Novel aspects of urokinase function in the injured lung: role of α2-macroglobulin

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Komissarov AA, Stankowska D, Krupa A, Fudala R, Florova G, Florence J, Fol M, Allen TC, Idell S, Matthay MA, Kurdowska AK. Novel aspects of urokinase function in the injured lung: role of α2-macroglobulin. Am J Physiol Lung Cell Mol Physiol 303: L1037–L1045, 2012. First published October 12, 2012; doi:10.1152/ajplung.00117.2012.—The level of active urokinase (uPA) is decreased in lung fluids of patients with acute lung injury/acute respiratory distress syndrome (ALI/ARDS) whereas α2-macroglobulin (α2-M), a plasma proteinase inhibitor, is a major component of these fluids. Since there have been reports describing the ability of α2-M to form complexes with uPA in vitro, we hypothesized that α2-M may interact with uPA in the lung to modulate its biological activity. Pulmonary edema fluids and lung tissues from patients with ALI/ARDS were evaluated for the presence of uPA associated with α2-M. Complexes between α2-M and uPA were detected in alveolar edema fluids as well as in lungs of patients with ALI/ARDS where they were located mainly in close proximity to epithelial cells. While uPA bound to α2-M retains its amidolytic activity towards low-molecular-weight substrates, it is not inhibited by its main physiological inhibitor, plasminogen activator inhibitor 1. We also investigated the functional consequences of formation of complexes between uPA and α2-M in vitro. We found that when α2-M:uPA complexes were added to cultures of human bronchial epithelial cells (BEAS-2B), activation of nuclear factor-κB as well as production of interleukin-6 and -8 was substantially suppressed compared with the addition of uPA alone. Our findings indicate for the first time that the function of uPA in patients with ALI/ARDS may be modulated by α2-M and that the effects may include the regulation of the fibrinolytic and signaling activities of uPA.

epithelial cells; lung; inflammation; α2-macroglobulin; urokinase

α2-MACROGLOBULIN (α2-M), a high-molecular-weight (HMW; >700 kDa) plasma glycoprotein, forms a tetramer composed of identical subunits, linked in pairs by disulfide bonds. The major source of plasma α2-M is the hepatocyte; however, other monocytes including macrophages synthesize and secrete this protein. α2-M is a “slow acting” proteinase inhibitor that binds proteinases from all major classes. The mechanism by which α2-M inhibits proteinases is unique. When proteinases hydrolyze the specific peptide bond localized in the so called “bait” region of α2-M, the resulting conformational change irreversibly traps the enzyme within the α2-M molecule. One or two molecules of proteinase are bound per molecule of α2-M depending on the association rate constant and possibly on the size of the proteinase. Further, among proteinase inhibitors, only α2-M-entrapped enzymes can still interact with low-molecular-weight (LMW) substrates and inhibitors, which are able to reach the active site of the enzyme located in a “molecular cage” type complex with α2-M.

Conformational change of the α2-M, which occurs upon reaction with proteinases, exposes binding sites for the LMW inhibitor/low-density lipoprotein receptor-related protein (LRP). LRP is present on the surfaces of many different cell types, including hepatocytes, macrophages, and dendritic cells. α2-M in its native form does not bind LRP whereas α2-M-proteinase complexes rapidly undergo endocytosis (6, 18, 25, 39). Moreover, LRP functions as a signaling receptor for many ligands, including α2-M (26, 27).

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is a complex syndrome arising in a variety of clinical settings and characterized by severe respiratory failure. The microvascular endothelium and the alveolar epithelium undergo inflammatory changes during ALI/ARDS. Damage to the pulmonary alveolar-capillary barrier leads to the influx of protein-rich edema fluid into alveolar spaces (40). Lung fluids from patients with ALI/ARDS contain significantly elevated concentrations of α2-M, due primarily to increased pulmonary alveolar-capillary membrane permeability. On the other hand, this protein is practically undetectable in normal lung lavage fluid (15, 23, 43).

