Dual Role for Plasminogen Activator Inhibitor Type 1 as Soluble and as Matricellular Regulator of Epithelial Alveolar Cell Wound Healing

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Epithelium repair, crucial for restoration of alveolo-capillary barrier integrity, is orchestrated by various cytokines and growth factors. Among them keratinocyte growth factor plays a pivotal role in both cell proliferation and migration. The urokinase plasminogen activator (uPA) system also influences cell migration through proteolysis during epithelial repair. In addition, the complex formed by uPAR-uPA and matrix-bound plasminogen activator inhibitor type-1 (PAI-1) exerts nonproteolytic roles in various cell types. Here we present new evidence about the dual role of PAI-1 under keratinocyte growth factor stimulation using an *in vitro* repair model of rat alveolar epithelial cells. Besides proteolytic involvement of the uPA system, the availability of matrix-bound PAI-1 is also required for an efficient healing. An unexpected decrease of healing was shown when PAI-1 activity was blocked. However, the proteolytic action of uPA and plasmin were still required. Moreover, immediately after wounding, PAI-1 was dramatically increased in the newly deposited matrix at the leading edge of wounds. We thus propose a dual role for PAI-1 in epithelial cell wound healing, both as a soluble inhibitor of proteolysis and also as a matrix-bound regulator of cell migration. Matrix-bound PAI-1 could thus be considered as a new member of the matricellular protein family. (*Am J Pathol* 2006, 169:1624–1632; DOI: 10.2353/ajpath.2006.051053)

Lung epithelium is exposed to various physical and chemical factors that can lead to acute lung injury syndrome. Alteration of epithelial integrity is one of the main characteristics of this syndrome. Insufficient repair of the alveolar epithelium results in slower restoration of the barrier integrity and leads to fibrosis.¹ Re-epithelization of denuded basement membrane occurs through cell migration and spreading followed by proliferation and specific differentiation of type II pneumocytes to type I cells.²

The process of wound closure is controlled by many soluble mediators such as growth factors.³ Particularly, keratinocyte growth factor (KGF), a member of the fibroblast growth factor family (FGF-7), is an important and powerful paracrine agent secreted by stromal fibroblasts and is targeted to the epithelial cells by means of the specific receptor KGF-R.⁴ Ware and Matthay⁵ showed *in vivo* that a biologically active concentration of KGF was present in pulmonary alveoli in acute lung injury. The clinical use of KGF to stimulate wound healing is currently being investigated⁶ and appears to be a promising therapeutic approach.

In various experimental models, the use of KGF promotes healing in different situations, both *in vivo* and *in vitro*.^{7,8} In epithelial alveolar cells, KGF was shown to induce proliferation, both *in vitro*⁹ and *in vivo*.¹⁰ A protective effect of KGF on alveolar epithelial cells was also reported,¹¹ manifesting as resistance to oxidative stress and hyperoxia as well as to irradiation or chemotherapy.^{12–14} However, this protection was not necessarily associated with cell proliferation as initially suggested by Barazzzone and colleagues.¹⁵ Indeed, during epithelial alveolar wound closure, KGF was shown to promote cell spreading, lamellipodia and filopodia emission, and migration.^{16,17} Altogether, the repair of the alveolar epithe-

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lium, crucial for the restoration of the integral barrier, appears to be more efficient in the presence of KGF. In this model, the first steps of the healing process appear to be better characterized by cell migration rather than proliferation.¹⁷ Migration and spreading involve a sequence of interdependent processes, including formation of cell protrusions in the direction of movement, adhesion of the cell to the extracellular matrix, and deadhesion (rupture of adhesive contacts) to allow cell translocation.¹⁸

The urokinase-dependent plasminogen activation system is known to be involved in cell migration mainly through extracellular matrix proteolysis¹⁹ but also through unconventional actions.^{20–24} The system includes a protease, uPA; a glycosylated phosphatidylinositol-anchored receptor, uPAR (CD87), which localizes proteolysis at the cell periphery; and two specific inhibitors, plasminogen activator inhibitor type 1 and type 2 (PAI-1 and PAI-2). Generation of pericellular plasmin by uPA induces direct or indirect matrix proteolysis and is thought to be essential in matrix remodeling, cell adhesion, and, therefore, cell migration. In addition to its well-known involvement in proteolysis, the complex formed by uPAR-uPA and matrix-bound PAI-1 exerts nonproteolytic roles²⁵ operative in adhesion and migration of various cell types, ie, kidney epithelial cells, human myogenic cells, or invasive breast cancer cells.^{26–28}

