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PRINCIPAL INVESTIGATOR: Paul J. Higgins, Ph.D.

CONTRACTING ORGANIZATION: Albany Medical College
Albany, New York 12208-3412

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Paul J. Higgins, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albany Medical College
Albany, New York 12208-3412

E-Mail: higginp@mail.amc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Findings in mice genetically-engineered to be deficient in the expression of genes that comprise the plasmin activation cascade confirmed the importance of plasminogen activator inhibitor type-1 (PAI-1) in tumor-induced angiogenesis. We found that endothelial cells must express PAI-1 to undergo angiogenesis upon co-culture with human breast tumor cells. PAI-1, thus, is an important candidate target for gene therapy of human cancers. Year 01 work determined the effects of expression vector-driven PAI-1 synthesis in endothelial cells (in which the endogenous PAI-1 gene was molecularly disrupted) on Matrigel-induced angiogenesis. This provided an assessment of the threshold level of PAI-1 required for the "angiogenic switch". A vector construct in which PAI-1 cDNA expression was driven by a CMV promoter was used to assess the consequences of varying levels of PAI-1 synthesis on induced angiogenesis. A panel of endothelial transfectant cell lines was created that varied (over 40-fold) in PAI-1 mRNA expression. While PAI-1 null parental cells, and transfectants expressing high levels of PAI-1, were incapable of forming angiogenic neworks when cultured on Matrigel, cells that produced low to moderate levels of PAI-1 were highly angiogenic. This expression 'window" provides a target for directed therapy

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INTRODUCTION

Continued growth of a malignant tumor beyond a certain critical size is dependent on the development a network of feeder blood vessels (1,2). Findings from a number of laboratories have indicated that critical for this "angiogenic switch" is 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 has 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 metastic spread, early recurrance and poor prognosis. Preliminary data from this laboratory (and presented in the original application) 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 will be 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) 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 01 of this investigation were directed to the goals described in Task 1 of the approved statement of work.

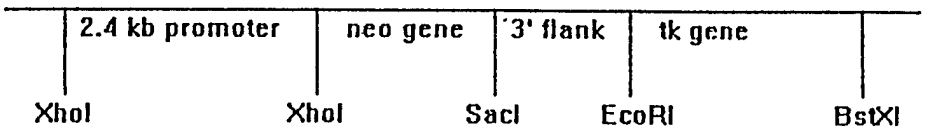
Task 1. To quantify the effects of vector-driven PAI-1 synthesis on the human breast tumor-induced angiogenic phenotype in transfectant T2-null endothelial cells (a clone in which the endogenous PAI-1 gene was disrupted by molecular targeting). This phase of the work is necessary to determine the threshold 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., form branched tubular networks and migrate 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 (8,9). 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 (9).

Since we were able to show 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.



Linear representation of the PAI-1 targeted disruption vector. The 2.4-kb PAI-1 5' flanking promoter and the 1.8-kb 3' non-coding flanking region are PAI-1-specific genomic segments used to direct the vector to the endogenous PAI-1 gene for homologous recombination. The vector backbone is pGEM-based (not shown). The neo and *Herpes simplex* tk genes provided positive/negative selection criteria. After transfection of T2 endothelial cell, G418-resistant clones were tested for subsequent insensitivity to gangcyclovir indicating elimination of the tk gene and increasing the likelihood of proper integration of the construct. One such clone (T2-null cells) was found to be incapable of PAI-1 mRNA or protein synthesis; Southern analysis indicated that these cells were indeed PAI-1 null.

The resulting cell line (T2-null) similarly failed to form branched angiogenic networks *in vitro* (data presented in the original application). 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 EC-1 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 to 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.

KEY RESEARCH ACCOMPLISHMENTS

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

- Confirmed that targeted ablation of endothelial cell PAI-1 gene expression, using either 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.
- 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.
- 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.
- Developed the Matrigel culture system to evaluate the angiogenic capability of our genetically-engineered endothelial cells using software programs that provide an opportunity to measure endothelial tube length and network branching complexity.
- Developed methods to harvest endothelial cells out of Matrigel culture in order to assess PAI-1 mRNA abundance (by Northern blotting) and protein synthesis (by Western blotting). This technique is required for the unambiguous assessment of PAI-1 expression status in the Matrigel/collagen co-culture model system.

REPORTABLE OUTCOMES

1. The following manuscripts are in press:

   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.

   Kutz, S.M., Hordines, J., McKeown-Longo, P.J., and Higgins, P.J. (2001) TGF-β_1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. Journal of Cell Science.

2. A new Idea proposal has been submitted to the DOD Breast Cancer Program. Title: "Inducible Anti-Angiogenic Gene Therapy of Breast Cancer".

3. 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.

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

2. PAI-1 expression is required for endothelial cells for form tubular structures when plated on Matrigel-coated surfaces (basal angiogenesis) or in breast carcinoma co-culture systems (stimulated angiogenesis).

3. 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.

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

5. 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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9. Providence, K.M., Kutz, S.M., Staiano-Coico, L., and Higgins, P.J. (2000) PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. Journal of Cellular Physiology 182, 269-280.

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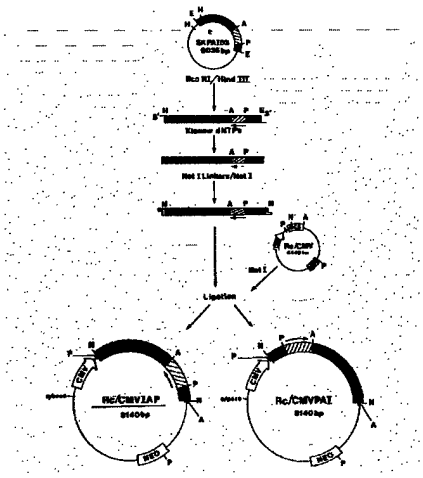
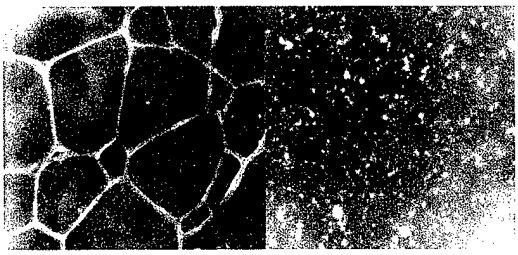


Figure 1. Construction of Rc/CMV vectors that drive expression of a PAI-1 cDNA insert cloned in sense (PAI) and antisense (IAP) orientations. Black region refers to insert (ref. 45 for cloning details) (left panel). T2 endothelial cells placed on Matrigel form capillary networks (bottom left) and express PAI-1 (bottom right). PAI-1 antisense transfectants (T2/IAP) do not migrate to form capillaries (bottom middle) or express PAI-1 (right)



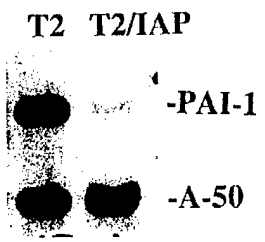


Figure 1

JOURNAL OF CELLULAR PHYSIOLOGY 182:269–280 (2000)

PAI-1 Gene Expression Is Regionally Induced in Wounded Epithelial Cell Monolayers and Required for Injury Repair

KIRWIN M. PROVIDENCE,[1] STACIE M. KUTZ,[1] LISA STAIANO-COICO,[2] AND PAUL J. HIGGINS[1]*

[1]*Cell & Molecular Biology Program, Albany Medical College, Albany, New York*
[2]*Department of Surgery, Weill Medical College of Cornell University, New York, New York*