Urokinase (uPA), a serine protease, catalyzes the activation of plasminogen to plasmin and is regulated by plasminogen activator inhibitor type 1 (PAI-1). Moreover, increased production of PAI-1 in patients with ALI/ARDS is partially responsible for the decrease in amount of active uPA and promotes fibrin deposition within the alveolar spaces of patients with ALI/ARDS (11, 16, 17, 38). Since our previous studies indicate that α2-M present in lung edema fluids from patients with ALI/ARDS retains its antiproteinolytic activity, and α2-M has been suggested to act as an uPA inhibitor, we tested the hypothesis that α2-M affects function of uPA in the injured human lung (23, 31).

Our study was undertaken to address gaps in the current understanding of the role of uPA in the pathogenesis of ALI/ARDS. We first sought to determine whether endogenous uPA is targeted solely by PAI-1 or whether it also interacts with α2-M in lung edema fluids of patients with ALI/ARDS. We next wanted to assess the possible effects of formation of endogenous α2-M:uPA complexes on the fibrinolytic and sig-
naling activities of uPA. Lastly, we sought to determine whether or not endogenous active α₂-M within edema fluids can form complexes with exogenous uPA, since treatment with fibrinolytic agents has been proposed as a useful therapeutic approach in ALI/ARDS (13, 14).

MATERIALS AND METHODS

Human subjects. Studies involving human lung tissues and pulmonary edema fluids were approved by the Institutional Review Board of the University of Texas Health Science Center in Tyler and the University of California, San Francisco. Human lung tissues were obtained from the Department of Pathology. Tissues remaining after pathological evaluation was completed were routinely de-identified and archived. Lung tissue sections from four patients with ALI/ARDS and three histologically normal lung tissues were evaluated in this study. In total 98 or 48 different fields were analyzed as described in Confocal microscopy in ALI/ARDS and normal lung tissue sections, respectively.

Pulmonary edema fluid samples were obtained as previously described (22, 23, 41). The fluids were collected after informed written consent had been obtained from the subjects or their representatives. Hydrostatic pulmonary edema (HE) was defined based on clinical evidence of cardiac dysfunction from an acute myocardial infarction, exacerbation of chronic heart failure, or volume overload with either a pulmonary arterial wedge pressure >18 mmHg or a two-dimen-
sional echocardiogram demonstrating a reduction in the left ventric-
ular ejection fraction plus the presence of a transudative pulmonary edema fluid to plasma total protein ratio < 0.65. ALI was diagnosed according to the following criteria: 1) a PaO₂/FiO₂ ratio ≤300 mmHg; 2) bilateral infiltrates on the chest radiograph; and 3) a pulmonary artery wedge pressure of ≤18 mmHg and/or no clinical evidence of elevated left atrial pressure (4). Alveolar edema fluid samples were obtained within 15 min of intubation and mechanical ventilation. A 14-French suction catheter (Becton-Dickinson, Lincoln Park, NJ) was passed through the endotracheal tube and wedged into the distal airways. Then, samples of >2 ml were suctioned gently from edema fluid through an endotracheal tube. Heparin (10 –100 U) was added to each sample, and samples were centrifuged at 3,000 g for 10 min to remove cells. All samples were aliquoted and stored at −70°C. Samples were thawed on ice and refrozen when additional aliquoting was necessary.

Reagents. Enzymatically active two chain uPA (HMW form, free of bacterial endotoxins) was obtained from American Diagnostica (Stamford, CT). α₂-M was purchased from either American Diagnostica or Biodesign (Saco, ME). α₂-M:uPA complexes were prepared by incubation of uPA with α₂-M at 1 to 70 molar ratio. Anti-α₂-M receptor (LRP) antibody was a generous gift from Dr. Dudley K. Strickland (University of Maryland School of Medicine, Baltimore, MD). The fast form of α₂-M (α₂-M*) was prepared as previously described (22).

Human recombinant PAI-1 was purified and characterized as de-
scribed elsewhere (24). The concentration of PAI-1 was calculated from absorbance at 280 nm, using extinction coefficient of 0.93 1·cm−1·mg−1 and Mf value of 43,000.

