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Endothelial cells must express plasminogen activator inhibitor type-1 (PAI-1) in order to undergo tumor-dependent angiogenesis. The PAI-1 gene has emerged, therefore, as an important candidate target for gene therapy of human breast cancer. In year 02, we continued work to define the effects of expression vector-driven PAI-1 in endothelial cells functionally PAI-1-null as well as in cells in which the gene was molecularly disrupted. Restoration of cell motility, a requirement for angiogenic network formation, was evident in cells transfected with a PAI-1 expression vector. To initially determine the level of PAI-1 required for cell migration, primary cultures were established from PAI-1 knockout (PAI-1-/-) and wild-type (PAI-1+/+) mice. PAI-1-/- cells were motile deficient relative to PAI-1+/+ cells; migratory ability could be "rescued" in the PAI-1-/- genetic background by exogenous addition of low (1 ug/ml) levels of recombinant PAI-1. Higher PAI-1 concentrations (10 ug/ml) inhibited migration consistent with the hypothesis that cellular motile processes such as angiogenesis require "balanced proteolytic activity" and occur within a "window" of PAI-1 concentrations. These data support our original contention that the PAI-1 gene is an accessible anti-angiogenic target.

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INTRODUCTION

Continued growth of a malignant tumor beyond a certain critical size is dependent on the development of a network of feeder blood vessels (1,2). Findings from a number of laboratories have indicated that this "angiogenic switch" is highly-dependent on the temporally-regulated and focalized localization of several extracellular proteases and protease inhibitors involving members of both the plasmin-based and metalloproteinase cascades (3,4). *In vitro* analysis of the requirements for the formation of endothelial tubular networks in various culture model systems implicated both urokinase plasminogen activator (uPA) and its fast-acting type-1 inhibitor (PAI-1) as necessary to achieve complete angiogenesis, consistent with the "balanced proteolysis" concept of endothelial cell migration (5-7). Recent data in mice genetically-engineered to be deficient in expression of genes that encode specific elements of the plasmin activation system has confirmed the critical importance of PAI-1 synthesis in tumor-induced angiogenesis (5-7). Indeed, the absence of host PAI-1 completely inhibited local invasion and vascularization of transplanted malignant tumors in PAI-1 null mice (5,6). This inability to mount an angiogenic response, moreover, prevented invasive growth by an aggressive and metastatic tumor type (5,6).

Breast tumors with high PAI-1 levels, in particular, are fast-growing carcinomas with a well-developed angiogenic network, a high incidence of metastatic spread, early recurrence and poor prognosis (8, reviewed in 9). PAI-1 as a determinant in aggressive growth behavior is particularly important in the progression of mammary carcinoma. Most recently, PAI-1 has been shown to be markedly promigratory for invasive breast cancer cells, an effect attenuated by direct inhibition of PAI-1 function (10). These data highlight the potential relevance of PAI-1 modulation as a means to treat human breast cancer and complement our own work on genetic targeting of PAI-1 mRNA transcripts with subsequent attenuation of cellular motile traits (11-13).

Preliminary data from this laboratory clearly indicate that endothelial cells must express PAI-1 in order to undergo angiogenesis in response to co-culture with human breast tumor cells. Thus, the PAI-1 gene has emerged as an important candidate target for anti-angiogenic-based gene therapy of human cancers. The goals of this investigation are to determine the level of endothelial cell PAI-1 expression necessary for development and maintenance of the breast tumor-induced angiogenic phenotype in a 3-D culture model of breast tumor-stromal-endothelial cell interactions that mimics the *in vivo* disease state. Gene therapy approaches using antisense vector constructs as well as homologous recombination methods previously developed in this laboratory are utilized to directly disrupt PAI-1 gene expression in cultured endothelial cells. The consequences of this targeted disruption on the ability of endothelial cells to form branching angiogenic networks in response to co-culture with human breast cancer cells will be evaluated. "Rescue" experiments were designed using PAI-1-null endothelial cells transfected with a sense expression vector (Rc/CMVPAI), or PAI-1 "add-back" approaches, to assess the level of PAI-1 synthesis required for initiation of the angiogenic switch (both basal and tumor-stimulated angiogenesis). Finally, a feasibility study will be initiated to evaluate the therapeutic usefulness of endothelial cell-specific targeting of anti-angiogenic constructs on the breast carcinoma-induced angiogenic response. Such investigations represent the initial necessary steps toward the design of clinically-relevant genetic constructs. This study will constitute the first comprehensive assessment of PAI-1 genetic therapy as an approach to inhibit growth of human breast cancers by targeting a gene essential for the angiogenic process.

