Participation of urokinase-type plasminogen activator receptor in the clearance of fibrin from the lung

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Hattori, Noboru, Thomas H. Sisson, Yin Xu, Tushar J. Desai, and Richard H. Simon. Participation of urokinase-type plasminogen activator receptor in the clearance of fibrin from the lung. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L573–L579, 1999.—In vitro studies have demonstrated that the binding of urokinase-type plasminogen activator (uPA) to its cell surface receptor (uPAR) greatly accelerates plasminogen activation. However, the role of uPAR in clearing abnormal fibrin deposits from the lung is uncertain. Knowing that uPA binding to uPAR is species specific, we used adenoviral vectors to transfer human or murine uPA genes into human or mouse epithelial cells in vitro and to mouse lungs in vivo. By measuring degradation of fluorescein-labeled fibrin, we found that uPA lysed fibrin matrices more efficiently when expressed in cells of the same species. A monoclonal antibody that blocks the binding of human uPA to human uPAR suppressed fibrin degradation by human cells expressing human uPA but not murine uPA. Importantly, 3 days after intratracheal delivery of the vectors, mice receiving murine uPA transgenes degraded fibrin matrices formed within their air spaces more efficiently than animals transduced with human uPA genes. These results show that uPA bound to uPAR increases the efficiency of fibrinolysis on epithelial cell surfaces in a biologically relevant fashion.

fibrinolysis; gene therapy; inflammation

Alterations in the fibrinolytic cascade are likely to play an important role during inflammation and repair within the lung. Evidence that fibrinolysis is impaired during lung inflammation is demonstrated by the presence of abnormal accumulations of fibrin within the alveolar and interstitial spaces (1, 2, 13, 20, 21). Furthermore, analysis of bronchoalveolar lavage (BAL) fluid from patients with diseases associated with fibrin accumulation such as acute respiratory distress syndrome and idiopathic pulmonary fibrosis have revealed that the normally present fibrinolytic activity within the alveolar space is inhibited by increased levels of plasminogen activator (PA) inhibitor (PAI)-1 (3, 9, 18, 19). Similar findings have been reported from experiments with animal models of pulmonary inflammation including bleomycin-induced lung injury and fibrosis (16, 17, 25). Furthermore, Eitzman et al. (11) found a close relationship between fibrinolytic activity and pulmonary fibrosis in experiments with transgenic mice with alterations in their PAI-1 genes. In particular, mice having a targeted deletion of the PAI-1 gene developed less fibrosis than wild-type animals after bleomycin administration. Conversely, mice with a constitutively expressed murine PAI-1 transgene developed an exaggerated fibrotic response to bleomycin injury.

These observations encouraged us to evaluate the use of gene transfer to upregulate alveolar fibrinolytic activity during inflammatory lung diseases in an effort to limit fibrosis. Accordingly, we created adenoviral vectors containing cDNAs for human (Ad.huPA) and murine (Ad.muPA) urokinase-type PA (uPA) and succeeded in upregulating fibrinolysis in vitro and in vivo (15). We selected uPA over other approaches of fibrin clearance because uPA binds to a specific cell surface receptor [uPA receptor (uPAR)] that is present on macrophages (23) and alveolar epithelial cells (14). When bound to uPAR, uPA has been shown to activate plasminogen more efficiently (12). However, before the present study, there was no direct evidence demonstrating that the binding of uPA to uPAR influenced fibrin clearance from the lung in a biologically relevant fashion.

To evaluate the role of uPA in fibrin removal from epithelial cell surfaces and the lung, we took advantage of the knowledge that the binding of uPA to uPAR is species specific (31). Using adenoviral vectors, we transferred human and murine uPA genes into cultured monolayers of epithelial cells of the same or opposite species. Similarly, we transferred and expressed murine and human uPA genes within mouse lungs. By forming plasma-derived fibrin matrices over the monolayers in vitro and within the lung air spaces in situ, we were able to compare the rates of fibrin degradation and evaluate the role of uPAR.

METHODS

Materials. Human Glu-plasminogen, antibodies against human uPAR (3936 and 3937), and recombinant PAI-1 (human and mouse) were purchased from American Diagnostica (Greenwich, CT). Fluorescein-labeled anti-mouse IgG, fluorescein isothiocyanate, plasmin, and α-casein were obtained from Sigma (St. Louis, MO). Human uPA, 5,5′-dithiobis-(2-nitrobenzoic) acid, and thiobenzyl benzyloxycarbonyl-L-lysinate were purchased from Calbiochem (La Jolla, CA). Human fibrinogen was obtained from Kabi (Molndal, Sweden). Texas Red-conjugated bovine serum albumin (BSA) was purchased from Molecular Probes (Eugene, OR).