Detection of uPA associated with α₂-M in edema fluid samples. Pulmonary edema fluid samples were subjected to nondenaturing polyacrylamide gel electrophoresis (5% Tris-borate) and Western blot analysis. The 5% Tris-borate gels are ideally suited for analysis of HMW proteins, such as α₂-M. The ratio of acrylamide to bisacryl-
amide is 29:1, which results in more open pore structure that is recommended for separation of proteins >150-kDa. Therefore, in this type of electrophoresis only large proteins, such as α₂-M or proteins/α₂-M complexes, can be detected but smaller proteins or protein complexes (such as uPA, uPA/PAI-1 complex, PAI-1, etc.) migrate quickly enough to exit the gel during electrophoresis. We used 5% Tris-borate gels to visualize complexes between α₂-M and uPA (uPA bound to α₂-M) in their native state. Significantly, such gels are routinely employed to study the function of α₂-M (22, 23).

Separated proteins were transferred to a polyvinylidene difluoride membrane (Pall, Pensacola, FL). The membrane was then blocked and incubated with anti-uPA antibody or anti-α₂-M antibody (both from American Diagnostica) followed by enhanced chemiluminescence reagents (PerkinElmer Life Sciences, Boston, MA). Finally, the mem-
brane was exposed to X-Ray film F- X810 (Phenix, Hayward, CA).

Analysis of amidolytic activity of uPA and detection of active α₂-M in human pulmonary edema fluids. Measurements of amidolytic ac-
tivity of uPA in pulmonary edema fluids were carried out and analyzed as described previously (21).

Cells and culture conditions. Human bronchial epithelial cells (BEAS-2B) were obtained from the ATCC (Manassas, VA). BEAS-2B were cultured in LHC-9 medium (GIBCO-BRL Laboratories, Grand Island, NY). At confluence, the medium was replaced with serum-free RPMI containing L-glutamine. The cells were then washed and incubated with uPA (10 ng/ml), α₂-M:uPA complexes containing 10 ng/ml of uPA, or α₂-M (10 μg/ml) alone in serum-free RPMI 1640 medium (for measurements of cytokines) or HBSS (for evaluation of cell signaling). In some experiments, BEAS-2B cells were pretreated with anti-α₂-M receptor (LRP) antibody or fast form of α₂-M (α₂-M*) for 30 min at 4°C before incubation with stimulants.

Confocal microscopy. Lung tissue sections from patients with ALI/ARDS and normal lung tissues were processed as previously described (2). The sections were incubated with anti-α₂-M antibody (INC Biomedicals, Solon, OH) and FITC-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA), then with anti-uPA antibody (American Diagnostica) followed by Texas Red-conjugated secondary antibody (Santa Cruz), and finally counterstained with Hoechst 33342 (Calbiochem, San Diego, CA). Anti-surfactant protein B antibody (Chemicon, Temecula, CA) and Cy5-conjugated secondary antibody (Chemicon) were used to visualize epithelial cells.

In addition, effect of α₂-M on function of uPA was studied in vitro using human bronchial epithelial cells (BEAS-2B; ATCC). For con-
focal microscopy experiments, BEAS-2B cells mounted on slides by cytospin centrifugation were incubated with antibodies against uPAR (urokinase receptor; American Diagnostica) and LRP (Santa Cruz), or with anti-phospho-nuclear factor (NF)-κB p65 antibody (Cell Signaling, Danvers, MA), or anti-phospho Rac1 antibody (Santa Cruz). In some experiments, anti-uPA and anti-LRP antibodies were also used. The slides were evaluated using a PerkinElmer Ultra VIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope (PerkinElmer, Wellesley, MA), using ×60 PlanApo immersion objective (numerical aperture 1.4) at room temperature. Ultra VIEW Imaging Suite software (version 5.5.0.4) and CorelDRAW 12 (version 12.0.0.536) were used for image processing.