BODY

Studies in year 02 of this investigation continued to be directed to the goals described in Task 1 of the approved statement of work.

Task 1. To quantify the effects of vector-driven PAI-1 synthesis on the human breast tumor-induced angiogenic phenotype in transfectant T2-null endothelial cells (a clone in which the endogenous PAI-1 gene was disrupted by molecular targeting). This phase of the work is necessary to determine the threshold level of PAI-1 expression required for cultured endothelial cells to undergo a switch to an angiogenic phenotype.

a. develop a panel of T2-null derived endothelial cells which synthesize differing levels of vector-driven PAI-1 mRNA and protein as a consequence of transfection with a constitutively active (CMV promoter-based) PAI-1 expression vector (months 1-8).

b. correlate levels of PAI-1 expression for each transfectant T2-null-derived endothelial cell line with the extent of the angiogenic response (i.e., ability to form branched tubular networks and migrate over planar surfaces or through matrix barriers) induced by co-culture with human breast tumor cells and tumor-derived stimuli (months 9-14).

We created PAI-1 functional "knockout" endothelial cells using T2 cells as the parental strain by transfection of a PAI-1 antisense expression vector (10,11). While T2 cells formed tubular networks when placed in culture over a Matrigel substrate and expressed high levels of PAI-1 mRNA, antisense vector-transfectants did not exhibit an angiogenic response (**Figure 1**). T2 wild-type endothelial cells did exhibit an angiogenic switch when plated on Matrigel-coated surfaces. A robust angiogenic response (complete with gel invasion, sprout formation from the lateral surface of the tubular structures, complex branch patterns in 3-D orientation), however, required co-culture with MDA-MB-231 human breast carcinoma monolayers. Northern blot analysis confirmed that the T2/IAP antisense cell line did not express PAI-1 transcripts (**Figure 1**) and that the down-regulation of expression achieved was, at the protein level, specific for PAI-1 (11). A similar approach was used to create the PAI-1 functionally-null cell line 4HH (12). While wild-type T2 cells were capable of forming extensively branched capillary networks in a complex 3-D gel consisting of a 3:1 mixture of Vitrogen-Matrigel, 4HH cells were incapable of lattice formation and effectively degraded the gel scaffold **(Figure 2)**.

Since these data provided proof of principle (i.e., that inhibition of PAI-1 ablated *in vitro* angiogenesis), we derived a stable PAI-1-null T2 cell line by molecularly-disrupting the endogenous gene with a targeting vector. The resulting cell line (T2-null) similarly failed to form branched angiogenic networks *in vitro*. T2-null cells were subsequently transfected with the PAI-1 sense expression vector Rc/CMVPAI (8,9) (**Figure 1**) and four neomycin (G418)-resistant cell lines were derived that varied in the level of vector-driven PAI-1 transcript expression and migratory ability (**Table 1**). Unlike T2 cells (or the related 4HH cell line) where

PAI-1 expression is ablated by antisense PAI-1 (9), antisense c-*fos* (10) constructs or by targeted gene disruption and which are poorly motile and non-angiogenic (**Table 1**), vector-mediated PAI-1 "rescue" restored (to varying extents) cellular motile ability. We are presently quantifying the PAI-1 protein synthesized by the four transfected lines and determining the actual extent to which these cells exhibit a basal angiogenic phenotype (in response to culture on Matrigel-coated surfaces) or a stimulated angiogenic response in the MDA-MB-231/Matrigel or collagen co-culture overlay system. This information is important as it will define for the first time the actual level of PAI-1 down-regulation (by antisense expression vector delivery) required to achieve a therapeutic response (i.e., inhibition of angiogenesis).

Table 1. Effect of PAI-1 expression targeting and vector "rescue" on cell motility using a quantifiable assay of planar locomotion[a]

Cell Line	Method of PAI-1 Expression Disruption	Relative Motility[b]
T2	None (wild-type)	100
T2/IAP	Rc/CMVIAP transfection	40 ± 8
T2-null	Targeted disruption vector	37 ± 5
T2-nullR1	Disruption vector⇨Rc/CMVPAI	56 ± 4
T2-nullR2	Disruption vector⇨Rc/CMVPAI	78 ± 9
T2-nullR3	Disruption vector⇨Rc/CMVPAI	93 ± 7
T2-nullR4	Disruption vector⇨Rc/CMVPAI	49 ± 3

