Urokinase Induces Expression of its Own Receptor in Beas2B Lung Epithelial Cells

By

Sreerama Shetty and Steven Idell

From the Department of Medical Specialties, The University of Texas Health Center at Tyler, Tyler, TX USA

Address all correspondence to:

Sreerama Shetty, Ph.D.
Associate Professor of Medicine
Department of Medical Specialties
The University of Texas Health Center at Tyler, Lab C-6
Biomedical Research Building
11937 US Highway 271
Tyler, TX, 75708

Telephone: 903-877-7668
Fax: 903-877-5627
Email: sreerama.shetty@uthct.edu
Summary

Interaction between the urokinase-type plasminogen activator (uPA) and its receptor (uPAR) localizes cellular proteolysis and promotes cellular proliferation and migration. The interaction between uPA and uPAR at the surface of epithelial cells thereby contributes to the pathogenesis of lung inflammation and neoplasia. In this study, we sought to determine if uPA itself alters uPAR expression by lung epithelial cells. uPA enhanced uPAR expression as well as $^{125}\text{I}$-uPA binding in Beas2B lung epithelial cells in a time and concentration-dependent manner. The uPA-mediated induction of uPAR is not accomplished through its receptor and requires enzymatic activity. The low molecular weight fragment of uPA, lacking the receptor binding domain, was as potent as intact two-chain uPA in inducing expression of uPAR at the cell surface. Plasmin, the end product of plasminogen activation did not alter uPA-mediated uPAR expression. Induction of uPAR by uPA represents a novel pathway by which epithelial cells can regulate uPAR-dependent cellular responses that may contribute to stromal remodeling in lung injury or neoplasia.

Running title: Expression of urokinase receptor by urokinase
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The abbreviations used are: uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2; GPI, glycosyl phosphatidylinositol; PMA, phorbol myristate acetate; TGF-β, transforming growth factor-beta; BSA, bovine serum albumin; DTT, dithiothreitol; HMW, high molecular weight; LMW, low molecular weight; ATF, aminoterminal fragment; APS, ammonium persulfate; SAEC, small airway epithelial cells; Beas2B, bronchial epithelial cells; Thr, α-thrombin; PMSF, phenylmethylsulfonyl fluoride.
Introduction

Extracellular proteolytic enzymes including serine proteinases and metalloproteinases have been implicated in remodeling of the extracellular matrix in lung injury and lung neoplasia (1-3). These enzymes influence inflammatory cell traffic or cancer cell invasiveness via the breakdown of basement membranes and extracellular matrix (4-7). Plasmin, a serine protease, is involved in the dissolution of extracellular matrix and basement membrane during tissue degradation. This protease is generated via the action of plasminogen activators such as urokinase (uPA) or tissue plasminogen activator (tPA) and can influence tissue remodeling either directly or through activation of latent collagenases. Urokinase is mainly involved in extravascular proteolysis in stromal remodeling in acute and chronic lung injury (8-10) and in metastatic neoplasia (7). During the last decade, evidence for involvement of the uPA system in lung injury and repair or lung neoplasia (1) has steadily increased and it now seems clear that uPA-dependent plasminogen activation is central to these processes. Increased expression of uPA or uPAR has been inversely correlated with prognosis in lung cancer (11,12).

uPA-mediated plasminogen activation is tightly regulated by several factors, including its high affinity receptor (uPAR), and two specific and fast acting inhibitors (PAI-1 and PAI-2) (13). Synthesis of fibrinolytic components (uPA, uPAR, plasminogen activator inhibitor –1; PAI-1, and PAI-2) is regulated by a variety of hormones, growth factors and cytokines either at the transcriptional or posttranscriptional level (13-18). The uPAR binds to both uPA and its proenzyme pro-uPA to enhance cell surface plasminogen activation several fold compared to that of fluid phase uPA (7,13). Receptor-bound uPA can be inhibited by PAI-1 and PAI-2 and uPAR provides a mechanism for internalization of PAI-1-inactivated uPA. The uPAR therefore plays an important role both in localizing and modulating cell surface plasminogen activation. Human uPAR is heavily glycosylated and is attached to the cell membrane by a glycosyl phosphatidyl inositol (GPI) anchor. Both uPA and uPAR, as well as PAI-1 and -2 are expressed by lung
epithelial cells (19-21), indicating that autocrine regulation of this fibrinolytic system by the epithelium could influence the course of either lung injury or lung cancer.