Moreover, several studies showed a tight correlation between the expression of uPA system components and re-epithelization.^{29–36} For example, injury triggers increasing expression of uPA and PAI-1 in rat tendon,²⁹ in human renal epithelial cells,³⁰ in human³¹ and mouse keratinocytes,³³ or in human bronchial epithelial cells.³⁴ Likewise, decreased expression of one of the components of the uPA system resulted in different alterations of wound healing depending on the cell type.^{31–33,35,36} The majority of these studies indicated PAI-1 as the major actor. However, the mechanisms underlying this process remain poorly understood. The aim of this study was thus to understand better the role of the uPA system and in particular the role of PAI-1, during cell migration in an *in vitro* model of epithelial wound healing. For these studies, we used a rat epithelial alveolar wound-healing model under controlled KGF stimulation.

We show here that the addition of exogenous plasmin, antibodies against uPA, or soluble PAI-1 at the time of wounding modifies the proteolytic action of urokinase and plasmin, which is required for efficient healing. The addition of soluble PAI-1 (sPAI-1) decreases the migration-dependant wound healing; however, the inhibition of endogenous PAI-1 by specific antibodies also results in an unexpected decrease of wound repair. In the first hours after wounding, immunolocalization and Western blotting of PAI-1 localized it as a cell- or matrix-bound protein. These results provide evidence for a dual role for PAI-1 in epithelial cell wound healing, as a soluble inhibitor of proteolysis and as a new matricellular regulator of cell migration and cell functions.³⁷

Materials and Methods

Reagents

DNase I and anti-thyroglobulin antibody were obtained from Sigma Chemicals (L'Île d'Abeau Chêne, France). Elastase was purchased from Worthington (Paris, France). Dulbecco's modified Eagle's medium, glutamine, fetal calf serum, newborn calf serum, and antibiotics used for cultures were obtained from Gibco BRL (Cergy-Pontoise, France). Plasmin, prepared from human plasma, has a specific activity ≥ 5 U/mg, and was purchased from Calbiochem-VWR International S.A.S. (Fontenay sous Bois, France). Rabbit anti-rat PAI-1 (no. 1062) and anti-rodent uPA (no. 1190) were obtained from American Diagnostica (Greenwich, CT). Human recombinant KGF was provided as a gift from Amgen, Inc. (Thousand Oaks, CA).

Isolation and Primary Cultures of Alveolar Epithelial Cells

Alveolar epithelial cells were isolated as previously described.^{17,38} After anesthesia, pathogen-free male Sprague-Dawley rats (175 to 200 g) were tracheotomized and exsanguinated. The lung vascular bed was perfused with solution I containing 140 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L phosphate-buffered saline (PBS), 10 mmol/L *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 mmol/L CaCl₂, and 1.3 mmol/L MgSO₄, pH 7.4, at 37°C. Lungs were then removed from the thorax, and bronchoalveolar lavages were then performed with solution II containing 140 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L PBS, 10 HEPES, 6 mmol/L D-glucose, and 0.2 mmol/L ethylenediaminetetraacetic acid to remove alveolar macrophages and then rinsed twice with solution I. Lungs were filled with 15 ml of elastase solution (porcine pancreas, crystallized twice, 60 IU/ml, prepared in solution II) and incubated in physiological saline at 37°C for 30 minutes. Lungs were subsequently minced in the presence of DNase I, and 5 ml of newborn calf serum was added to inhibit the effect of elastase. Lungs were then sequentially filtered through 150-, 50-, and 10- μ m nylon mesh. The filtrate was centrifuged at $300 \times g$ for 8 minutes. The cell pellet was resuspended in Dulbecco's modified Eagle's medium containing 25 mmol/L D-glucose at 37°C. The cell suspension was plated on bacteriological plastic dishes to help remove macrophages by differential adherence. After a 1-hour incubation at 37°C in a 5% CO₂ to 95% air incubator, unattached cells in suspension were removed and centrifuged at $300 \times g$ for 8 minutes. The resulting cell pellet ($>95\%$ viability, 20 to 30×10^6 cells/rat) was plated at a density of 1×10^6 cells/cm² on 24-well culture plates (Nunc, Rochester, NY). Culture medium was Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mmol/L L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin. Cultures plates were incubated in a 5% CO₂ to 95% air incubator. After 36 hours, the culture medium was changed for a serum-free medium composed of Dulbec-