[a] Reference #9 has been appended regarding details of the directed planar migration system used. Cells were grown to confluency and the media changed to fresh DMEM/10 for maintenance in a post-confluent condition for an additional 3 days. Alternatively, confluent cultures were maintained in serum-free DMEM for 3 days for initiate a contact-inhibited/serum-deprivation state of growth arrest. Wounds were created by pushing the narrow end of a sterile P1000 plastic pipette tip (Continental Laboratory Products, San Diego, CA) through the monolayer. Cultures were incubated in the existing media for times indicated in the text. Wound closure was assessed by time-lapse photomicroscopy and injury repair rates calculated, as a function of time, from measurements made utilizing an inverted microscope fitted with a calibrated ocular grid.

[b] Relative motility = distance migrated in 24 hours compared to wild-type T2 cells.

The data presented in **Figures 1** and **2** as well as in **Table 1** implicate PAI-1 as an important element in the cellular motile process. To establish this conclusively, cells were isolated from PAI-1-null (PAI-1$^{-/-}$) mice and their wild-type (PAI-1$^{+/+}$) counterparts for direct comparison in planar motility assays (12,13). PAI-1$^{-/-}$ cells were clearly deficient in planar motility exhibiting a migratory rate <20% that of PAI-1$^{+/+}$ cells **(Figure 3)**. "Rescue" experiments were designed using our standard *in vitro* model of monolayer denudation injury and ocular grid measurements of cellular migration. Recombinant PAI-1 was added to "wounded" cultures of PAI-1$^{-/-}$ cells in final concentrations of 1 and 10 μg/ ml. Low concentrations of PAI-1 (i.e., 1 μg) effectively stimulated migration of PAI-1$^{-/-}$ cells with rescued motile rates closely approximating that of wild-type PAI-1$^{+/+}$ cells **(Figure 3)**. Higher concentrations of PAI-1 actually reduced the migratory response consistent with the need for "balanced proteolysis" to achieve an optimal angiogenic response (13).

KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments achieved during the report period are as follows:

1. Confirmed that targeted ablation of endothelial cell PAI-1 gene expression, using antisense expression vectors (Rc/CMVIAP) or homologous recombination, resulted in marked inhibition of cell motility and an inability to form angiogenic networks on Matrigel-coated surfaces and in the MDA-MB-231 human breast carcinoma co-culture system.

2. Developed transfection techniques to introduce sense PAI-1 expression vectors (Rc/CMVPAI) into endothelial cells in which the PAI-1 gene was disrupted by homologous recombination (T2-null) to assess the effects of expression "rescue" on cell motility and Matrigel-induced angiogenesis.

3. Created 4 such rescued cell lines (T2-nullR1-4); each line exhibited a unique pattern of locomotion in the monolayer denudation injury model of induced cell motility that was statistically significantly different (Student's t-test) from the rate of migration characteristic of the T2-null cells.

4. Developed culture of cells from both PAI-1$^{-/-}$ and wild-type PAI-1$^{+/+}$ mice for use in planar migration assays. Direct comparisons indicated that primary cells derived from PAI-1 knockout mice had a marked migratory deficit compared to their wild-type counterparts.

5. Addition of recombinant PAI-1 to cultures of PAI-1$^{-/-}$ cells restored their migratory rate to approximate that of wild-type cells. This genetic approach to motility assessments confirmed that PAI-1 is a critical element in the cellular motile program. These finding support the likelihood that the overall experimental strategy to target PAI-1 expression in tumor angiogenesis will result in the design of genetic approaches that will have a defined therapeutic applicability.

REPORTABLE OUTCOMES

1. The following manuscripts have been published or are in press and cite support from grant DAMD17-00-1-0124.

   Kutz, S.M., Hordines, J., McKeown-Longo, P.J., and Higgins, P.J. (2001) TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. Journal of Cell Science 114:3905-3914.

Providence, K.M., Staiano-Coico, L., and Higgins, P.J. (2001) A quantifiable *in vitro* model to assess the effects of PAI-1 gene targeting on epithelial cell motility. In: ;Wound Healing: Methods and Protocols (DiPietra, L., Editor), Humana Press.

Samarakoon, R. and Higgins, P.J. (2002) MEK/ERK pathway mediates cell-shape-dependent plasminogen activator inhibitor type-1 gene expression upon drug-induced disruption of the microfilament and microtubule networks. Journal of Cell Science 115 (accepted for publication).