Induced expression of plasminogen activator inhibitor type-1 (PAI-1), a major negative regulator of pericellular plasmin generation, accompanies wound repair in vitro and in vivo. Since transcriptional control of the PAI-1 gene is superimposed on a growth state–dependent program of cell activation (Kutz et al., 1997, J Cell Physiol 170:8–18), it was important to define potentially functional relationships between PAI-1 synthesis and subpopulations of cells that emerge during the process of injury repair in T2 renal epithelial cells. Specific cohorts of migratory and proliferating cells induced in response to monolayer trauma were spatially as well as temporally distinct. Migrating cells did not divide in the initial 12 to 20 h postinjury. After 24 h, S-phase cells were generally restricted to a region 1 to 2 mm from, and parallel to, the wound edge. Proliferation of wound bed cells occurred subsequent to wound closure, whereas the distal contact-inhibited monolayer remained generally quiescent. Hydroxyurea blockade indicated, however, that proliferation (most likely of cells immediately behind the motile "tongue") was necessary for maintenance of cell-to-cell cohesiveness in the advancing front, although the ability to migrate was independent of proliferation. PAI-1 mRNA expression was rapidly up-regulated in response to wounding with inductive kinetics approximating that of serum-stimulated cultures. Differential harvesting of T2 cell subpopulations, based on proximity to the injury site, prior to Northern assessments of PAI-1 mRNA abundance indicated that PAI-1 transcripts were restricted to cells immediately bordering the wound or actively migrating and not expressed by cells in the distal contact-inhibited monolayer regions. Such cell location–specific distribution of PAI-1-producing cells was confirmed by immunocytochemistry. PAI-1 synthesis in cells that locomoted into the wound field continued until injury closure. Down-regulation of PAI-1 synthesis and matrix deposition in renal epithelial cells, stably transfected with a PAI-1 antisense expression vector, significantly impaired wound closure. Transfection of the wound repair–deficient R/A epithelial line with a sense PAI-1 expression construct restored both approximately normal levels of PAI-1 synthesis and repair ability. These data indicate that PAI-1 induction is an early event in creation of the wound-activated phenotype and appears to participate in the regulation of renal epithelial cell motility during in vitro injury resolution. J. Cell. Physiol. 182:269–280, 2000. © 2000 Wiley-Liss, Inc.

Extracellular matrix (ECM) restructuring following tissue injury is regulated (both temporally and spatially) by the plasmin-based pericellular proteolytic cascade and by members of the metalloproteinase family (Dano et al., 1985; Laiho and Keski-Oja, 1989; Pilcher et al., 1997; Pollanen et al., 1991). Recent in situ and genetic analyses of the repair process suggest that the migratory, proliferative, and ECM remodeling stages of in vivo wound healing are dependent, to a significant extent, on plasminogen activation (i.e., the conversion of plasminogen to the active broad-spectrum protease plasmin by urokinase plasminogen activator [uPA]) (Pollanen et al., 1987, 1991; Schafer et al., 1994; Okada et al., 1995; Pappot et al., 1995; Jensen and Lavker, 1996; Romer et al., 1996; Carmeliet et al., 1997; Hasenstab et al., 1997; Carmeliet and Collen,

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*Correspondence to: Dr. Paul J. Higgins, Cell & Molecular Biology Program (Mailcode-165), Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208.
E-mail: phiggins@ccgateway.amc.edu

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1998). Studies in mice genetically engineered to be deficient in one or more elements in the plasmin activation cascade, for example, have confirmed the importance of uPA and plasmin in cell migration at specific injury sites (e.g., Romer et al., 1996; Carmeliet et al., 1997). Plasminogen activator inhibitor type-1 (PAI-1) functions in this process to negatively regulate plasmin generation by complexing with and inhibiting the catalytic activity of free as well as receptor-bound uPA (e.g., Ellis et al., 1990; Laiho and Keski-Oja, 1989) modulating, thereby, uPA-dependent motility in vivo (Carmeliet and Collen, 1995; Carmeliet et al., 1997). Indeed, it appears that various aspects of an efficient response to trauma, including the growth factor–dependent recruitment of endothelial cells into the wound field (Sato and Rifkin, 1988), require precise control over both the expression and localization of particular proteases and protease inhibitors (Andreasen et al., 1997; Pepper et al., 1993).

Cell type–specific synthesis and subcellular targeting of PAI-1 and uPA appear to be important considerations in the modulation of the pericellular proteolytic balance. Following injury, uPA and PAI-1 are initially produced by cells immediately adjacent to the wound edge in vivo as well as in vitro (Romer et al., 1991; Pawar et al., 1995; Reidy et al., 1995; Staiano-Coico et al., 1996; Carmeliet et al., 1997), where PAI-1 is likely stabilized in an active conformation with ECM-associated vitronectin (Declerck et al., 1988; Mimuro and Loskutoff, 1989a,b; Seiffert et al., 1990; Vassalli et al., 1991). Temporal changes in the expression, focalization, and/or relative activity levels of this protease/inhibitor pair may influence cell migration either as a direct consequence of ECM barrier proteolysis or by modulating cellular adhesive interactions with the ECM (Ciambrone and McKeown-Longo, 1990; Blasi, 1996, 1997; Jensen and Lavker, 1996; Carmeliet et al., 1997). Furthermore, recent findings indicate that PAI-1, specific integrins, and uPA function coordinately to influence adhesive events important in the control of cell movement (Carmeliet and Collen, 1995, 1996; Stefansson and Lawrence, 1996; Blasi, 1997; Waltz et al., 1997).

Proliferation is also an essential, albeit presumed independent, component in the closure of epithelial monolayer wounds (Pawar et al., 1995). The exact relationship between regenerative growth and trauma repair, however, remains to be defined (Jensen and Lavker, 1996; Zahm et al., 1997). Within the setting of induced cellular proliferation and migration, stimulated expression of PAI-1 appears associated with both processes (e.g., Bade and Feindler, 1988; Pepper et al., 1992; Thornton et al., 1994; Ryan et al., 1996; Kutz et al., 1997). Transcriptional regulation of the PAI-1 gene, moreover, is superimposed on a growth state–dependent program, which culminates in a proliferative response (Ryan et al., 1996; Kutz et al., 1997). Kinetic assessments indicate that PAI-1 transcription and mRNA expression, similar to that of uPA (Grimaldi et al., 1986), occur early and in immediate-early response (IER) fashion on addition of serum to quiescent cells (Ryan and Higgins, 1993; Ryan et al., 1996; Uno et al., 1997), thereby mimicking regenerative events in vivo (Schneiderman et al., 1993; Thornton et al., 1994). The PAI-1 gene, however, exhibits a complex mode of regulation on entry of G_0-arrested cells into a cycling G_1 condition (Ryan et al., 1996). Serum-induced PAI-1 transcription is maximal in mid-G_1 and declines abruptly prior to the onset of DNA synthesis (White et al., 1999). The amplitude as well as maintenance of expression through mid-G_1 phase is anchorage responsive and this latter adhesion-dependent requirement, unlike initial induction, involves secondary (i.e., protein synthesis–dependent) transcriptional-level events (Ryan et al., 1996). These data provided for a model of PAI-1 gene control in serum-stimulated cells, which incorporates both IER and secondary regulatory influences within an "activated" G_1 state (Kutz et al., 1997; Mu et al., 1998). Such fine control over the kinetics of PAI-1 expression appears to be one modulating aspect in the complexity of G_1 progression. In this regard, PAI-1 may regulate cell-to-substrate adhesion (a necessary prerequisite for G_1/S transition [Guadagno and Assoian, 1991; Guadagno et al., 1993]) or cell shape (and shape-dependent metabolic pathways [e.g., Higgins et al., 1994; Hawks and Higgins, 1998]) by directly influencing the immediate pericellular proteolytic microenvironment (Laiho and Keski-Oja, 1989). PAI-1-dependent ECM stabilization, moreover, may indirectly facilitate the formation of cell-ECM interactions necessary for cellular adhesion and/or migration (e.g., Planus et al., 1997).