co's modified Eagle's medium supplemented with 2 mmol/L L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin $\pm$ KGF (100 ng/ml).

This method of pneumocyte isolation allows us to reach a cell culture purity of 95% type II pneumocytes as reported previously in our laboratory.¹⁷ Cell culture purity was confirmed by immunostaining of epithelial Na⁺ channel (EnaC) (identification of epithelial cells) and vimentin (identification of mesenchymal cells). The vimentin antibody was a mouse monoclonal anti-rat antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 and revealed with fluorescein isothiocyanate-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) in a 1:400 dilution. The ENaC antibody was a rabbit polyclonal anti-rat antibody diluted 1:400 (a generous gift of P. Barby, Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Valbonne, France).

In Vitro Wound Healing Assay

The *in vitro* wound-healing assay was performed as previously described.³⁹ Freshly isolated rat alveolar epithelial cells were seeded on 24-well tissue culture dishes (2×10^6 cells/well). Except in controls, 100 ng/ml KGF was added immediately after cell seeding. When confluence was reached, cell monolayers were then gently scratched with a pipette tip across the entire diameter of the well and extensively rinsed with medium to remove all cellular debris. The area of denuded surface (150 to 300 μ m of width) was quantified immediately after wounding and again 20 hours later, and the extent of wound closure determined by calculating the ratio between the surface area of the wound after 20 hours incubation and the surface of the initial wound. These data were then expressed as the percentage of wound closure relative to the control conditions for each experiment. The plate was placed on an inverted microscope (Zeiss, Rueil-Malmaison, France), and an image was obtained with the use of a charge-coupled device camera (CCD-IRIS; Sony, Paris, France) connected to the microscope. The image was subsequently captured by an image-analyzing frame-grabber (Video Enhancer FA320; Futek, Irvine, CA) and analyzed by image analysis software (Image J 1.32e; National Institutes of Health, Bethesda, MD). Wounds that appeared to be too large ($>300 \mu$ m) or too narrow ($<150 \mu$ m) were ignored. Blocking antibodies were used at different concentrations, and controls were obtained using an appropriate dose of nonrelevant antibodies (anti-thyroglobulin antibodies).

Western Blot Analysis and Extracellular Matrix Isolation

Rat alveolar epithelial cells were wounded (see above), and after 24 hours, the cell supernatants were collected. Epithelia were washed two times with PBS at 37°C. Cells were removed by washing on ice with 1% PBS/Triton. Extracellular matrix was scraped off and extracted in sodium dodecyl sulfate sample buffer (1% sodium dode-

cyl sulfate). Samples were boiled 3 minutes at 100°C. Sodium dodecyl sulfate gel electrophoresis in a 10% polyacrylamide was performed under reducing conditions. The proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane. PAI-1 was detected with polyclonal rabbit anti-rat PAI-1 (no. 1062; American Diagnostica) diluted 1:200. The secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG (DakoCytomation, Trappes, France) diluted 1:800. The blot was developed by chemiluminescence using ECL Western Blotting Analysis System (Amersham Bioscience, Piscataway, NJ).