Providence, K.M., White, L.A., Tang, J., Gonclaves, J., Staiano-Coico, L., and Higgins, P.J. (2002). Epithelial monolayer wounding stimulates binding of USF-1 to an E box motif in the plasminogen activator inhibitor type-1 gene. Journal of Cell Science 115 (accepted for publication.

Samarakoon, R. and Higgins, P.J. (2002) pp60$^{c\text{-}src}$ integrates cytoskeletal dynamics, MAP kinase activation and PAI-1 gene regulation. In: Recent Research Developments in Biochemistry (in press).

1. A new Idea proposal has been submitted to the DOD Breast Cancer Program entitled "Inducible Anti-Angiogenic Gene Therapy". This grant was recently funded.

1. New cell lines have been created (T2-null Rc/CMVPAI-transfectants) that can be distributed to DOD investigators involved in angiogenesis research.

CONCLUSIONS

Several important conclusions were derived as a result of work initiated and completed during the period covered by this report.

- PAI-1 expression is required for optimal endothelial cell migration *in vitro*.

- Endothelial cell motile deficits, produced as a consequence of PAI-1 expression targeting, can be restored to approximately wild-type levels by transfection with the Rc/CMVPAI expression vector.

- Primary PAI-1$^{-/-}$ cells derived from the PAI-1 knockout mouse are markedly compromised in their motile ability (at least in planar 2-D models of induced cell migration), compared to PAI-1$^{+/+}$ isolated from wild-type control mice.

- The migratory defect evident in the PAI-1$^{-/-}$ genetic background can be "rescued" by addition of exogenous PAI-1 protein.

- These data are inconsistent with the emerging realization that "balanced proteolysis", in general, is an essential aspect of a successful angiogenic response and that PAI-1, in particular, is a major regulator of tumor-dependent angiogenesis.

- It is possible to design targeted genetic therapies to manipulate expression of an important pro-angiogenic gene (PAI-1) under defined *in vitro* conditions.

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transcripts inhibits serum and TGF-β1-stimulated PAI-1 gene expression and directed motility in renal epithelial cells. Cell Motil. Cytoskel. 48: 163-174.

APPENDIX

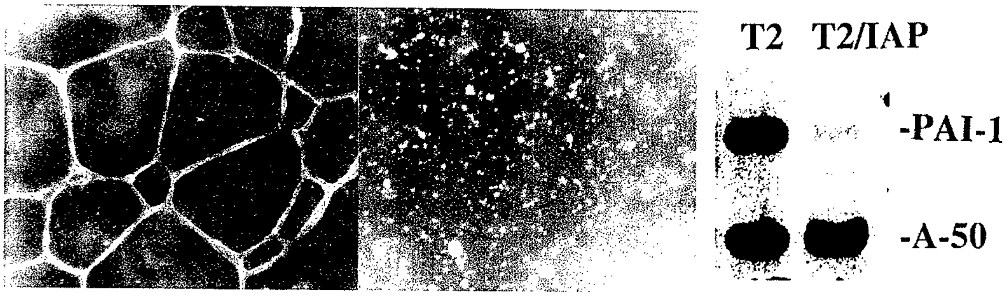

Figure 1. Capillary formation on Matrigel requires migration to achieve tubular coalescence and provides an easily accessible culture system to deliver plasmids, antibodies, reagents to a differentiated capillary network. Plating of T2 cells onto Matrigel results in the formation of tubular networks with luminal spaces (left panel). Northern analysis of harvested tubular structures indicated that cells in these networks expressed abundant PAI-1 transcripts (right panel). In contrast, T2 cells stably-transfected with the Rc/CMVIAP antisense construct expressed relatively low levels of PAI-1 mRNA (right panel) and failed to migrate and coalesce into tubular structures (middle panel). T2/IAP cells remained as single cells (without any evidence of a migratory track) or formed small multicellular aggregates in Matrigel. PAI-1 expression , thus, is required for tubular differentiation in Matrigel.