Analysis of effect of α₂-M on uPA activity in vitro. Amidolytic activity of uPA was estimated from changes in the fluorescence emission at 440 nm (excitation 344 nm) of fluorogenic 7-amino-4-methylcoumarin (AMC)-based substrate (Pefaffur; Bz-β-Ala-
Gly-Arg-AMC AcOH; Centerchem, Norwalk, CT) as described previously (21).

uPA (0.4 μM) was incubated in the presence or absence of 27 μM α₂-M at 37°C in 50 mM HEPS/NaOH. Aliquots of the reaction mixture were withdrawn at 0, 10, 20, 40, 80, 160, and 240 min, after which amidolytic activity of uPA was assayed in the presence or absence of a fourfold molar excess (over uPA) of human PAI-1. Both total and PAI-1-resistant activities of uPA were plotted vs. time and analyzed as previously described (21). In addition, α₂-M:uPA complexes were subjected to nonreduced SDS-PAGE (4–12% gradient NuPage gel; Invitrogen) and stained with Simple Blue (Invitrogen, Carlsbad, CA).

Cytokines. The concentrations of interleukin-6 (IL-6) and -8 (CXCL8) were measured using specific ELISA kits (eBioscience, San Diego, CA or Peprotech, Rocky Hill, NJ, respectively) according to the manufacturers’ instructions.
**RESULTS**

α2-M:uPA complexes in pulmonary edema fluids and lung tissues from patients with ALI/ARDS. The purpose of the first part of this study was to determine whether uPA is associated with α2-M in lungs of patients with ALI/ARDS. Edema fluid samples from 10 different patients with ALI/ARDS and preformed α2-M:uPA complexes were first run on the native gel (5% Tris-borate), and then Western blot analysis was performed using either a specific antibody directed against uPA or anti-α2-M antibody. Preformed α2-M:uPA complexes (uPA bound to α2-M) were visualized using anti-uPA antibody (Fig. 1A), suggesting that the anti-uPA antibody recognizes uPA associated with α2-M. In edema fluids bands visible after staining with the anti-uPA antibody migrate like preformed α2-M:uPA complexes, so it can be assumed that a portion of the uPA present in lung edema fluids is bound to α2-M (Fig. 1A). The same membranes were also incubated with the anti-α2-M antibody to confirm presence of α2-M in the samples (Fig. 1B).

Further, we evaluated normal lung tissues and tissues from patients with ALI/ARDS for the presence of uPA associated with α2-M. As shown in Fig. 2, there is a robust staining for α2-M (green) in ARDS tissue (Fig. 2B) and less in normal tissue (Fig. 2A). The latter finding is in agreement with a previously published study showing the presence of α2-M in normal lung (10). uPA is also easily detectable in normal lung tissue (Fig. 2A) though significantly more staining for uPA is visible in lung tissue from patient with ARDS (red) (Fig. 2B, red). The presence of colocalization (yellow) in ARDS tissue indicates that a portion of the uPA is associated with α2-M forming α2-M:uPA complexes. In addition, these complexes are detected mainly in close proximity to alveolar epithelial cell (Fig. 2B, magenta). It should be noted that Wang et al. (42) reported the presence of uPA alone associated with epithelial cells in lungs of normal mice and mice treated with lipopolysaccharide (LPS)-induced ALI by immunohistochemistry. Moreover, pulmonary expression of uPA was substantially increased in these mice.

BEAS-2B cells, a commonly studied lung epithelial model system (29, 35), express both uPAR (receptor for uPA) and LRP (α2-M receptor; see Fig. 6). uPA interacts with uPAR (35) in these cells while complexes between proteinases and α2-M normally bind to LRP (25).

Our findings indicate that α2-M:uPA complexes interact more readily with BEAS-2B cells than uPA alone (Fig. 2C). The cells were incubated either with uPA only or with the complexes, and the extent of binding was detected using anti-uPA antibody (red).

The binding of α2-M:uPA complexes to BEAS-2B cells has not been studied before. Therefore, we performed a series of experiments to show that α2-M:uPA complexes associate with LRP in BEAS-2B cells (Fig. 2D). The cells were incubated with α2-M:uPA complexes, and the degree of the colocalization (yellow) between LRP (green) and the complexes (red) was evaluated. The correlation coefficient was estimated to be ~0.90, suggesting that there is colocalization/interaction between LRP and α2-M:uPA complexes.