Immunocytochemistry

Media were aspirated and cells washed three times in PBS before fixation/permeabilization in -20°C methanol for 10 minutes. Cells were subsequently washed three times in PBS, specific sites saturated 45 minutes in PBS and 1% bovine serum albumin, and incubated with antibodies against uPA 1:400 (rabbit anti-rodent uPA IgG, no. 1190; American Diagnostica) or PAI-1 1:200 (rabbit anti-rat PAI-1 IgG, no. 1062; American Diagnostica) overnight. Primary antibodies were revealed 30 minutes with fluorescein isothiocyanate secondary antibodies (anti-rabbit IgG 1:400; Biomed, Foster City, CA). Coverslips were mounted in mounting media PBS/glycerol 1:1 (v/v).

Statistical Analysis

Statistical analysis was done using nonparametric Fisher test (Statview 5.0; SAS Institute Inc., Cary, NC). All *P* values <0.05 were considered statistically significant.

Results

Involvement of Plasmin in Epithelial Alveolar Wound Healing

To study the molecular involvement of the uPA system in epithelial alveolar wound healing *in vitro*, rat epithelial alveolar cells were grown in serum-free medium with or without effectors as described in Materials and Methods. The rate of wound closure was measured during KGF-induced healing and compared with control conditions. In accordance with the previously published data,¹⁷ we showed that 100 ng/ml KGF increases the rate of wound healing by 48% ($P < 0.0001$) (Figure 1). The effect of added plasmin (10^{-7} mol/L) on control or KGF-treated wounds was assayed (Figure 1). In serum-free conditions without KGF treatment, plasmin increased wound closure by 13%. In KGF-stimulated wounded cultures, plasmin induced an increase in wound closure, by 37% compared with control, suggesting that the uPA system plays an important role in KGF-induced wound healing.

Figure 1. Effect of KGF and plasmin on the rate of *in vitro* alveolar epithelial repair. Rat alveolar epithelial cells were grown to confluent monolayers, and a mechanical wound was made using a pipette tip. Wound closure was observed during healing, and photographs were taken at time 0 and 20 hours after wounding. For KGF conditions, cells were treated before injury and during the healing with 100 ng/ml KGF. Plasmin at 1.10^{-7} mol/L was added just before injury with or without KGF (100 ng/ml). KGF-free, $n = 22$; KGF, $n = 22$; KGF-free + plasmin, $n = 4$ ($*P < 0.001$); KGF + plasmin, $n = 6$ ($*P < 0.0001$, mean $\pm$ SEM). Pictures of wound closure were taken on living cultures, at $\times 25$ magnification, and analyzed to evaluate rate of wound closure. KGF favors healing by 48%. In KGF conditions, plasmin increases wound healing by 37%.

Involvement of Urokinase in Epithelial Alveolar Wound Healing

To better understand the role of the uPA system, we have used two different approaches. In the first one, we blocked the action of the urokinase plasminogen activator (uPA) by using anti-uPA antibodies in wounded cultures under KGF treatment (Figure 2A). These data indicated that wound closure was decreased by 12% with 1 μ g/ml of antibodies and 27% with 10 μ g/ml. In the second approach, we used soluble PAI-1. PAI-1, the physiological inhibitor of uPA, was added using the same culture conditions as described above. Soluble PAI-1 decreased the rate of wound closure by 30% (50 μ g/ml of soluble PAI-1) in KGF conditions (Figure 2B). Together, these data indicate that uPA is essential for wound healing in KGF-stimulated wounded cultures.

Involvement of Matrix-Bound PAI-1 in Epithelial Alveolar Wound Healing

PAI-1 is a serine protease inhibitor essential for the control of extracellular matrix proteolysis, but it has also been described as a matrix component promoting cell matrix interactions and thereby influencing cell migration.^{20,21,28,33,40-42} Here we hypothesized that matrix-bound PAI-1 could also be involved in the epithelial alveolar cell migration during wound closure. To address this possibility, we treated the injury with polyclonal anti-PAI-1 antibodies that have two targets in this experiment. First, anti-PAI-1 antibodies inhibit soluble PAI-1 and support proteolysis. As shown above (Figure 1), added plasmin increased proteolysis and had a positive influence on wound closure, as stated by other studies.¹⁹ However, on treatment with anti-PAI-1 antibodies, which should raise