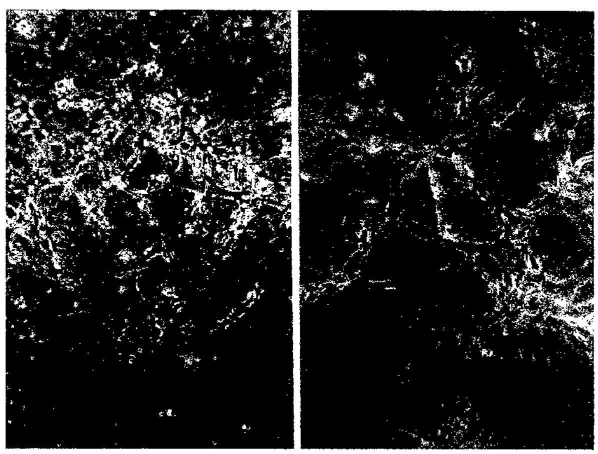

Figure 2. Visual analysis of the ability of the PAI-$1^{-/-}$ 4HH (left panel) and wild-type T2 (right panel) cells to form capillary networks in a complex "in gel" support matrix consisting of a 3:1 mixture of Vitrogen-Matrigel. Cultures were photographed 7 days after initiation of suspension culture. 4HH cells failed to form any real network structure and extensively degraded the gel matrix. T2 cells, in comparison, constructed highly branched and anastomizing capillary networks. Many of these tubular processes had lumen-like structures. Extensive sprout formation was evident at the tips of T2 branches indicating that T2 cell in-gel cultures formed both invasive and differentiated compartments.

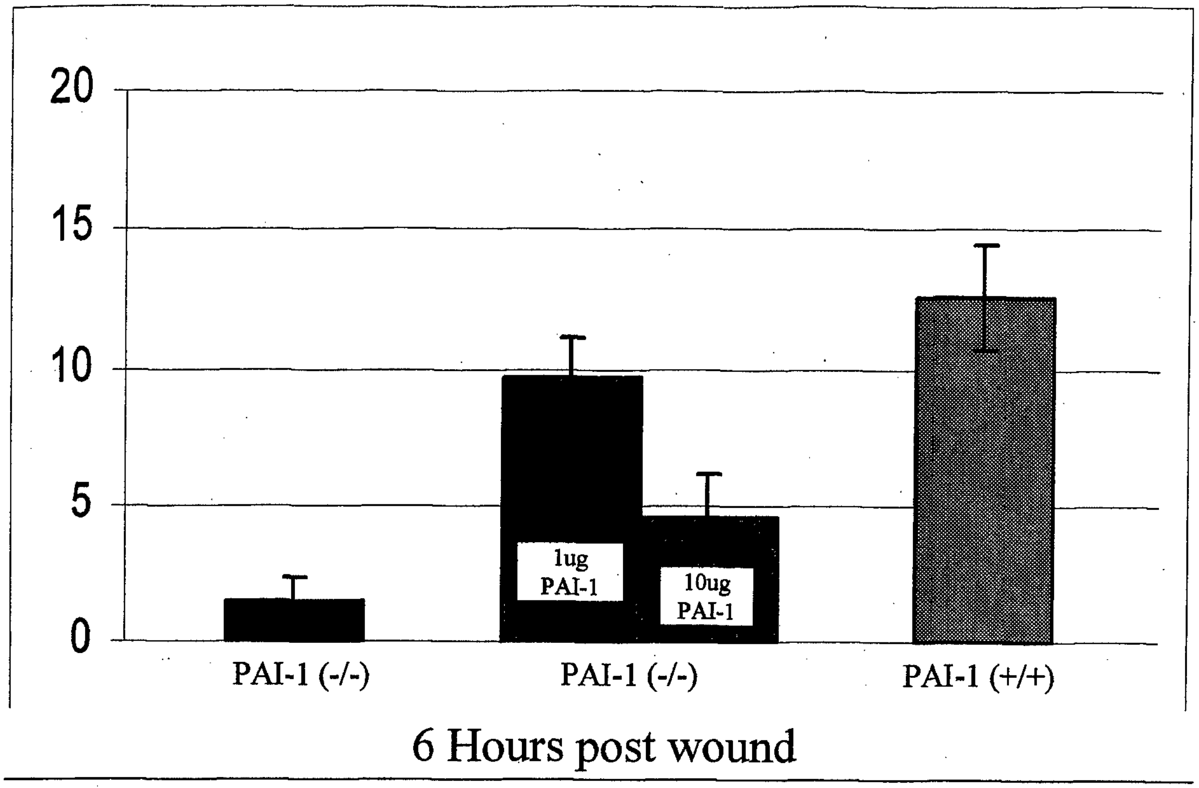

Figure 3. Relative migratory ability of primary cells isolated from PAI-1 knockout mice ($PAI\text{-}1^{-/-}$) compared to wild-type ($PAI\text{-}1^{+/+}$) control cells. The marked migratory deficient evident in cells derived from the PAI-1-null genetic background was efficiently restored to approximately wild-type levels by exogenous addition of recombinant PAI-1 at a concentration of 1 μg/ml.