Such in vitro observations, in fact, do have in vivo correlates. The morphology of the flattened regenerating renal epithelium, for example, is dramatically different from the normally quiescent highly polarized tubular cell (e.g., Wallin et al., 1992) and PAI-1 expression occurs specifically in regenerating proximal tubular cells following ischemia-reperfusion renal injury (Basile et al., 1998), suggesting a functional role in tissue repair. Clearly, the association between the activated phenotype and targeted accumulation of PAI-1 in the cellular undersurface in close proximity to newly formed focal adhesions is consistent with this function (Kutz et al., 1997). The time course of induced PAI-1 expression and the involvement of the PAI-1-synthesizing cellular cohort in the response to injury, however, are poorly understood. It is important, therefore, to clarify the kinetics of PAI-1 expression with regard to the emergence of specific cell subpopulations (i.e., migratory, proliferating) involved in repair growth and to assess the role of this protease inhibitor in the regenerative process.

MATERIALS AND METHODS

Cell lines and in vitro repair assay

The independent clonal isolates (EC-1 and T2), derived from an early-passage culture of normal rat kidney (NRK) epithelial cells (Ryan and Higgins, 1993), were maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) (DMEM/10). Cells were grown to confluency and the media changed to fresh DMEM/10 for maintenance in a postconfluent condition for an additional 3 days. Alternatively, confluent cultures were maintained in serum-free DMEM for 3 days to 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.

Growth "activation" of cells arrested in serum-deficient medium

The growth medium in low-density 150-mm dish cultures was aspirated, the cells rinsed twice in Hanks' balanced salt solution (HBSS) (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.14 M NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose), and serum-free DMEM added (which initiates a rapid G_0 arrest of NRK cells; Kutz et al., 1997). After 3 days, cultures were left untreated or exposed to actinomycin D (5 μg/ml), puromycin (20 and 100 μg/ml), or genistein (50 and 100 μM) for 30 min prior to, and during, stimulation with FBS (added directly to the quiescence maintenance medium to a final concentration of 20%). Cells were harvested for analysis of PAI-1 transcripts (described later) at times indicated in the text.

RNA analysis of cell subpopulations

Cells immediately adjacent to the wound (termed *edge-isolates*) were harvested by pushing the blunt (wide) end of a P1000 plastic pipette tip along the existing wound tract, displacing cells directly at, and 5 mm from, the wound edge; such scrape-released cells were subsequently collected by centrifugation at 1400 × g. Cells located between 10 and 40 mm from the wound border (i.e., in the intact monolayer regions) were collected in the same manner and termed *monolayer-isolates*. In some cases, the entire culture population was harvested with a cell scraper (*total dish–isolates*). Cellular RNA was extracted (Kutz et al., 1997) and denatured by incubation at 55°C for 15 min in 1X MOPS, 6.5% formaldehyde, and 50% formamide prior to electrophoresis (10 μg RNA/lane) on agarose/formaldehyde gels (1.2% agarose, 1.1% formaldehyde, 1X MOPS, pH 8.0) for 3 h at 70 V in 1X MOPS. Fractionated RNA was transferred to positively charged nylon membranes via downward capillary action using the turboblotter system (Schleicher & Schuell, Keene, NH) and UV crosslinked. Hybridization with [^{32}P]-dCTP-labeled cDNA probes to rat PAI-1 and mouse A50 was as described (Ryan et al., 1996). Blots were exposed to X-OMAT AR-5 film (Kodak, Rochester, NY) or analyzed with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for visualization and quantitation of mRNA species.

Immunocytochemistry

Cultures were washed twice in Ca^{2+}/Mg^{2+} free phosphate–buffered saline (CMF-PBS; 2.7 mM KCl, 1.2 mM KH_2PO_4, 0.14 M NaCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$) and fixed for 10 min at room temperature in 10% formalin/CMF-PBS. Following permeabilization with cold (4°C) 0.5% Triton X-100/CMF-PBS for 10 min at 4°C, cells were washed three times (5 min each) with CMF-PBS prior to incubation (3 h at room temperature) with rabbit antibodies to rat PAI-1 (Kutz et al., 1997) diluted in BSA (3 mg/ml)/CMF-PBS. After three CMF-PBS washes, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA; diluted 1:20 in BSA/CMF-PBS) for 30 min at 37°C, washed, and coverslips mounted with 100 mM *n*-propygalate in 50% glycerol/CMF-PBS. Where indicated, 4 μM monensin (Sigma, St. Louis, MO) was added to wounded monolayers prior to fixation. For identification of DNA-synthesizing cells, 5-bromo-2′-deoxyuridine (BrdU) (200 μmol/l final concentration) was added to cultures at time of wounding. The number and location of cells that incorporated the analogue (S-phase cells) were assessed by indirect immunofluorescence, using monoclonal antibodies to BrdU (Mu et al., 1998).

Construction and transfection of sense and antisense PAI-1 expression vector

pBluescript, containing a full-length rat PAI-1 cDNA (Higgins and Ryan, 1992), was digested with *Eco*RI and *Hind*III to generate a 2.6-kb insert (representing nucleotides −118 to +2572 relative to the start site of transcription). Agarose gel–purified DNA was blunt-ended with Klenow fragment/dNTPs using a fill-in reaction. *Not*I linkers were ligated, the fragments digested with *Not*I, and purified by agarose gel electrophoresis. Flanked inserts were ligated to *Not*I-digested calf intestinal phosphatase-treated gel-purified Rc/CMV expression vector DNA and subsequently transformed into competent INVαF′ *E. coli*. Plasmid DNA was isolated from ampicillin-resistant colonies; restriction endonuclease digestion and Southern blot analysis, using a 726-bp *Pst*I/*Apa*I-digested cDNA fragment as a probe, confirmed sense (Rc/CMVPAI) and antisense (Rc/CMVIAP) insert orientation (Higgins et al., 1997). Insert template activity was assessed in vitro for both constructs using T7 polymerase to initiate PAI-1 antisense and sense transcripts (confirmed by hybridization analysis and coupled STP3 transcription-translation [Novagen]/Western blotting, respectively). Cells were transfected with 20 μg of plasmid DNA using the calcium phosphate technique (Higgins et al., 1991). Stable transfectants were selected with G418 (150 μg/ml), and resistant clones were isolated and expanded in growth medium containing G418. Since T2 cells were highly resistant to G418, stable antisense-expressing clones were derived using the EC-1 line.