Cell culture. Human lung-derived epithelial cells (AS49) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Mouse keratinocytes (308 cells) were kindly provided by Dr. A. A. Dlugosz (University of Michigan, Ann Arbor, MI) and cultured...
in 0.05 mM Ca$^{2+}$ minimum essential medium supplemented with 8% FBS that had been passed over a Chelex column (32).

RT-PCR. Total RNA was extracted from A549 cells and 308 cells with TRIzol Reagent (GIBCO BRL, Life Technologies, Grand Island, NY). One microgram of total RNA was submitted to first-strand cDNA synthesis by oligo(dT) priming with the Ready-to-Go First Strand Kit (Pharmacia, Uppsala, Sweden). Five microliters of reaction sample were used for PCR amplification. To compare expression levels of mRNA in human and mouse cells, primers were chosen to match conserved sequences between the human and mouse cDNAs for β-actin and uPAR. The primers used were β-actin sense, 5'-CAGGAGGCCCAGAGCAAGAG-3' and antisense, 5'-AGCCAGTGCCAGGGCAGGA-3', and uPAR sense, 5'-CAGGACCCTCTGCAAGAG-3' and antisense, 5'-CGGGCGCCTCTCA-CAGCTC-3'. Sizes of the amplified products were 267 bp for human uPAR, 270 bp for murine uPAR, and 378 bp for both human and mouse β-actin. A cycle number of 25 was chosen because this level of amplification was found to be within the linear range (data not shown). The PCR products were electrophoresed on 1.2% agarose gels.

Flow cytometric analysis. Monolayers of A549 cells were washed with phosphate-buffered saline (PBS) and incubated with 2 mM EDTA in PBS at 37°C for 5 min. The detached cells were centrifuged and suspended in PBS with 5% FBS. One million cells were incubated with the anti-human uPAR antibodies 3936 and 3937 at 10 µg/ml for 1 h on ice. The cells were washed in PBS and then reacted with fluorescein-labeled mouse IgG for 30 min on ice. The stained cells were washed again, resuspended in PBS with 5% FBS, and analyzed by flow cytometry.

Adenovirus-mediated uPA gene transfer to monolayers of cultured epithelial cells. Construction and purification of the adenoviral vectors Ad.huPA and Ad.muPA, which contained human and murine uPA cDNAs, respectively, were previously described (15). As a control condition, a recombinant adenovirus containing no expression cassette (Ad.BglII) was also prepared. Viral titers (in plaque-forming units) were determined by infection of monolayers of 293 cells with serial dilutions of the recombinant adenoviruses. A549 cells and 308 cells were grown to confluence in 24-well plates and incubated with adenoviral vectors diluted in serum-free DMEM at varying doses [multiplicity of infection (MOI)]. The viral suspension was removed, and complete medium was placed into each well. After 16 h of incubation, the cells were washed with PBS four times, and serum-free DMEM (200 µl) was placed into each well. After incubation for varying times, the conditioned medium was collected. The cells were lysed in 0.1% Triton X-100 in PBS and centrifuged, and the supernatants were collected. PA activity in the conditioned medium and supernatants of cell lysates was measured by a chromogenic assay.

Adenovirus-mediated uPA gene transfer to mouse lungs in vivo. Specific pathogen-free male C57Bl/6 mice (body weight 20–25 g) were purchased from Charles River (Wilmington, MA). Vectors were introduced intratracheally to anesthetized mice as previously described (15). Higher doses of Ad.muPA compared with Ad.huPA were required because the murine construct routinely generated lower levels of transgene expression. Three days after viral instillation, the mice were killed by an overdose of pentobarbital sodium. The trachea of each animal was cannulated with an 18-gauge needle, and the lungs were lavaged with 1 ml of PBS. The BAL fluid was cleared of cells by centrifugation after which PA activity was measured.

PA activity assay. PA activity was measured with an indirect chromogenic assay as previously described (14). Briefly, samples were incubated with human Glu-plasminogen at 37°C for 30 min, and color reagent consisting of 5,5’-dithiobis-(2-nitrobenzoic acid) and thiobenzyl benzoyloxy-carbonyl-L-lysinate was added. The rate of change of absorbance at 414 nm was monitored, and the PA activity was calculated from a standard curve that was generated with known amounts of human uPA.