Active uPA forms complexes with α2-M, which protects the enzyme from inactivation by PAI-1. Next, we evaluated the ability of uPA bound to α2-M to react with active PAI-1 (Fig. 3). Incubation of uPA with α2-M results in a time-dependent increase in uPA amidolytic activity that is resistant to inactivation by PAI-1 (Fig. 3). Normally PAI-1 rapidly interacts with uPA and forms a covalent inhibitory complex stable under SDS-PAGE (7). Preincubation with α2-M resulted in diminishing ability of PAI-1 to interact with uPA (Fig. 3). The results shown in Fig. 3 indicate that uPA in complex with α2-M resists inhibition by PAI-1, which is in accord with previously published observations (31).

Further, SDS-PAGE analysis of the reaction mixture (Fig. 3, inset) demonstrates that the free uPA band (50 kDa) vanishes after 100 min of incubation with α2-M. When PAI-1 resistant amidolytic activity approaches saturation, free uPA disappears from the reaction mixture. These results clearly illustrate the formation of the “molecular cage” type complexes between the proteinase and α2-M, where the enzyme retains amidolytic activity.

Statistical analysis. Differences between groups were evaluated by a simple one-way ANOVA using SIGMASTAT (SPSS Science, Chicago, IL). The direct comparison between any two treatment groups was performed using the Student’s t-test, or nonparametric Mann-Whitney test when the data sets were not normally distributed, or by the Fisher Exact test. A P value <0.05 was considered significant.
activity toward a LMW fluorogenic substrate is protected from interaction with HMW inhibitor PAI-1 due to steric restrictions.

Incubation of exogenous uPA with pulmonary edema fluids protects the enzyme from inactivation by PAI-1. Pulmonary edema fluids from patients with ALI/ARDS or HE were incubated with exogenous urokinase in the presence or absence of excess of PAI-1, and the amidolytic activity of uPA was analyzed (Fig. 4). The data shown in Fig. 4 clearly demonstrate that a portion of amidolytic activity of uPA becomes resistant to inhibition by PAI-1 after incubation with edema fluids, possibly due to the formation of α₂-M:uPA complexes. In addition, this portion is significantly higher in pulmonary edema fluids from patients with ALI/ARDS ($P < 0.05$).

To test whether α₂-M forms “molecular cage” type complexes with endogenous urokinase, the amidolytic activity of uPA (in the presence or absence of exogenous PAI-1) was measured in the pulmonary edema fluids. The data presented in Fig. 5 show that the level of endogenous amidolytic activity of uPA is comparable in patients with ALI/ARDS and HE. Further, incubation of edema fluids with uPA results in the protection of the enzyme from inactivation by an excess of PAI-1, indicating the formation of “molecular cage” type complexes between the α₂-M of edema fluid and uPA. The amount of endogenous activity of uPA resistant to inhibition by PAI-1 was higher ($P < 0.01$) in edema fluids from patients with ALI/ARDS.

Expression of uPAR and LRP on BEAS-2B. Since α₂-M:uPA complexes found in lungs of patients with ARDS were located in the epithelial layer, we used BEAS-2B cell line, a commonly studied model of lung epithelial system (29, 35), in subsequent experiments. Data presented in Fig. 6 confirm the presence of uPA on BEAS-2B cells (36) and show that LRP is also expressed by these cells.

Activity of α₂-M:uPA complexes in vitro. Data presented in Fig. 2D indicate that uPA bound to α₂-M can associate with