Metabolic labeling, cell extraction, and gel electrophoresis

Cells were washed with HBSS, then labeled in serum-/methionine-free RPMI 1640 medium containing 50 μCi [^{35}S]methionine/ml (specific activity = 1100 Ci/mmol) (Ryan and Higgins, 1993). The conditioned labeling medium was aspirated and monolayers washed with CMF-PBS prior to extraction with 0.2% (w/v) saponin in CMF-PBS to isolate cell-substratum contact regions and associated undersurface proteins (Higgins et al., 1990, 1991). Saponin-resistant (SAP fraction) residues were scraped into sample buffer (50 mM Tris/HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol) and boiled. For electrophoresis, 25,000 cpm trichloroacetic acid–insoluble SAP-fraction protein were separated on SDS/9% acrylamide slab gels (Ryan and Higgins, 1988). Labeled protein bands were visualized by fluorography and quantified by computerized densitometry (Smith et al., 1992). Identification

TABLE 1. Kinetics of wound closure in scrape-injured T2 cell monolayers

Hours postwounding[1]	Wound width[2]	% Injury repair	Closure rate[3]
0	9.68 ± 0.20	0	—
2	9.21 ± 0.22	4.8	2.4
4	8.66 ± 0.29	10.5	2.6
6	8.35 ± 0.33	13.7	2.3
9	7.23 ± 0.39	25.3	2.8
12	6.32 ± 0.43	34.0	2.8
21	3.97 ± 0.51	58.9	2.8
26	2.18 ± 0.58	77.0	2.9
30	1.62 ± 0.45	83.3	2.8
36	0	100	—

[1]A contact-inhibited monolayer of T2 cells was scrape-wounded and the extent of injury repair (measured by a calibrated ocular grid and expressed as percent closure) plotted as a function of time postinjury.
[2]Width of the remaining unhealed region was measured in arbitrary units with a calibrated ocular grid. Data represent the mean ± SD of 10 individual assessments.
[3]Closure rate = $\frac{\text{mean \% repair}}{\text{time (h) postwounding}}$

of the rat PAI-1 protein in one-dimensional electrophoretic separations utilized criteria described previously (Higgins et al., 1989, 1990) as well as by immunochemical reactivity with PAI-1-specific antibodies (Higgins et al., 1990).

RESULTS

Kinetics of the repair response

Cellular movement into the wound "bed" occurred relatively quickly (i.e., within 1 h after monolayer scraping). Trauma site closure proceeded at a constant rate and was complete by 30 to 36 h after injury in this model (Table 1). Time-lapse videomicroscopy and examination of fixed, acridine orange-stained, T2 cell cultures confirmed that relatively close cell-to-cell contact in the migrating tongues, as well as within the distal monolayer regions, was maintained throughout the repair process (i.e., solitary migratory cells did not enter the denuded zone; see later discussion). An absence of mitotic cells in the migratory cohort was also apparent in the time-lapse assessments, suggesting that the mobile population does not divide (at least during the initial 12 to 20 h after wounding). Mitotic cells were evident, however, in regions of the monolayer approximately 1 to 2 mm from, and parallel to, the edge of the original scrape injury (at 24 h after wounding) and within the confines of the initial injury site (only after the opposing fronts made contact) but not in the distal monolayer.

This regional compartmentalization of T2 cells with differing proliferative kinetics, moreover, suggests that a relationship exists between entry into the division cycle and distance from the wound. To assess this possibility, the culture medium was supplemented with 200 μmol/l BrdU at the time of scraping specifically to identify cells that enter S phase in response to injury. This quantitative approach, when applied over the entire time course of monolayer repair, confirmed that (1) confluent T2 cells are, in fact, contact inhibited; (2) 24 h postinjury, the majority of S-phase cells concentrate in a region approximately 1 to 2 mm from, and parallel to, the wound edge and (to a lesser extent) in some leading edge cells; and (3) proliferation of cells that had entered

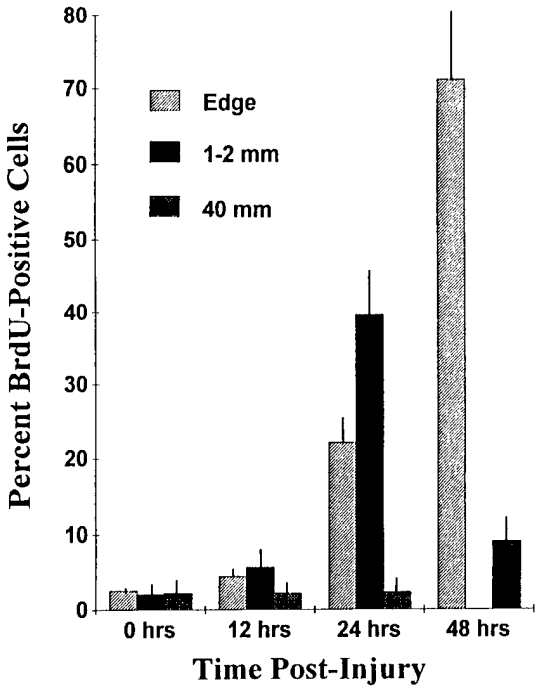

Fig. 1. T2 cells synthesize DNA as a function of their spatial relationship to the wound site. The fraction of S-phase cells is low in uninjured monolayers or in scraped cultures at early times (i.e., ≤12 h) postinjury. By 24 h after wounding, most proliferating cells (as determined by immunocytochemical detection of BrdU incorporation) occur within the first 1 to 2 mm from, and parallel to, the wound edge, although S-phase cells also begin to appear at the leading edge. Cells in the distal monolayer (i.e., 40 mm from the injury site) remain generally quiescent throughout the repair process. The fraction of DNA-synthesizing cells in the distal regions increases only slightly (i.e., comprising <10% of the total population) after complete closure (by approximately 36 h). The healed wound bed population (i.e., "Edge" cells at the 48-h time point) proliferate vigorously in the postclosure period. Percent S-phase (BrdU-positive) cells was determined by counting 10 random fields representative of each of the indicated culture areas for three independent experiments; data plotted represent the mean ± SD of such assessments.

the wound bed occurs subsequent to scrape closure (Fig. 1). The presence or absence of conditioned serum had no significant effect on repair regional kinetics (i.e., the rate of closure differed by only 10 to 15% for cultures wounded in serum-containing as compared to serum-free medium).

To ascertain whether migration alone is sufficient to achieve wound closure, T2 monolayers were scrape-injured in the presence of 0.4 mM hydroxyurea (HU) to inhibit ribonucleoside diphosphate reductase activity (Krakoff et al., 1968; Wang et al., 1997). Preliminary experiments indicated that this concentration of HU effectively inhibited serum-induced DNA synthesis in NRK cells. HU-treated T2 cultures exhibited the same initial morphologic response to wounding as control populations (i.e., membrane ruffling and cell spreading occurred within 30 min of injury followed by migration into the denuded area). By 12 h postinjury, however, the integrity of the migrating sheet was lost in HU-treated cells; after 24 h (a time point that correlated with a significant increase in the fraction of BrdU-positive cells in control cultures; e.g., Fig. 1), there was a complete HU-associated loss of cell-to-cell contact at

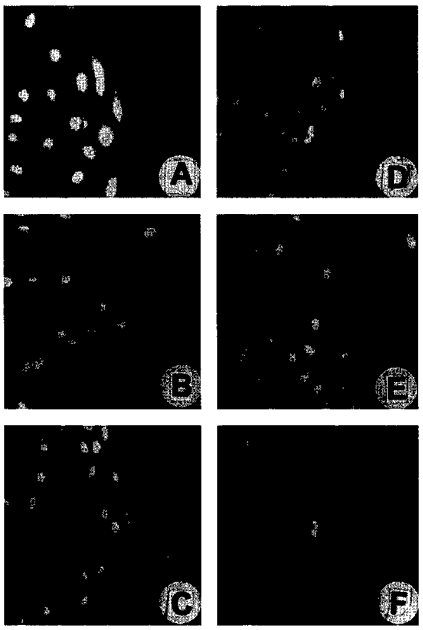

Fig. 2. Effects of hydroxyurea (HU) on the repair response by T2 cells. In control wounded monolayers, the migratory tongue advanced as a generally uniform sheet of closely juxtaposed cells (**A, B, C** = 6, 12, 24 h, respectively, after scraping). Such cohesion was lost in HU-treated cultures (**D, E, F** = 6, 12, 24 h, respectively, postinjury). HU-associated loss of cell-to-cell contact was evident within 12 h (E) and clearly obvious by 24 h (F) postwounding; cell morphology was unaffected by HU. In each panel, the direction of migration is from left to right into the denuded zone. For acridine orange histochemistry, cells were permeabilized in 0.08 N HCl, 0.15 M NaCl, and 0.1% Triton X-100 for 30 sec, then incubated in staining buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 6.0, 1 mM disodium EDTA, 0.15 M NaCl, 6 μg acridine orange/ml). UV light microscopy, ×50.