Assessment of PAI production by epithelial cells infected with adenoviral vectors. A549 cells and 308 cells were grown to confluence in 24-well plates and incubated with Ad.BglII at varying MOIs for 2 h. Serum-free DMEM was placed into each well, and the cells were incubated for 24 h, after which the conditioned medium was harvested. Forty microliters of conditioned medium were added to 10 µl of reagent human uPA (final concentration 0.625 Ploug unit/ml). After 15 min of incubation at 37°C, PA activity was measured with the chromogenic assay described in PA activity assay. As a positive control for PA inhibition, known amounts of human PAI-1 or mouse PAI-1 were added to fresh DMEM, after which the reagent uPA was added.

Zymography. The molecular sizes of PAs were assessed with zymography by the method of Powell-J ones (27). The samples were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% slab gels containing 5 mg/ml of α-casein and 20 µg/ml of Glu-plasminogen. After the gels were washed in 1% Tween 80 for 1 h at 37°C, the gels were incubated in 0.1% Tween 80 in PBS overnight at room temperature. The gels were stained with Coomassie blue and destained in 10% acetic acid and 50% methanol.

Preparation of plasma-derived fibrin-rich matrices. For each experiment, venous blood drawn from a single volunteer was immediately chilled to 4°C without the addition of anticoagulant and centrifuged at 2,500 g for 20 min. The plasma was mixed with three volumes of DMEM, and fluorescein-labeled fibrinogen was added (0.1 mg/ml). The plasma was then used immediately to form fibrin-rich matrices. For experiments measuring fibrinolysis within intact lungs, Texas Red-conjugated BSA (0.25 mg/ml) was added to serve as an internal control to correct for dilutional differences between samples. Labeling of fibrinogen with fluorescein was performed as previously described (30).

Measurement of fibrinolysis by the monolayers of epithelial cells. Fibrin degradation was assessed by measuring the solubilization of fluorescein-labeled fibrin as previously described (30). A549 cells and 308 cells were grown in 24-well plates and infected with adenoviral vectors as described in Adenovirus-mediated uPA gene transfer to monolayers of cultured epithelial cells. The cells were washed four times in serum-free DMEM, and 200 µl of plasma with fluorescein-labeled fibrinogen was added to each well. In <20 min, we routinely observed that the fibrinogen within the plasma underwent polymerization to fibrin. After 6 h, fibrinolysis of the matrices was stopped, and the contents of the wells were centrifuged at 10,000 g for 10 min. The fluorescence in the supernatant was measured in a spectrofluorometer with excitation and emission wavelengths of 485 and 515 nm, respectively. To measure the level of background fluorescence from samples that had undergone no fibrinolysis (0% lysis), fibrin-rich matrices were formed in wells containing no cells. After 6 h, the matrices were transferred to 1.5-ml tubes and centrifuged, after which the fluorescence level of the supernatants was measured. To determine the level of fluorescence from fully lysed matrices (100% lysis), fibrin-rich matrices were treated with 0.1 ml of 10 mg/ml plasmin and processed as indicated above. Percent fibrinolysis of an unknown sample was determined from the fluorescence of its supernatant.
with the assumption that there is a linear relationship between the fluorescence of the 0 and 100% lysis samples.

Measurement of fibrinolysis within lungs. Three days after intratracheal adenoviral vector instillation, the mice were killed and 1 ml of 1:3 (vol/vol) diluted plasma containing fluorescein-labeled fibrinogen and Texas Red-conjugated BSA was injected intratracheally. We had previously found (personal observations) that fibrinogen rapidly polymerizes to fibrin after intratracheal instillation. The Texas Red-conjugated BSA was included in the instilled fluid to allow for correction for any differences in dilution that may occur between samples during subsequent tissue processing. After the plasma was instilled, the trachea of each animal was ligated and the lungs were placed into a 50-ml tube and incubated at 37°C. After 3 h, the lungs were minced and centrifuged at 10,000 g for 10 min. The fluorescent and Texas Red levels in 100 µl of supernatant were determined. Texas Red fluorescence was measured with excitation and emission wavelengths of 590 and 620 nm, respectively. During each experiment, separate aliquots of labeled plasma were also placed into plastic tissue culture wells. The fibrin matrices in one set of wells were allowed to remain intact (0% lysis), and another set were lysed with excess plasmin (100% lysis). The level of fluorescein fluorescence was divided by the level of Texas Red fluorescence. The differences in dilution, the level of fluorescein fluorescence in the supernatants after 3 h of incubation. To correct for correction for any differences in dilution that may occur during subsequent tissue processing. After the plasma was instilled, the trachea of each animal was ligated and the lungs were placed into a 50-ml tube and incubated at 37°C. After 3 h, the lungs were minced and centrifuged at 10,000 g for 10 min. The fluorescent and Texas Red levels in 100 µl of supernatant were determined. Texas Red fluorescence was measured with excitation and emission wavelengths of 590 and 620 nm, respectively. During each experiment, separate aliquots of labeled plasma were also placed into plastic tissue culture wells. The fibrin matrices in one set of wells were allowed to remain intact (0% lysis), and another set were lysed with excess plasmin (100% lysis). The level of fluorescein fluorescence was divided by the level of Texas Red fluorescence. The amount of fibrinolysis in the lung was calculated from the ratios of fluorescein to Texas Red fluorescence. The assumption that percent fibrinolysis followed a linear relationship between the values measured for the 0 and 100% lysis in vitro control samples.