Fig. 2. A: association of uPA with α₂-M in normal lung. Left: from the top: α₂-M (green), uPA (red), Nuclei (blue; staining with Hoechst 33342), and Epith. (magenta; epithelial cells stained with anti-surfactant protein B antibody). Middle: colocalization of α₂-M (green) and uPA (red), and of Nuclei and Epith. in normal lung tissue. Right: selected areas where colocalization was present were enlarged. No significant colocalization (yellow) of uPA and α₂-M is visible in normal lung tissue section. B: association of uPA with α₂-M in lungs of patients with ALI/ARDS. Left: from top: α₂-M (green), uPA (red), Nuclei (blue; staining with Hoechst 33342), and Epith. (magenta; epithelial cells stained with anti-surfactant protein B antibody). Middle: colocalization of α₂-M (green) and uPA (red), and of Nuclei and Epith. in the ARDS lung tissue. Right: selected areas where colocalization was present were enlarged. α₂-M:uPA complexes (yellow) are visible in close proximity to the epithelial cells (magenta) in ALI/ARDS tissue. Further, in case of lung tissue sections from patients with ALI/ARDS (4 patients in total), 98 distinct fields were analyzed using confocal microscopy. C: association of uPA or α₂-M:uPA complexes with BEAS-2B cells. Cells were incubated either with uPA alone or α₂-M:uPA complexes, and uPA associated with BEAS-2B cells detected with anti-uPA antibody (red). D: association of α₂-M:uPA complexes with low-density lipoprotein receptor-related protein (LRP) in BEAS-2B cells. Cells were incubated with α₂-M:uPA complexes. LRP was visualized using a specific anti-LRP antibody (green) and α₂-M:uPA complexes associated with BEAS-2B cells were detected with anti-uPA antibody (red). Colocalization between LRP and α₂-M:uPA complexes is shown in yellow, and colocalization coefficient equals 0.90.
and 0.020 uPA alone but not B activation (26, 32). Our results demonstrate that H9260 induce NF- H9262 H9251 order rate constant for H9251 and H9249 withdraw at the indicated time points and uPA amidolytic activity was H9251 after 100 and 240 min of incubation with or without H9251 estimated as 1 mol of uPA per 32 H9251 human PAI-1. Solid lines represent the best fit (H9251 described (21). HE, hydrostatic pulmonary edema.

In summary, uPA alone but not α2-M:uPA complexes induce NF-κB activation (Fig. 7). To extend these observations we evaluated release of cytokines, i.e., IL-6 and CXCL8, from BEAS-2B cells. Production of these cytokines is regulated by α2-M:uPA complexes compared with cells cultured with uPA alone (Table 1). Further, α2-M alone had no effect on cytokine release from BEAS-2B cells (Table 1).

Moreover, it has been previously reported that engagement of LRP or of uPAR leads to activation of Rac1 (27). We observed Rac1 activation (green) in cells incubated with α2-M:uPA complexes (B vs. A = Medium, Fig. 8) [P < 0.001] that could be suppressed by blockade of LRP (Fig. 8C). On the other hand, the active form of Rac1 (green) could be detected in cells stimulated with uPA alone regardless of LRP status.
It should be stressed that although uPA:uPAR complexes bind to and are internalized via LRP they also may undergo endocytosis by interacting with ENDO180 (endocytic receptor 180) or by other routes that are not dependent on LRP (7, 34).

These findings support our hypothesis that uPA in complex with α₂-M interacts with LRP associated with lung epithelial cells.

(not blocked or blocked; Fig. 8, D vs. E; P < 0.001 vs. medium for both).
urokinase (uPA):

In cells incubated with uPA

<table>
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<tr>
<th></th>
<th>IL-8</th>
<th>uPA</th>
<th>uPA/α2-M Complex</th>
<th>α2-M</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>13.44 (10.35–15.96)²</td>
<td>57.11 (51.58–97.06)²</td>
<td>16.88 (15.57–17.27)²</td>
<td>13.09 (10.05–16.85)²</td>
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<td>0.00 (0.00–2.78)²</td>
<td>15.97 (7.54–58.42)²</td>
<td>0.00 (0.00–22.98)²</td>
<td>0.00 (0.00–13.71)²</td>
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α2-MACROGLOBULIN-UROKINASE COMPLEXES IN THE LUNG

**DISCUSSION**

It is well recognized that the fibrinolytic system does not function properly in patients with ALI/ARDS (16, 17). Accordingly, the level of enzymatically active uPA is decreased in lungs of these patients, partially due to the increased production of PAI-1. Moreover, abnormal fibrinolysis leads to fibrin deposition within the alveolar space in lungs of these patients, partially due to the increased production of PAI-1. Furthermore, after incubation with an excess of exogenous enzyme (Figs. 5 and 4, respectively) allowed us to estimate the fraction of α2-M bound to endogenous uPA to be ~14% of active α2-M. Since ~50% of α2-M present in alveolar edema fluids exists in an active form (23), α2-M:uPA complexes would likewise comprise about 14% of the inactive (“fast” form) of α2-M.