Expression of uPA and uPAR controls several cellular functions, including epithelial cell adhesion, signaling and mitogenesis, and most of the biological activities of uPA are dependent on its association with the uPAR (4,13,22). uPA is reported to generate intracellular signals either by uPAR-dependent or uPAR–independent mechanisms (23). The expression of these components by the lung epithelium is tightly regulated during normal physiological processes and is disordered in lung injury or lung cancer. It is noteworthy that the signaling pathways activated by uPA/uPAR seem to be the same pathways that induce their own expression (23). We therefore postulated that interactive regulation between uPA and uPAR was plausible, albeit not previously described. This possibility was investigated using cultured Beas2B cells and primary bronchial epithelial cells as a model system. In these studies, we describe a novel regulatory pathway by which uPA induces uPAR expression by Beas2B as well as primary bronchial epithelial cells.
Experimental Procedures

Materials
Culture media, penicillin, streptomycin, fetal calf serum (FCS) were purchased from Gibco BRL laboratories (Grand Island, NY); tissue culture plastics were from Becton Dickinson Labware (Lincoln Park, NJ). α-thrombin, herbimycin, genistein, bovine serum albumin (BSA), ovalbumin, Tris-base, aprotinin, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), silver nitrate, ammonium persulfate (APS) and phorbol myristate acetate (PMA) were from Sigma Chemical Company (St. Louis, MO). Acrylamide, bisacrylamide and nitrocellulose were from BioRad laboratories (Richmond, CA). Recombinant high molecular weight (HMW) uPA was a generous gift from Drs. Jack Henkin (Abbott Laboratories, Abbott Park, IL) and Andrew Mazar (Angstrom Pharmaceuticals, San Diego, CA). The low molecular weight (LMW), amino-terminal fragment (ATF), anti-uPA and anti-uPAR antibodies were obtained from American Diagnostics (Greenwich, CT). A5 and B428 compounds were generous gifts from Dr. Andrew Mazar. XAR X-ray film was purchased from Eastman Kodak (Rochester, NY).

Cell cultures
Small airway epithelial cells (SAEC) were obtained from Clonetics (San Diego, CA). Human bronchial epithelial cells (Beas2B) or lung epithelial tumor cells, including H1395 and A549 human lung adenocarcinoma cells, H157 human lung squamous cells, H460 large cell lung carcinoma cells and H146 human lung small cell lung carcinoma...
cells were obtained from the ATCC. Human pleural mesothelial (MeT5A) and spindle-shaped (M33K) or epithelioid (M9K) pleural malignant mesothelioma cells were obtained from Dr. Brenda Gerwin, NIH. These cells, as well as primary cultures of human pleural mesothelial cells obtained from pleural fluid aspirates, were maintained in RPMI 1640 medium containing 10% heat-inactivated FCS, 1% glutamine and 1% antibiotics as previously described (24).

**Total cellular membrane extraction and Western blotting**

Cells grown to confluence were serum starved overnight with RPMI-glutamine media containing 0.5% BSA. The cells were treated with or without various agents for indicated times and were washed with PBS. Receptor bound uPA was removed by glycine-HCl treatment as described earlier (25). We used SDS gel electrophoresis and Western blotting to measure functional uPAR at the cell surface. Membrane proteins isolated as described earlier (26) from Beas2B, SAEC, MeT5A, selected lung tumor cell lines and pleural mesothelioma cell lines were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 1% BSA in wash buffer for 1 h at room temperature followed by overnight hybridization with uPAR monoclonal antibody in the same buffer at 4°C, washed and uPAR proteins were detected by enhanced chemiluminescence (ECL).
\textbf{125}I-uPA binding

Recombinant uPA (5 µg/ml) was treated with 2 mCi of \textsuperscript{125}I-Na and 50 mg iodogen for 5 min at 4°C. The reaction was stopped by addition of excess KI, and unincorporated label was removed by passage through a sephadex-25 column as described earlier (27).

In a separate experiment, membrane proteins of Beas2B cells treated with uPA for varying time (0-24 h) periods, were separated on SDS-PAGE and transferred to nitrocellulose membrane as described above. The nitrocellulose membrane was subjected to ligand blotting assay, using \textsuperscript{125}I-uPA as described earlier (27).

We also measured binding of \textsuperscript{125}I-uPA by the method of Waltz et al. (28) with modifications. Beas2B cells grown to confluence in 24 well plates were treated with or without uPA for 24 h in serum-free media containing 0.5% BSA. The cells were acid-treated and subjected to \textsuperscript{125}I-uPA binding as we described earlier (27). The non-specific binding was measured in the presence of a 400-fold molar excess of cold uPA. Activation of plasminogen by Beas2B cells was measured by a modification of the esterolytic method as we previously described (27).

\textbf{Plasmid Construction}

Plasmid uPAR/pBluescript was obtained from the ATCC. The human uPAR mRNA template containing a complete sequence of uPAR cDNA (nucleotides −16 to1144) from uPAR pBluescript was subcloned to Hind III and Xba I sites of pRC/CMV (Invitrogen) and the sequences of the clones were confirmed by sequencing. The uPAR insert was released by Hind III or Xba I, purified on 1% agarose gels, extracted with phenol/chloroform and used as a cDNA probe for Northern blotting.
Random priming of uPAR cDNA

The full length template of uPAR was released with Hind III or Xba I, purified on 1% agarose gels and labeled with $^{32}$P-dCTP using a rePi Prime labeling kit (Amersham, Arlington Heights, IL). Passage through a Sephadex G-25 column removed unincorporated radioactivity. The specific activity of the product was $6 \times 10^8$ cpm/µg.