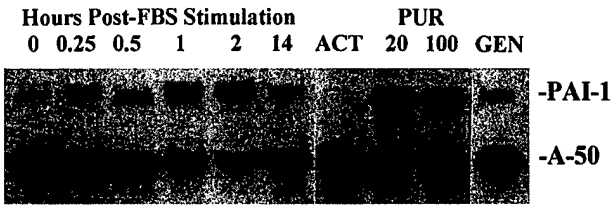

Fig. 3. Time course and metabolic characteristics of serum-induced PAI-1 gene expression. Quiescent T2 cell cultures were stimulated by direct addition of serum (to a final concentration of 20%); control cultures were maintained in serum-free DMEM (time 0). RNA was isolated at the times indicated post-FBS addition. For assessment of the metabolic requirements of serum-induced PAI-1 expression, cells were pretreated with actinomycin D (ACT; 5 μg/ml), puromycin (PUR; 20 or 100 μg/ml), or genistein (GEN; 100 μM) prior to addition of FBS; RNA was extracted from inhibitor-treated cultures 4 h after serum stimulation. Northern blots were hybridized with ^{32}P-labeled PAI-1 and A-50 cDNA probes simultaneously. PAI-1 transcriptional activation in response to serum had IER characteristics and was genistein-sensitive. Identical results were obtained in four different experiments.

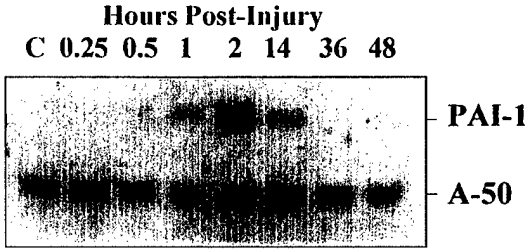

Fig. 4. Induction of PAI-1 in mRNA response to wounding. Confluent T2 monolayers were scrape-wounded and RNA from total dish-isolates extracted at the times indicated. Northern blots were hybridized with ^{32}P-labeled PAI-1 and A-50 cDNA probes simultaneously. PAI-1 transcripts were evident as early as 30 min to 1 h after injury, expression was maximal at 2 h, and declined to levels comparable to quiescent controls by 36 h. C = quiescent nonwounded T2 cells. Blot shown is representative of three individual experiments.

the leading edge (Fig. 2). Migratory activity, however, appeared unaffected by HU, since cells from opposing, albeit noncohesive, "fronts" made contact within the same time frame (i.e., 30 to 36 h) as in untreated populations. Migration, therefore, occurs independently of proliferation, although proliferation (most likely of cells immediately behind the mobile tongue) is necessary for maintenance of monolayer integrity in the zone of repair.

Characteristics of induced PAI-1 expression in wound-"activated" cultures

The temporally and spatially distinct phases of migration and proliferation are associated with the wound-"activated" phenotype (Figs. 1 and 2). It was, therefore, important to assess whether induced PAI-1 expression was an element in this repair response and, if so, to determine both the time course of PAI-1 transcript synthesis and in situ distribution of PAI-1 expressing cells relative to the site of injury. Similar to serum-stimulation of quiescent NRK cell cultures (used for a kinetic comparison; e.g., Fig. 3), wounding-induced PAI-1 mRNA transcripts are first detectable in total dish–isolates of T2 cells within 30 min to 1 h after monolayer scraping (Fig. 4). PAI-1 mRNA is maximally expressed between 1 and 2 h posttrauma, declining dramatically by 14 h (Fig. 4) and closely approximating the changing transcript abundance typical of serum-stimulated cultures (Fig. 3). To ascertain specifically which cells within injured cultures express PAI-1, RNA was extracted from individual edge- and monolayer-isolates at various times postscraping. PAI-1 mRNA was rapidly induced (by 2 h) after injury in edge-isolates, declined quickly thereafter, and by 24 h posttrauma PAI-1 mRNA levels in cells at the injury site approximated that of nonwounded controls (Fig. 5). PAI-1 transcript abundance in distal monolayer-isolates, in contrast, did not change over the time course of repair, remaining similar to that of quiescent cultures.

In situ assessments of PAI-1 accumulation confirmed the RNA analysis. Consistent with the Northern blot data (Figs. 4 and 5), contact-inhibited T2 cells expressed only low levels of PAI-1 protein. Continuous migration into the denuded area, combined with the observation that the distal monolayer does not express PAI-1 mRNA (Fig. 5), suggests that PAI-1 protein synthesis may be restricted to leading edge cells and cells proximal to the site of injury (i.e., within the region of the migrating tongue). To address this issue, monensin (a Na^+/K^+ ionophore that interferes with several metabolic functions, including protein secretion but not synthesis [Ledger et al., 1980; Uchida et al., 1980]) was

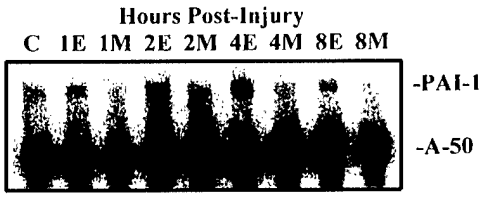

Fig. 5. Regional specificity of induced PAI-1 mRNA expression. Confluent T2 monolayers were wounded and RNA isolated at various times postinjury from cells bordering the wound edge (E = edge-isolates) or in the distal monolayer region (M = monolayer-isolates). Northern blots were hybridized with ^{32}P-labeled PAI-1 and A-50 cDNA probes simultaneously; blot shown is representative of triplicate experiments. PAI-1 transcript expression was restricted to cells at the wound edge. The time course of PAI-1 induction in edge-isolates was similar to that of total dish-isolates (e.g., Fig. 4).

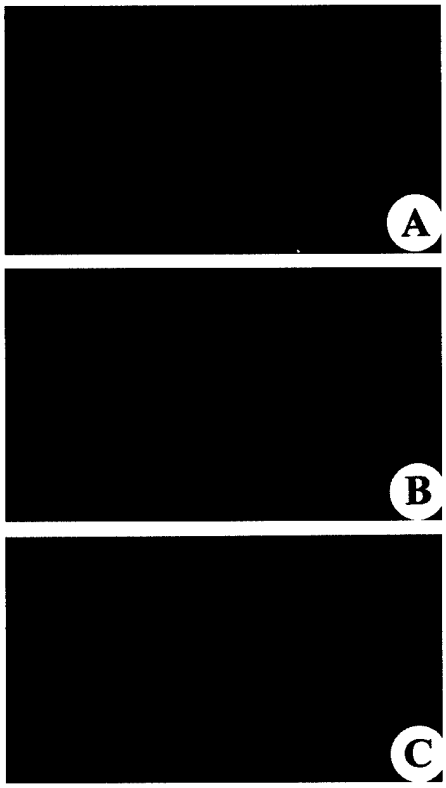

Fig. 6. Relationship between PAI-1 immunoreactive T2 cells and site of injury. Monensin treatment was used to inhibit protein secretion to maximize the likelihood of detection of de novo synthesized PAI-1 protein. In response to wounding, PAI-1 protein specifically localizes to cells adjacent to the wound edge with minimal synthesis by distal monolayer region cells. (**A**) nonwounded monolayer; (**B**) edge, 4 h postinjury; and (**C**) monolayer, 4 h postinjury. Indirect immunofluorescence microscopy, ×185.

added to cultures at the time of wounding. Immunocytochemical evaluation of monensin-treated cultures confirmed the Northern assessments; de novo PAI-1 protein synthesis occurs primarily by cells immediately adjacent to the wound edge (Fig. 6). Coincident with the wound-induced expression of PAI-1 mRNA transcripts (e.g., Fig. 5), and at every time point thereafter, immunoreactive PAI-1 protein was resolved both at the migrating edge and within cells proximal to the closing wound bed. PAI-1 synthesis continues until wound closure with only minimal expression in the distal monolayer.