Figure 2. Inhibition of wound closure in KGF and serum-free condition by blocking uPA. Rat alveolar epithelial cells were grown to confluent monolayer in serum-free medium or pretreated by KGF 24 hours before injury. Monolayers were scraped using a pipette tip as described in experimental procedure. **A:** Wounded monolayers of rat primary alveolar epithelial cells were incubated with uPA antibodies at indicated concentrations, and wound closure was measured after 20 hours. Anti-thyroglobulin antibodies were used at 10 µg/ml as an internal control. Data are means $\pm$ SEM from five (1 µg/ml) and six (10 µg/ml) independent experiments ($*P < 0.025$). For KGF-free conditions, four independent experiments were performed in the presence of anti-uPA antibodies ($P = 0.0028$). Inhibition of uPA by anti-uPA antibodies decreased wound healing (by 12.4% for 1 µg/ml and 26.8% for 10 µg/ml). **B:** Wounded monolayers of rat primary alveolar epithelial cells were incubated with soluble recombinant active PAI-1 at 50 µg/ml, and wound closure was measured throughout 20 hours. Data are means $\pm$ SEM from three independent experiments for KGF-treated culture ($*P = 0.0007$) and serum-free condition ($*P = 0.0005$). **Inset** shows the behavior of wound closure under KGF-free conditions, with or without anti-uPA antibodies (10 µg/ml) or soluble PAI-1 (50 µg/ml). Inhibition of uPA, by soluble PAI-1, decreased wound healing (by 29.7% for 50 µg/ml).

proteolysis, the rate of wound closure did not increase as expected, but instead the opposite occurred, and healing was dramatically decreased by anti-PAI-1 antibodies. A reduction of wound closure by 19% was seen with 10 µg/ml of anti-PAI-1 antibodies, 30% with 20 µg/ml, and 40% with 50 µg/ml (Figure 3). This decrease prompted us to look for a second mechanism of wound closure inhibition. Anti-PAI-1 antibodies also bind matrix PAI-1 (mPAI-1), which has been shown to promote cell matrix interactions, adhesion, and anchorage.^{21,25–27} These data together with the results presented in Figure 2, A and B, strongly suggest an alternative nonproteolytic role of mPAI-1 as a partner committed in the migration process.

Localized Expression of PAI-1 and uPA in the First Steps of Epithelial Alveolar Wound Healing

In the KGF wound repair assay, PAI-1 expression appeared restricted to the outermost edge of cells of the wound as shown by immunocytochemistry (Figure 4, A

Figure 3. Anti-PAI-1 antibodies inhibit wound closure under KGF conditions. Monolayer wounding was delayed when anti-PAI-1 antibodies (10 to 50 µg/ml) were added during wound healing compared with KGF condition. Wound closure was observed and quantified 20 hours after injury, as described previously. Data are means $\pm$ SEM from six (10 µg/ml), five (20 µg/ml), and four (50 µg/ml) independent experiments. **Inset** shows the behavior of wound closure under KGF-free conditions, with or without anti-PAI-1 antibodies (20 µg/ml). Anti-PAI-1 antibodies decreased wound closure, and this effect appeared to be dose-dependent. With nonrelevant antibodies (anti-thyroglobulin, 50 µg/ml), rate of wound closure was similar to control KGF-treated cultures. $*P < 0.05$.

and B). These results suggest that the leading cells deposit new matrix on the denuded substrate (just at their outer edge to which PAI-1 is attached). A similar distribution pattern was also observed for uPA (Figure 4C). Immunolabeling of uPA appeared diffused on all cells, but a much stronger signal was observed on wound edge cells. Finally, by Western blotting, we showed that PAI-1 expression was dramatically increased under KGF conditions (Figure 4D). PAI-1 was preferentially found in matrix extracts rather than cell supernatants (not detectable). Our results provide evidence for the dual role of PAI-1 during the wound healing of epithelial alveolar cells, on the one hand as proteolytic inhibitor and on the other as matrix-bound protein.