RESULTS

uPAR expression in A549 cells and 308 cells. Flow cytometric analysis was used to confirm that A549 cells express uPAR under our culture conditions. As shown in Fig. 1A, anti-uPAR antibody bound to the surface of A549 cells, demonstrating the presence of cell surface uPAR. Because an antibody against murine uPAR was not available to us, RT-PCR was used to determine whether uPAR message is expressed in 308 cells. The primers for uPAR and β-actin were chosen to bind to sequences that are identical between human and mouse cDNAs, enabling us to compare the expression levels of uPAR and β-actin mRNA between mouse 308 cells and human A549 cells. This semiquantitative RT-PCR analysis revealed that mRNA for uPAR was expressed in 308 cells at a level slightly higher than that in A549 cells (Fig. 1B).

Transduction of PA activity by epithelial cells infected with uPA adenoviruses. To determine the kinetics of uPA expression, we examined PA activity present in conditioned medium from A549 cells and 308 cells infected with uPA adenoviruses at an MOI of 10. Sixteen hours after infection, the cells were washed and fresh serum-free DMEM was placed onto the monolayers. At varying times thereafter, the conditioned medium was collected and PA activity was measured. As shown in Fig. 2A, PA activity accumulated progressively in the conditioned medium. To characterize the PA activity, casein-plasminogen zymography was performed (Fig. 2B). Large lytic bands were seen at molecular masses appropriate for the products of the transferred uPA genes (45 kDa for murine uPA and 54 kDa for human uPA). Under the conditions of the assay, the relatively small amount of uPA generated by endogenous uPA genes was not detectable.

To determine whether differences between A549 cell and 308 cell PA activity could be due to differential induction of PAIs by adenoviral vectors, we tested the conditioned medium for inhibitors. Epithelial cells were infected with varying doses of Ad.BglII, and conditioned medium was collected as above. Next, a known amount of reagent uPA was added to the medium. After the samples were incubated for 15 min at 37°C, residual PA activity was measured. We found that infection with Ad.BglII did not induce detectable levels of PAIs (Fig. 2C). Our assay conditions were sufficient to detect an inhibitor equivalent to <0.1 µg/ml of PAI-1 (see Fig. 2C, inset), and no significant difference in inhibition between human and mouse PAI-1 was observed (data not shown).

Lysis of fibrin matrices by 308 cells and A549 cells transduced with murine or human uPA. To compare the efficiency by which different epithelial cell monolayers lysed fibrin matrices, the level of PA activity produced by vector-transduced cells (conditioned medium plus cell lysate) was measured. By expressing the amount of fibrin degradation by a monolayer relative to the total amount of PA activity produced by the cells, the efficiency of fibrinolysis by monolayers infected with different vectors could be compared. As shown in Fig. 3, A549 cell monolayers were more efficient at degrading fibrin matrices when expressing human uPA than when expressing murine uPA. Conversely, 308 cell monolayers were more efficient when expressing murine uPA than human uPA. These results suggest that the ability of uPA to bind uPAR augments the efficiency of fibrin matrix degradation. The same conclusions would have been reached if fibrin degradation had been expressed relative to conditioned-medium PA activity rather than
conditioned-medium plus lysate activity. The fibrin degradation observed in these experiments was totally dependent on the presence of uPA because the addition of a uPA-specific inhibitor, amiloride (1 mM), completely blocked fibrin degradation (data not shown). Furthermore, the observed fibrin degradation could be ascribed entirely to the activity of the transgenic uPA because the amount of fibrin degradation by nontransfected cells was undetectable during the 6-h assay (data not shown).