TABLE 2. Inhibition of wound closure by genistein

Serum[1]	Hours postwounding	Treatment[2]	% Injury repair[3]
None	2	DMSO	4.43 ± 0.62
		50 μM	4.92 ± 0.92
		100 μM	3.61 ± 1.69
None	12	DMSO	35.01 ± 6.37
		50 μM	22.62 ± 3.33
		100 μM	15.62 ± 3.34
None	24	DMSO	76.97 ± 0.83
		50 μM	41.05 ± 6.59
		100 μM	27.67 ± 6.19
10%	2	DMSO	4.72 ± 0.83
		50 μM	4.18 ± 0.48
		100 μM	3.58 ± 0.54
10%	12	DMSO	39.83 ± 2.99
		50 μM	27.66 ± 4.38
		100 μM	20.61 ± 3.24
10%	24	DMSO	92.27 ± 3.41
		50 μM	61.59 ± 5.72
		100 μM	46.89 ± 7.73

[1]Contact-inhibited monolayers (in serum-free or 10% FBS–containing DMEM) of T2 cells were scrape-wounded and the extent of injury repair (measured by a calibrated ocular grid and expressed as percent closure) plotted as a function of time postinjury (as in Table 1).
[2]Cultures were incubated in DMSO (solvent control) or the indicated concentrations of genistein by direct addition to the maintenance medium immediately after wounding.
[3]Data represent the mean ± SEM for three independent experiments.

Consequences of PAI-1 expression manipulation on in vitro wound repair

Location-specific induced expression of PAI-1 mRNA and protein within the wound field (e.g., Figs. 4 to 6) suggested that, if PAI-1 was functionally related to the repair process, inhibition of synthesis by cells at the site of trauma might affect either the time course of injury resolution or the recruitment of cells into the denuded zone. Monensin was not appropriate for this evaluation. Initial studies indicated that this drug did reduce, but not completely inhibit, PAI-1 secretion and accumulation in the cellular undersurface region. Thus, monensin was useful for in situ studies (e.g., Fig. 6) but of limited value for functional assessments. Additional pharmacologic and molecular genetic approaches were designed, therefore, to address this issue. The tyrosine kinase inhibitor genistein was selected as the pharmacologic agent, since cell shape–dependent PAI-1 transcription (Hawks and Higgins, 1989), serum-induced PAI-1 expression (Fig. 3), and cell proliferation/angiogenic responses (Fotsis et al., 1993) are sensitive to genistein treatment. Initial experiments indicated that the time course of PAI-1 mRNA expression by NRK cells bordering the wound margin was similar to that of serum-stimulated cells (Figs. 5 and 6); moreover, PAI-1 induction in response to serum (e.g., Fig. 3) and scrape injury (data not shown) was inhibited by genistein. Genistein-mediated suppression of PAI-1 transcription was evident at concentrations of inhibitor that effectively reduced migration (e.g., Table 2).

A molecular genetic approach was devised, therefore, in which EC-1 cells were stably transfected with an

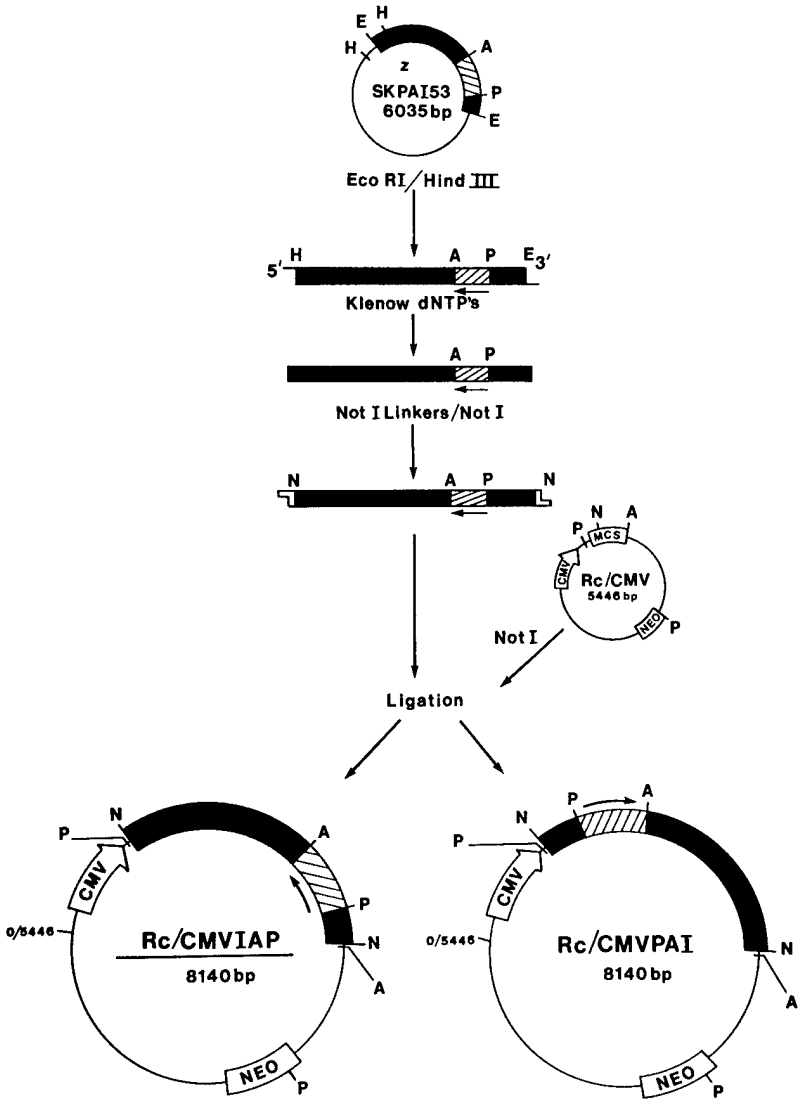

Fig. 7. Construction of sense (PAI) and antisense (IAP) expression vectors. Cloning of PAI-1 cDNA (nucleotides −118 to +2572) into the *Not*I site of the 5.4-kb Rc/CMV vector was as detailed (Materials and Methods). Topography of known restriction sites (P, *Pst*I; N, *Not*I; A, *Apa*I) was used to assess insert orientation.

expression vector (Rc/CMVIAP) bearing a PAI-1 cDNA insert in antisense configuration (Fig. 7) to specifically assess the relationship between wound-induced PAI-1 expression and cell migration. One derived line (Rc/CMVIAP-4HH) was selected for analysis since gel electrophoresis of the SAP fraction protein complement indicated that the 4HH clone was functionally PAI-1 "null" (Fig. 8). Injury site closure by the 4HH derivative was significantly impaired relative to EC-1 parental cells and Rc/CMV transfectants (Fig. 9). Wounds in 4HH monolayers were less than 60% "healed," even at the protracted time of 84 h postscraping, compared to collaterally evaluated controls for which more than 92% repair was typically achieved within the 30 to 36 h window (Fig. 9) and closure complete by 40 h or less. It was not possible to "rescue" the 4HH healing-deficient phenotype by transfection with the Rc/CMVPAI sense expression vector; surviving clones consisted of morphologically aberrant cells and, as such, were unsuited for wounding assays. A previous screen of NRK cells (Higgins et al., 1991), however, resulted in the derivation of one clone (NRK-R/A) that expressed significantly reduced levels of PAI-1 compared to parental EC-1 cells (Fig. 8). NRK-R/A cells also exhibited a marked inability to close monolayer scrape wounds, although this phenotype was not as severe as in the 4HH derivative and may reflect the fact that, unlike the 4HH clone, NRK-R/A cells express at least some PAI-1 (Fig. 8). Transfection of the NRK-R/A line with the Rc/CMVPAI vector restored both approximately normal levels of PAI-1 synthesis and wound repair ability (Fig. 10).