Discussion

Today, KGF appears as an efficient protector of the lung. This well-known member of the FGF family (FGF-7)⁴ is indeed present *in vivo* in normal lung and released at the wound site,⁶ where it has been shown to protect epithelium against oxidative stress in mice¹¹ and to keep the type II pneumocytes in a secretory phenotype.⁴³ Mice treated intravenously with KGF show attenuated lung damage in a situation in which proliferation is not involved, ie, oxygen exposure.¹⁵ In humans, during acute lung injury, Stern and collaborators⁴⁴ showed the protective role of KGF and even proposed, together with other groups,⁶ treatment with KGF for acute or chronic lung injury. Recently, Finch and Rubin⁴⁵ suggested the use of KGF for human epithelial protection and repair. For these reasons, it was of interest to us to study the role of the uPA system in KGF-treated cells. We thus used a model in which the rate of wound closure under KGF conditions was increased compared with serum-free conditions¹⁷ by 49% in our hands. Previous studies showed that KGF has no effect on proliferation during the first 24 hours of the experiment but rather has an effect on cell motility.¹⁷ To determine the role of the uPA system in epithelial

Figure 4. Localization of PAI-1 and uPA by immunocytochemistry and Western blotting in KGF-treated cultures. **A** and **B**: PAI-1 expression in scrape-wounded rat alveolar epithelial cells. PAI-1 expression was evident 24 hours after injury and restricted to cells immediately bordering the edge of the wound. **C**: Expression of diffused uPA, specifically localized to the cells of the outermost edge of the wound. **D** (before wounding) and **E** (wounding site): Controls treated with the secondary antibody only. The same secondary antibody was used for both PAI-1 and uPA staining. **Arrowheads** in **E** underline wound margin. In **F**, matrix extract from serum-free or KGF-wounded cultures was assessed for PAI-1 expression. Matrix-bound PAI-1 (mPAI-1) remained undetectable on serum-free condition; under KGF treatment, expression of mPAI-1 was dramatically increased, whereas soluble PAI-1 (ie, in supernatant) was undetectable.

alveolar wound healing, short-time experiments were thus performed during the first steps of closure in which cell migration is pivotal.

In the presence of KGF, we showed that plasmin increased wound closure (37%), confirming an essential role of the uPA system.^{20,40} The inhibition of uPA,

either by specific antibodies or by soluble PAI-1 induced a decrease in wound healing (down 27 and 30%, respectively), suggesting that uPA is a key actor in this process. These results imply that the proteolytic action of the uPA system is crucial in wound healing and is operative under KGF stimulation condition. It is noteworthy that KGF has been shown to increase uPA in human epithelial keratinocytes.⁴⁶ Generally speaking, the uPA system is increased overall in lung wound healing⁴⁷ or during transdifferentiation of type II pneumocytes into type I pneumocytes, an indispensable step for a complete healing in the lung. Our experiments were performed in the frame of wound healing under KGF stimulation.

In contrast, when the inhibition of PAI-1 by neutralizing antibodies, leads to a decrease in wound healing, it suggests that in this case it is not the proteolytic role of the uPA system that is involved.⁴⁸ The effect of inhibiting the uPA inhibitor would be expected to result in an increase in proteolysis and, as a consequence, an increase in healing. Thus, another explanation is needed. PAI-1 is found either soluble or matrix-bound,^{26,49–51} where it plays diverse roles in adhesion depending on the cell type or the experimental conditions. Matrix-bound PAI-1 can either promote cell adhesion^{21,25,27} and subsequent migration^{24,28,40,52} or deadhesion.^{20,22,23,53}

The inhibition of healing in the presence of blocking antibodies against PAI-1, dependent on the concentration (up to 40% with 50 $\mu\text{g/ml}$), together with the dramatic increase in matrix bound PAI-1 (Figure 4D) suggests that a nonproteolytic action of the uPA system is involved in the KGF-induced wound healing. Indeed, we previously showed and others recently confirmed that mesenchymal cells,^{25,27} epithelial cancer cells,²⁸ or normal cornea cells⁴⁰ can adhere on immobilized PAI-1. Matrix-bound PAI-1 would then serve as a hook for cell spreading and migration (Figure 5A). These cells have to bear on their surface the appropriate counterpart, ie, uPAR-uPA. Indeed, the cells at the wounding edge require the maximum spreading: this is where both uPA and PAI-1 proteins are precisely immunolocalized (Figure 4).