To confirm the involvement of uPAR in accelerating the lysis of fibrin matrices, we measured the effects of conditioned-medium plus lysate activity. The fibrin degradation observed in these experiments was totally dependent on the presence of uPA because the addition of a uPA-specific inhibitor, amiloride (1 mM), completely blocked fibrin degradation (data not shown). Furthermore, the observed fibrin degradation could be ascribed entirely to the activity of the transgenic uPA because the amount of fibrin degradation by nontransfected cells was undetectable during the 6-h assay (data not shown).

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To confirm the involvement of uPAR in accelerating the lysis of fibrin matrices, we measured the effects of
anti-uPAR antibodies on the fibrinolytic activity of A549 cells. Monolayers of A549 cells that had been infected with varying doses of adenoviral vectors were incubated with anti-human uPAR antibodies, and their ability to degrade fibrin matrices was measured (Fig. 4A). An anti-uPAR antibody (3936) that competitively inhibits the binding of human uPA to human uPAR (10, 24, 29) reduced the rate of fibrin matrix degradation by A549 cells transduced with human uPA. A different antibody (3937) that binds to uPAR but does not block uPA binding to uPAR had no such inhibitory effect. Neither antibody altered the fibrinolytic activity of A549 cells transduced with murine uPA, which is known not to bind to human uPAR. Because 3936 and 3937 antibodies do not bind to murine uPAR, we expected and found no effect of these antibodies on mouse cells transduced with either murine or human uPA (data not shown).

To further characterize the effects of anti-uPAR antibodies on epithelial cell-induced fibrin degradation, we determined dose-response relationships for the antibodies. We found that the anti-human uPAR antibody 3936 caused a dose-dependent inhibition of fibrin degradation by A549 cells infected with Ad.huPA (Fig. 4B). No effect was seen with any other combination of vector and anti-uPAR antibody. These results demonstrate that the binding of uPA to uPAR accelerates fibrin matrix degradation.

A549 cells transduced with human uPA. A different antibody (3937) that binds to uPAR but does not block uPA binding to uPAR had no such inhibitory effect. Neither antibody altered the fibrinolytic activity of A549 cells transduced with murine uPA, which is known not to bind to human uPAR. Because 3936 and 3937 antibodies do not bind to murine uPA, we expected and found no effect of these antibodies on mouse cells transduced with either murine or human uPA (data not shown).

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Lysis of fibrin matrices by mouse lungs transduced with murine uPA. To determine whether the in vitro observations in Lysis of fibrin matrices by 308 cells and A549 cells transduced with murine or human uPA could be extrapolated to the intact lung, varying doses of adenoviral vectors were administered to mice intratracheally. Three days later, the mice were killed, and one group underwent BAL to measure PA activity and the other group had their lungs instilled with plasma containing fluorescein-labeled fibrinogen and Texas Red-conjugated albumin. The percent fibrin degradation was measured for each virus dose, and the level was compared with the amount of PA activity generated in the BAL fluid (Fig. 5). We found that mice transplanted with murine uPA were more efficient at degrading fibrin matrices than mice expressing transgenic human uPA.
DISCUSSION

The PA cascade appears to be centrally involved in the removal of fibrin from damaged tissue. As mentioned in the introduction, depression of the fibrinolytic system within the lung occurs frequently during inflammatory conditions. Importantly, genetic manipulations in mice that enhance fibrinolysis, namely targeted deletion of PAI-1 genes, were found to reduce the fibrosis that occurred after bleomycin administration (11). Of the two types of endogenous PAs, uPA appears to be more active in extravascular and pericellular fibrinolysis than tissue-type PA (tPA) (4, 28). One of the unique characteristics of uPA is its ability to bind to a specific cell surface receptor, uPAR, expressed by a variety of cells including alveolar epithelial cells and macrophages. When uPA is bound to uPAR, it is brought into close proximity with plasminogen, which also binds to the cell surface by its own receptors. The proximity of the two molecules greatly accelerates the rate of plasminogen activation. Furthermore, plasmin formed on the cell surface is relatively resistant to inactivation by its major inhibitor α2-antiplasmin (12, 26). Taken together, uPAR is well suited to play an important role in cell-mediated fibrinolysis because it provides a means to precisely focus fibrinolysis on the cell surface. This physical arrangement could have substantial biological benefits during inflammatory lung diseases when fibrin deposits are formed on epithelial cell surfaces. An indication that these processes are important was suggested in previous experiments by Simon et al. (30) in which physical contact between fibrin matrices and epithelial cells was found necessary for optimal cell-induced fibrinolysis to occur.