IL-6: 0.00 (0.00–2.78)* 15.97‡ (7.54–58.42)* 0.00 (0.00–22.98)* 0.00 (0.00–13.71)*

Analysis of lung tissue sections from patients with ALI/ARDS also shows that uPA forms complexes with α2-M. It should be noted that these are random specimens that were archived after pathological evaluation and diagnosis of diffuse alveolar damage or ALI/ARDS (3).

α2-M most likely interacts with uPA via a two-step mechanism: fast formation of a noncovalent complex, followed by slow reaction resulting in a covalent complex between α2-M and proteinase. In contrast to noncovalent complexes, the active (uPA/α2-M) complexes in comparison with cells cultured with uPA alone.

**Table 1. Cell cultures**

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<tr>
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<th>Medium</th>
<th>uPA</th>
<th>uPA/α2-M Complex</th>
<th>α2-M</th>
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α2-M:uPA complexes in comparison with cells cultured with uPA alone.

"Results are expressed as median values with 25 to 75 percentiles. †Levels of CXCL8 were significantly decreased (P < 0.001) in cells incubated with urokinase (uPA)/α2-macroglobulin (α2-M) complexes in comparison with cells cultured with uPA alone. ‡Levels of IL-6 were significantly decreased (P < 0.01) in cells incubated with uPA α2-M complexes in comparison with cells cultured with uPA alone.

Fig. 8. A: effect of blocking of LRP on Rac1 phosphorylation induced by α2-M:uPA complexes, and by uPA. BEAS-2B cells were incubated with medium only (A), or α2-M: uPA complexes (α2-M:uPA; B) or anti-LRP antibody (100 μg/ml) and α2-M:uPA complexes (α2-M:uPA) [C]. Positive cells were counted, and the percentage of positive cells presented in graphical form. *P < 0.001 for the difference between α2-M:uPA complexes (B) and medium (A), and α2-M:uPA complexes without and with blocking of LRP (B vs. C). Representative results from 2–4 independent experiments are shown. Similar data was obtained whether LRP was blocked using the specific antibody or by preincubation with its ligand, fast form of α2-M (α2-M*, 10 μg/ml) (not shown). BEAS-2B cells were also incubated with uPA (D) or anti-LRP antibody (100 μg/ml) and uPA (E). Positive cells were counted, and the percentage of positive cells presented in graphical form. *P < 0.001 for the difference between uPA (D) and medium (A), and ‡P < 0.001 for uPA with blocking of LRP (E) and medium (A). We also scanned all the cells and plotted intensity of signal for every condition which corresponds directly to the number of positive cells (*P < 0.001 for the difference between α2-M:uPA complexes (B) and medium (A), and α2-M:uPA complexes without and with blocking of LRP (B vs. C); †P < 0.001 for the difference between uPA (D) and medium (A), and ‡P < 0.001 for uPA with blocking of LRP (E) and medium (A)). Representative results from 2–4 independent experiments are shown. Similar data was obtained whether LRP was blocking using the specific antibody or by preincubation with its ligand, fast form of α2-M (α2-M*, 10 μg/ml; not shown).
covalent interactions are stable under conditions of nonreduc-
ing SDS-PAGE. Further, uPA complexed with α2-M becomes
unable to interact with PAI-1. Thus, in the presence of an
excess of PAI-1, uPA bound to α2-M retains its amidolytic
activity, cannot form a SDS-PAGE stable inhibitory complex
with PAI-1, or cleave this serpin via the substrate pathway.
Thus formation of complexes with α2-M makes uPA inacces-
sible to HMW inhibitors such as PAI-1.

