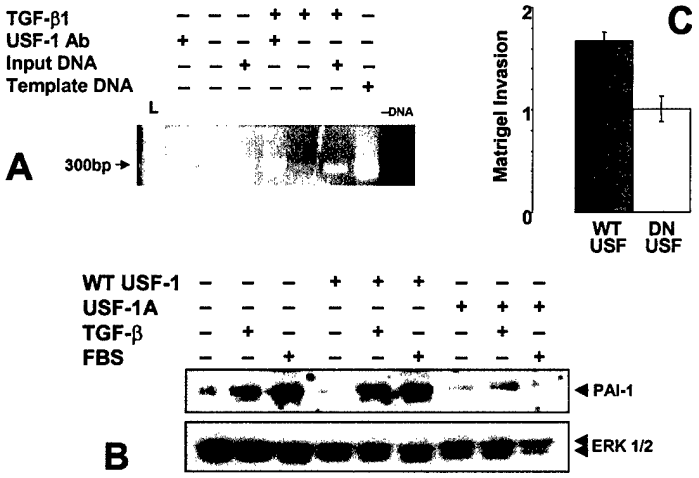

Figure 1. Dominant-negative USF-1 (USF-1A) attenuates TGF-β1-induced PAI-1 protein expression and barrier invasion. USF-1 was confirmed to be an endogenous PAI-1 promoter PE2 region DNA-binding factor by chromatin immunoprecipitation using antibodies to USF-1 **(A)**. Controls included addition of sonicated cellular DNA but without immunoprecipitation (input DNA), addition of PAI-1p806-Luc DNA as a control template for PCR (template DNA) and PCR reaction mixtures with H_2O in place of DNA (–DNA). L = sizing ladder. To evaluate the effects of molecular genetic interference with USF function, RK cells were untransfected or transfected with CMV-driven WT USF-1 or CMV-driven dominant-negative USF-1A. Whole cell lysates from quiescent cultures or cells stimulated with 20% FBS or 1 ng/ml TGF-β1 were collected, separated on 9% SDS-PAGE, proteins transferred, and blots probed with anti-rat PAI-1 antibody **(B)**. Transfers were reprobed with antibodies to ERK1/2 to assess protein loading. Invasion of RK cells expressing either WT (USF-1) or dominant-negative (USF-1A) expression constructs was compared ± TGF-β1 **(C)**. Data in **(C)** specifically represent TGF-β1-induced fold-invasion of Matrigel-coated barriers (i.e., TGF-β1-associated invasion/untreated controls); histogram represents mean±standard error from 8 migration evaluations.

Key Research Accomplishments

It was confirmed (in immortalized cells) that genetic targeting of PAI-1 transcripts resulted in attenuation of PAI-1 synthesis and inhibition of capillary network formation.

Human (HMEC-1) and primary mouse endothelial cells were successfully "tagged" with an expression vector encoding a chimeric PAI-1 green fluorescent protein marker driven by PAI-I promoter sequences.

"Tagged" endothelial cells were capable of producing hybrid capillary networks when co-cultured with tubulogenic T2 cells on Matrigel-coated surfaces.

PAI-1 gene expression was positively regulated by upstream stimulatory factor-1 (USF-1), a member of the helix-loop-helix-leucine zipper transcription factor family. Use of a dominant-negative USF-1 construct effectively attenuated growth factor-induced PAI-1 expression. These findings suggest that combinational approaches using antisense PAI-1 and dominant-negative USF-1 constructs may form the basis for more efficient anti-angiogenic gene therapy.

Tet-inducible constructs are presently under development.

Reportable Outcomes

All genetically-engineered immortalized (HMEC-1, T2) cells will be maintained in the laboratory of the PI. The pEGFP-1 and dominant-negative USF-1A plasmids are also stored as frozen stocks in the laboratory. Tet-inducible systems are currently being developed in human breast cancer cell lines (MCF-7), human epithelial cells (HaCaT) and in the T2 + HMEC-1 cells as well as primary endothelial cells. These resources will be made available upon request to members of the scientific community engaged in breast cancer research. Dr. Stacie Kutz has devoted 30% of her time/effort to this project during the report period. She is largely responsible for PAI-1 gene control studies in T2 cells including development of USF-1 controls on PAI-1 expression. Qunhiu Ye has replaced Jianzhong Tang on this project.

Conclusions

The present work is based largely on our continuing hypothesis that molecular targeting of PAI-1 expression in angiogenic vessels represents a unique gene therapy approach that has the distinct advantages of (1) potential cell-specific construct targeting and (2) a high likelihood of success when directed to established angiogenic "beds". Our laboratory has had considerable experience in the construction and utilization of both sense and antisense PAI-1 expression vectors (summarized in the **Introduction**), and more recently, in the design of small molecule inhibitors of PAI-1 function and PAI-1 gene expression in endothelial cells incorporated into formed capillary structures, constitutes an important approach to address the critical question of whether PAI-1 targeting can have a therapeutic bvenefit on existing angiogenic networks. We envision that a multifaceted attempt to target PAI-1 gene expression in both breast carcinoma cells (see **Introduction**) and in the collateral tumor vascular network would likely require cell type-specific expression modulation control. The goals described in this funded program, in conjunction with the general scope of work ongoing in the laboratory of the PI, reflect these separate but focused efforts to utilize gene therapy approaches to maximize a positive outcome for the management of human breast cancer.

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