To assess the roles of the various components of the fibrinolytic system, mice with targeted deletions in uPA, tPA, and/or uPAR genes have been studied [reviewed by Carmeliet and Collen (7)]. Mice deficient in both uPA and tPA develop extensive fibrin deposits in several organs including the liver, lung, gastrointestinal tract, and reproductive organs. However, a deficiency in uPA alone caused only mild fibrin deposition in the liver and intestine, whereas deficiency of tPA alone was not associated with any abnormal accumulations of fibrin (8). These data indicate that uPA and tPA can substitute for each other under many, but not all, circumstances. Therefore, to isolate the contribution of uPA-uPAR interactions to fibrin removal, mice deficient in tPA and uPAR were genetically engineered (5). These mice had fibrin accumulation in the liver, but other organs appeared normal. The conclusion from these observations is that uPAR contributes little to prevent spontaneous deposition of fibrin in most organs except the liver. However, a potential contribution for uPAR in removing fibrin deposits during pathological conditions has not been evaluated.

In the present study, we demonstrated that transfected uPA caused degradation of fibrin matrices more efficiently on cultured monolayers of epithelial cells when the uPA was of the same species of origin as the cells. This observation suggests a role for uPAR because uPA binding to uPAR is species specific. This interpretation is supported by our finding that antibody-mediated blocking of uPA binding to uPAR inhibited fibrin degradation in a dose-dependent manner. However, our data also showed that when high levels of uPA are produced, fibrin degradation can occur in the absence of binding to uPAR. The relevance of these in vitro studies to the participation of uPAR in fibrin degradation within the intact lungs was demonstrated with mice transfected with human or murine uPA. The efficiency of fibrin degradation was superior for murine uPA relative to human uPA, again compatible with a role for uPAR. These results also show that a complete understanding of the processes responsible for fibrin clearance requires more information than what can be learned from studies of BAL fluid alone. The participation of solid-phase components, e.g., uPAR on alveolar cells, must also be considered. Although the kinetics of events occurring within fibrin matrices are difficult to measure due to the complexities of liquid- to solid-phase interactions, our results clearly demonstrate the contribution of uPAR to fibrin clearance from epithelial-lined surfaces.

Comparisons of the rates of fibrin degradation were made while taking into account the levels of uPA activity generated within the experimental systems. This allowed us to correct for differences in fibrinolytic rates due to differences in the efficiency of uPA gene transfer and expression induced by the different adenoviral vectors (15). For the in vitro studies, we used total PA activity generated by the epithelial monolayers by summing the levels measured in conditioned medium and cell lysates. For studies involving intact lungs, comparisons were made at equivalent levels of PA activity contained within the BAL fluid. Unfortunately, it is not feasible to measure epithelial cell-associated PA activity within the intact lung. However, studies performed on animals with a genetic deficiency in uPAR suggested that the amount of PA protein contained on the cell surface is relatively small compared with the total amount secreted (6). Our data also show that the PA activity responsible for initiating fibrin degradation in our experiments was generated almost entirely from the transgenes. The amount of uPA produced by the endogenous genes was insufficient to be detected under the conditions of our zymographic assay.

Our in vitro chromogenic assay of PA activity used human plasminogen as the substrate. Published studies have reported small but significant differences in the catalytic efficiencies between human and murine uPA for plasminogen activation (22). In particular, the catalytic efficiency $k_{cat}/K_m$ from analysis of Lineweaver-Burk plots of human uPA for human plasminogen is $0.20 \text{ M}^{-1} \cdot \text{s}^{-1}$, whereas that of murine uPA for human plasminogen is $0.1 \text{ M}^{-1} \cdot \text{s}^{-1}$. However, this difference is of no consequence because the measurements of both PA activity and fibrin degradation that were used in our studies employed human plasminogen as the PA substrate. Thus all the measurements were internally consistent. We also assessed whether our results might have been influenced by a species-specific induction of PAIs caused by adenoviral infection. We detected no such increase in production of inhibitors to human uPA.
by infected mouse or human epithelial cells. We did not assess whether mouse cells might produce inhibitors that preferentially interact with murine uPA.

In summary, our results show that uPA binding to uPAR increases the efficiency of fibrin degradation by cultured monolayers of epithelial cells and by the intact lung. These findings support our choice of uPA as the PA for our gene transfer experiments in which we wish to upregulate pulmonary fibrinolysis in a localized fashion to limit inflammation-induced fibrosis.

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