Exogenous uPA added to human pulmonary edema fluids
forms α2-M/zyme complexes in a similar manner to that
which occurs in rabbits with tetracycline-induced pleural injury
treated with single chain urokinase (21). Further, active α2-M
in the human alveolar space forms “molecular cage” type
complexes with endogenous uPA. α2-M bound to endogenous
uPA, does not significantly alter uPA activity towards LMW
substrates but inhibits its ability to interact with HMW inhib-
itors (PAI-1). Therefore, the reaction between endogenous uPA
and α2-M could limit endogenous free uPA activity in the lung.
The α2-M:uPA complexes are observed in pulmonary edema
fluids from patients with ALI/ARDS and HE fluids and carry
the same ramifications for the regulation of local fibrin turn-
over. However, the level of these complexes is increased in
pulmonary edema fluids from patients with ALI/ARDS com-
pared with fluids from patients with HE.

Our previously published data (21) concur with the current
findings. We reported that binding to rabbit α2-M does not
significantly affect the ability of scuPA or tcuPA to cleave
LMW substrates, but it considerably suppresses interactions
with PAI-1-2 (21). We have now confirmed that tcuPA (referred
to in the current study as uPA) complexed with α2-M is
protected from inactivation by PAI-1 (Fig. 3). This is true for
complexes between endogenous α2-M and endogenous (Fig. 5)
or exogenous uPA (Fig. 4) as well as interactions of uPA with
α2-M in vitro (Fig. 3).

On the other hand, α2-M:scuPA complexes purified from
pleural fluids of rabbits with tetracycline-induced pleural injury
treated with scuPA retain their ability to activate plasminogen.
This is in contrast to the findings in rabbits that received tcuPA
[ uPA] (21). α2-M:uPA complexes purified from pleural fluids of
these rabbits exert substantially lower plasminogen activating
activity (21). Physiological significance of these observations
has yet to be established.

Apart from its proteolytic functions, uPA is capable of
activating intracellular signaling pathways (27, 32). Impor-
tantly, our results demonstrate for the first time that uPA
triggers activation of NF-κB in human epithelial cells and that
this activation is abrogated in cells stimulated with α2-M:uPA
complexes. In agreement with the former observation Prager et
al. (32) reported that uPA induces activation of NF-κB in
endothelial cells. On the other hand, uPA had no effect on
nuclear translocation of NF-κB in neutrophils (1). It should be
stressed that activity of uPA towards epithelial cells cannot be
due to endotoxin contamination of uPA since α2-M:uPA com-
plexes containing the same amount of this protein do not
activate NF-κB.

Our studies also indicate that both uPA and α2-M:uPA
complexes induce phosphorylation of Rac1 in human epithelial
cells. Similarly, Kian et al. (20) showed that uPA was able to
trigger activation of Rac1 in smooth muscle cells. However,
the ability of uPA and α2-M:uPA complexes to phosphorylate
Rac1 in epithelial cells has not been reported before.

Finally, our study is the first to show production of cytokines
by epithelial cells stimulated by uPA. We have also dem-on-
strated that formation of complexes between uPA and α2-M
suppresses ability of uPA to trigger the release of cytokines
from these cells.

It should be noted that BEAS-2B cells are widely used to
study functions of epithelial cells in relation to lung inflam-
mation/injury (12, 28, 29, 35) as these cells display many of
the phenotypic characteristics of primary cells when proper culture
conditions are applied (ATCC). However, BEAS-2B cells lack
several epithelial differentiation markers and do not form tight
epithelial barriers (37).

Overall our findings indicate that α2-M may control the
ability of uPA to induce cellular activation, including signal
transduction events. We also show that complexes between
uPA and α2-M bind to and modify cellular signaling via LRP.
The latter triggers cellular responses that differ from those
induced by uPA through interaction with uPAR (26, 32).
Notably, α2-M:uPA complexes do not activate NF-κB, which
is in contrast to uPA alone. At this moment it is difficult to
determine which of these responses are more important in
the context of lung injury (8, 33). However, formation of α2-M:
uPA complexes represents a newly recognized phenomenon in
ALI/ARDS that can contribute to the regulation of injury and
repair in patients affected by this condition.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.A.K. and A.K.K. conception and design of re-
search; A.A.K., D.S., A.K., R.F., G.F., J.F., and M.F. performed experiments;
prepared figures; T.C.A., S.I., M.A.M., and A.K.K. edited and revised manu-
script; T.C.A. and S.I. approved final version of manuscript.

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