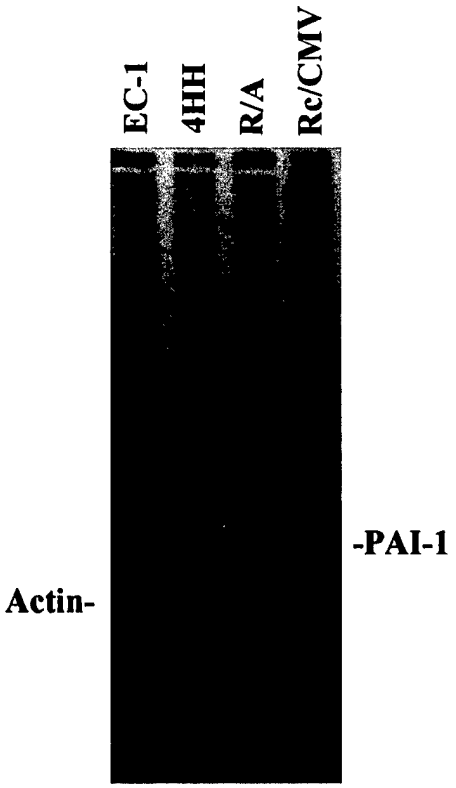

Fig. 8. Relative PAI-1 protein content in the saponin-resistant fraction of control and transfected NRK cells. Cellular SAP fractions (25,000 cpm trichloroacetic acid–insoluble protein) were separated on SDS/9% acrylamide slab gels. Bands corresponding to actin and the 52-kDa PAI-1 protein are indicated. Compared to parental EC-1 cells and insertless vector-transfected controls (Rc/CMV), de novo synthesized PAI-1 protein was virtually undetectable in an antisense PAI-1-expressing derivative (4HH). The R/A isolate (Higgins et al., 1991), which expresses relatively low levels of PAI-1 protein, is also included for comparison. Although actin and PAI-1 are the predominant protein species resolved, it is apparent that the almost complete loss of and reduction in SAP fraction PAI-1 deposition for the 4HH and R/A cell lines, respectively, is highly specific.

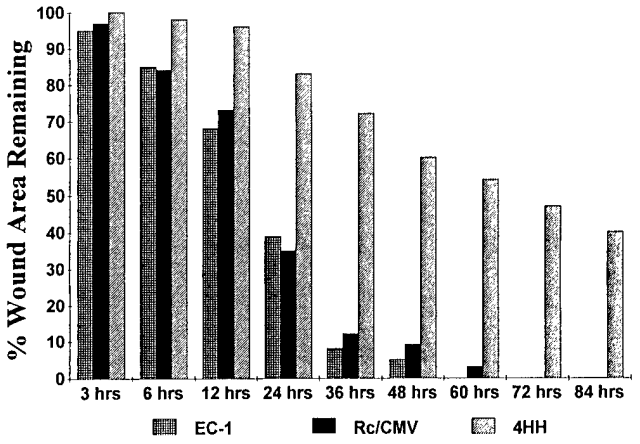

Fig. 9. Kinetics of monolayer scrape wound repair by Rc/CMVIAP-4HH cells compared to parental EC-1 cells and Rc/CMV control transfectants. Confluent monolayers of EC-1, Rc/CMV, and Rc/CMVIAP-4HH cells were maintained in serum-free medium for 3 days, then wounded, and the time course of injury repair assessed as described (Materials and Methods). Whereas the repair rate for EC-1 and Rc/CMV cells was virtually identical, wounds created in monolayers of 4HH cells exhibited little migratory activity and remained open for as long as 84 h postinjury. Data represent the average of duplicate wound repair assays.

DISCUSSION

In vitro migration into scrape-denuded areas is accomplished by the lateral movement of surviving cells across relatively uncomplicated substrates (e.g., Pepper et al., 1987; Sato and Rifkin, 1988; Ando and Jensen, 1996) unlike in vivo transit through fibrin-rich barriers or a provisional wound matrix (Clark et al., 1995; Yamada and Clark, 1995). Despite these considerable physiologic differences, certain commonalites are evident between the two models. Data presented here indicate that cohorts of migrating and proliferating T2 epithelial cells induced in response to monolayer trauma are, at least initially, spatially as well as temporally distinct. Proliferation is required to maintain the integrity of the migrating front, although inhibition of DNA synthesis (with HU) does not affect cell motility. Similar functional compartmentalization occurs as part of the wound repair response in vivo, suggesting that several important phases of injury resolution (regional PAI-1 expression, spatial/temporal distinctions between the motile and proliferative phenotypes) (e.g., Romer et al., 1991; Pawar et al., 1995; Reidy et al., 1995; Staiano-Coico et al., 1996; Zahm et al., 1997) are recapitulated in the T2 epithelial cell system. Migration may be, in fact, a prerequisite to trauma-associated proliferation as the impaired ability of 4HH cells to heal monolayer wounds was reflected in a substantially reduced number of S-phase or mitotic cells in the 1- to 2-mm region distal to the site of injury (data not shown).

Cycles of (leading edge) adhesion and (trailing edge) detachment must be maintained for cells to locomote effectively; such motile cells utilize focal contact–like structures to form transient attachments with the ECM (e.g., Woods and Couchman, 1988). The levels of cell surface uPA and newly synthesized or matrix-bound PAI-1, therefore, may regulate movement by influencing the extent of disruption of integrin-ECM adhesions (Duband et al., 1991; Okedon et al., 1992; McGuire and Alexander, 1993; Deng et al., 1996; Stefansson and Lawrence, 1996; Blasi, 1997; Chapman, 1997). Subcellular targeting of de novo synthesized PAI-1 to the cellular undersurface in close proximity to focal contact sites (Kutz et al., 1997), moreover, can influence uPA-dependent proteolysis and cell attachment, the latter as a consequence of interactions between the uPA/PAI-1/uPA receptor (uPAR) system and vitronectin or between PAI-1 and vitronectin/αv integrins (Loskutoff et al., 1999). Indeed, the uPAR associates with β_2/β_1 integrins, binds to vitronectin (Wei et al., 1994, 1996; Bohuslav et al., 1995; Kanse et al., 1996; Chapman, 1997), and focalizes uPA (Wilcox et al., 1997). Vitronectin absorbed from the medium or newly synthesized (Underwood et al., 1993) at the injury site may serve as the initial "matrix" onto which cells adjacent to the wound can migrate.