Our data are also in agreement with the results of Staiano-Coico's group,⁵⁴ who showed an overexpression of PAI-1 in preconfluent cells in human keratinocytes in culture. In our experiments, the potential targets of blocking antibodies against PAI-1 are on the one hand soluble PAI-1 and on the other hand matrix-bound PAI-1. If these antibodies were only directed to sPAI-1, we should increase the uPA proteolysis and increase its favorable action in the uPAR/vitronectin ligation as proposed by Waltz and colleagues.²⁶ Alternatively, the PAI-1 antibodies might inhibit the recycling of the uPA-uPAR-integrin complex as proposed by Czekay,^{22,53} and it may also enhance the ligation of the uPA-uPAR-integrin complex to the matrix.²² These polyclonal antibodies against PAI-1 should also increase integrin-dependent cell locomotion because without PAI-1, the binding between integrins and vitronectin is allowed.²¹ Blocking antibodies against PAI-1, if only directed on sPAI-1, might be expected overall to increase wound healing. However, because we observed the opposite, the best explanation is that an-

Figure 5. Schematic representation of the molecular bridge involving uPA, uPAR, and PAI-1 as soluble (A) or matricellular protein (B). PAI-1 could be involved at least in two different processes during KGF-enhanced rat alveolar epithelial wound healing. On the one hand, soluble sPAI-1 (A) plays its well-known role of inhibitor of the proteolytic pathway by preventing plasminogen activation via uPA. On the other hand, bound to extracellular matrix proteins (ie, vitronectin), mPAI-1 (B) acts as a part of transitory anchoring complex involved in cell adhesion and migration. Moreover, in those two cases, the molecular complex [including low-density lipoprotein receptor-related protein (LRP)] mediates internalization of uPAR-uPA-PAI-1 complex and may initiate signal transduction modulating migration. Various signal transduction pathways inducing migration and involving this complex have been described, ie, diacylglycerol (DAG) in epidermal cells by Del Rosso et al⁶⁰; MEK/ERK by Ossowski and Aguirre-Ghiso⁶¹; the Rac 1 pathway for murine embryonic fibroblasts (MEFs) and 1929 cells by Ma et al⁶²; the raft pathway by Carlin et al⁶³ in human airway smooth muscle cells; and finally, in a mPAI-1-enriched matrix, another pathway might be involved (M.M., unpublished data).

other target exists for these antibodies. We propose that this target is matrix-bound PAI-1. In Figure 5, we schematically show the putative modes through which PAI-1 and the PA system may influence cell movement. First, when PAI-1 is soluble (Figure 5A), it acts only as the inhibitor of the proteolysis. Second, (Figure 5B) it acts as a matrix anchor of the molecular complex formed by the uPA system components.

Finally, in its matrix-bound form, PAI-1 appears as a possible member of the matricellular proteins defined by Bornstein and colleagues.^{55–58} These proteins primarily

modulate cell functions such as adhesion or migration.³⁷ Indeed, PAI-1 meets a majority of requirements to be considered as such because PAI-1 is expressed at high level in response to injury,^{31,33,59} it does not serve a structural role of the matrix but functions contextually as a modulator of cell-matrix interactions^{25,27} and of cell functions,^{24,28} and it binds to uPAR-bound uPA. It is this bound PAI-1 that serves adhesion by contributing to a molecular complex (uPAR-uPA-PAI-1) involved in cell adhesion, spreading, and migration,^{24,25,28} or serves deadhesion by inactivating the integrin/vitronectin link,^{21,49,53} impairing the uPA-uPAR-integrin-matrix link or the uPAR/vitronectin link. Thus, the results we show in this study suggest that PAI-1 could be considered as a matricellular protein.

Taken together these results place the uPA system in both a conventional role (ie, proteolysis) and nonconventional role (ie, hooking cells via matrix-bound PAI-1 for spreading and migration) as central in alveolar cell wound healing under KGF induction. This suggests that, more generally, the uPA system could be a target for subtle, new therapeutic approaches.

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