Binding of vitronectin to the uPAR, and the formation of uPAR-dependent adhesions, requires uPA (Waltz and Chapman, 1994; Wei et al., 1994). Such

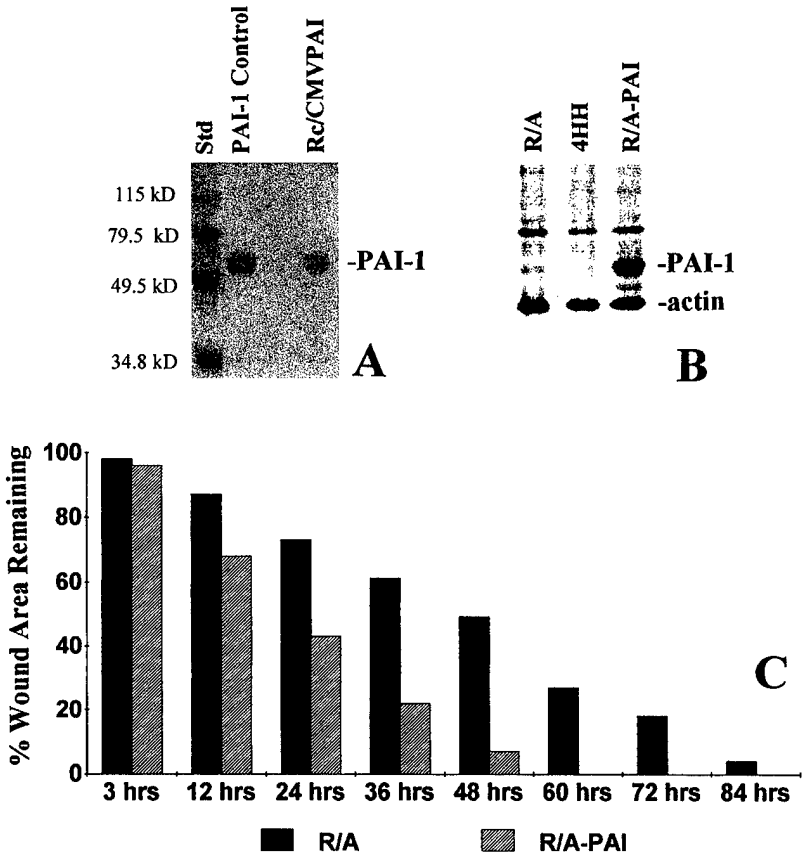

Fig. 10. Effect of vector-driven PAI-1 expression on wound repair by transfectant R/A cells. Coupled in vitro transcription/translation assay confirmed that PAI-1 protein was synthesized using the Rc/CMV-PAI vector as template. When the coupled reaction was complete, the translation products were resolved on a SDS/10% acrylamide gel followed by immunoblotting with PAI-1 antibodies (**A**). Protein standards (Std) and purified PAI-1 protein provided internal controls. Transfection of the Rc/CMVPAI plasmid into R/A cells resulted in derivation of the R/A-PAI line. Gel electrophoresis of the saponin fraction of ^{35}S-methionine-labeled cultures indicated that SAP fraction PAI-1 protein levels expressed by R/A-PAI cells was greater then 11-fold that of the parental R/A line (**B**). The time course of wound closure for R/A-PAI transfectants was significantly reduced relative to R/A cells (**C**), although injury repair by R/A cells (which expressed low levels of PAI-1 compared to EC-1 cells but more PAI-1 than the 4HH derivative; e.g., panel C and Fig. 8) was inhibited relative to EC-1 cells and more effective than 4HH cells (Fig. 9).

uPAR–vitronectin interactions involve the second and third domains of the receptor and are stimulated by pro-PA, uPA, and the isolated A-chain of uPA (Andreasen et al., 1997; Blasi, 1996). PAI-1 binds to the somatomedin B domain of vitronectin, which is the same region that interacts with the uPAR (Deng et al., 1996). The approximately 30-fold greater affinity of PAI-1 for vitronectin, as compared to the affinity of vitronectin for uPAR, suggests a mechanism whereby this SERPIN may effectively dissociate bound vitronectin from uPAR and can detach cells that utilize uPAR as a matrix anchor from a vitronectin substrate (Deng et al., 1996; Loskutoff et al., 1999). Receptor-associated uPA/PAI-1 complexes, moreover, are internalized by endocytosis, which promotes formation of a vacant uPAR on the cell surface by either the internalization of the complex alone or the complex bound to the uPAR followed by receptor recycling (Andreasen et al., 1994, 1997; Blasi, 1996). The available uPAR may bind newly absorbed vitronectin, further supporting adhesive interactions between the cell and the "matrix" to promote lateral migration into the denuded area. Alternatively, PAI-1 may directly inhibit αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site (Stefansson and Lawrence, 1996; Loskutoff et al., 1999).

Wound healing appears to be managed by the temporal induction of genes associated with the plasminogen activation cascade, changes in cellular adhesive characteristics, and by the spatial relationship between surviving cells and their distance from the injury site. Enhanced uPA/plasmin activity is important for cell invasion through complex matrices (Meissauer et al., 1991; Kariko et al., 1993; Stahl and Mueller, 1994; Kawada and Umezawa, 1995; Liu et al., 1995). The requirement for uPA activity to transverse over a substratum, however, is less clear and may be cell type dependent. Although both keratinocytes and endothelial cells express increased levels of uPA and PAI-1 in response to monolayer wounding, uPA is apparently not required for keratinocyte locomotion in vitro, whereas occupancy of uPAR by uPA, but not uPA catalytic activity, facilitates wound-responsive endothelial

cell motility (Pepper et al., 1987; Sato and Rifkin, 1988; Okedon et al., 1992; Ando and Jensen, 1996). Present data indicate that PAI-1 expression is an early response to injury and necessary for normal rat kidney–derived epithelial cells to effectively repair monolayer wounds. Since PAI-1 is also critical for invasive growth (Liu et al., 1995; Bajou et al., 1998), the function of PAI-1 in wound repair is likely to be complex. Whether this SERPIN stimulates or inhibits cell motility is likely dependent on both the level and focalization of uPA activity, the composition of the ECM, and the integrin complement of the cell (Kjoller et al., 1997).

These orchestrated events suggest that wounding may be a stimulus that indirectly leads to (genistein-sensitive) signal-transduction events that manage wound healing. In addition to the involvement of local growth factors in cellular reprogramming (e.g., Sato and Rifkin, 1998), uPA binding to its receptor may also transduce signals to the cell interior independent of plasmin generation (Andreasen et al., 1997). uPA–uPAR interactions induce c-*fos* gene expression (Dumler et al., 1994), whereas pro-uPA binding to the u-PAR has been reported to inhibit cell cycle progression of HL-60 cells (Howell et al., 1994), explaining, perhaps, the delay in proliferation by cells of the migratory front. The role of uPA in migration across a denuded zone may be cell type related (Pepper et al., 1987; Sato and Rifkin, 1988; Ando and Jensen, 1996) and appears complicated by differential utilization of uPA/uPAR vs. vitronectin/integrin targets as PAI-1-sensitive motors (e.g., Chapman, 1997; Loskutoff et al., 1999). Regardless of the precise mechanism(s) involved, the present findings, using the complementary approaches of molecular genetic targeting of PAI-1 expression and rescue of a repair-deficient phenotype, strongly suggest that PAI-1 regulates renal epithelial cell motility in response to monolayer wounding. The associated changes in the temporal expression and site-specific localization of PAI-1, moreover, would likely influence the stability of both preexisting and newly formed cell-to-ECM adhesive complexes (Ciambrone and McKeown-Longo, 1990), thereby consistently modulating cellular migratory traits over the time course of injury repair (Blasi, 1993, 1996, 1997).

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