Nuclear Run-on Transcription Activation Assay

Cells grown to confluence in two T182 flasks were serum starved overnight in RPMI-BSA media. The cells were later treated with PBS, uPA or TGF-β (2 ng/ml) for 12 h at $37^\circ$ C, then washed with ice-cold PBS. Cells were resuspended in 0.4 ml lysis buffer (10 mM Hepes buffer pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 0.5 mM PMSF, 2 µg/ml each leupeptin and aprotinin and 0.5 mg/ml benzamidine). The cells were homogenized and incubated on ice for one h to release the nuclei. The nuclear pellet was subjected to sucrose-gradient centrifugation by careful layering on top of 7.5 ml sucrose buffer (1.3 M) followed by centrifugation at 10,000 X g for 15 min at 4 °C. The nuclear pellet was resuspended in 200 µl lysis buffer. The nuclei (10-20 x 10^6) were subjected to the transcription reaction in the presence of 250 µCi $^{32}$P-UTP at 30° C for 30 min. $^{32}$P-labeled nuclear RNA was isolated using TRI reagent and unincorporated radioactivity was removed by repeated precipitation and washing by cold ethanol. RNA pellets were dissolved in 40 µl RNase-free water. Recombinant plasmid DNA (10 µg/slot) was denatured by heating at 95° C in NaOH/EDTA solution for 5 min and chilling on ice. Denatured DNA solution was spotted on a nitrocellulose membrane using
a slot blot apparatus under vacuum. The membranes were vacuum-baked, prehybridized overnight at 42 °C in a hybridization buffer (50 mM Hepes pH 7.0, 50% formamide, 4 x SSC, 2 x Denhards solution, 2 mM EDTA, 0.1% SDS, 0.225 mg/ml tRNA, 0.1 mg/ml poly A⁺ and 0.2 mg/ml salmon sperm DNA). ³²P-labeled RNA (10 X 10⁶ cpm/ml) was added to the hybridization solution and the membranes were incubated in a hybridization oven for 48 h. The filters were washed with 2-0.1 X SSC with in between RNase digestion, air dried and exposed to X-ray film.

**Northern blotting of uPAR mRNA**

A Northern blotting assay was used to assess the level of uPAR mRNA. Beas2B cells grown to confluence were serum-starved overnight in RPMI-BSA media, then treated with uPA for varying times (0-24 h) in the same media. Total RNA was isolated using TRI reagent. RNA (20 µg) was isolated on agarose/formaldehyde gels. After electrophoresis, the RNA was transferred to Hybond N⁺ according to the instructions of the manufacturer. Prehybridization and hybridization were done at 65°C in NaCl (1M) /SDS (1%) and 100 µg/ml salmon sperm DNA. Hybridization was performed with a uPAR cDNA probe (1 ng/ml) labeled to approximately 6 X 10⁸ cpm/µg of DNA overnight. After hybridization, the filters were washed twice for 15 min at 65°C, with: 2 X SSC, 1% SDS; 1 X SSC, 1% SDS, and 0.1% SSC, 1% SDS, respectively. The membranes were next exposed to X-ray film at –70°C overnight. The intensity of the bands was measured by densitometry and normalized against that of β-actin.

**Overexpression of uPA: Transfection of Beas2B cells with uPA cDNA**

The uPA cDNA (29) was subcloned to eukaryotic expression vector pRc/CMV2 (Invitrogen) containing the CMV promoter at Hind III/Not I sites. The orientations and sequences were confirmed by sequencing. Beas2B cells were transfected with the prepared chimeric plasmid constructs by lipofection using lipofectamine (Gibco BRL). Stable cell lines were made by treating Beas2B cells with neomycin for three months.
Cells carrying plasmid DNA which survived after neomycin treatment were scrapped off from 6-well plates and grown in T75 flasks and the presence of plasmid DNA was confirmed by PCR using specific primers. The over-expression of uPA by cDNA transfected cells was confirmed by Western blotting Beas2B cell lysates as well as conditioned media using a uPA monoclonal antibody. The effect of endogenous uPA overexpression on uPAR expression was measured by Western blotting for uPAR as described above.

Results

Expression of uPAR in lung epithelial cells

Since we previously found that MeT5A mesothelial cells and MS-1 mesothelioma cells both express uPAR in vitro (25,27), we initially wanted to determine if uPAR is differentially expressed in cultured nonmalignant, bronchial epithelial (Beas2B) cells or small airway epithelial (SAE) cells versus an array of malignant lung carcinoma-derived cells. Pulmonary artery smooth muscle cells (PASMC) and human pulmonary microvascular endothelial cells (HPMVEC) were also analyzed. Western blotting assays were used to determine the level of uPAR expression at the surface of primary cultures of lung small airway epithelial cells, Beas2B cells, human lung carcinoma derived cells (H1395, A549, H157, H460, H146) cells as well as cells from human pleural malignant mesothelioma cells (M33K, M9K, MS-1). Basal expression of uPAR in unstimulated cells was greatest in H157, H460 and A549 cells among the lung cancers (Fig. 1).
Time-dependent expression of uPAR by uPA

Signaling pathways activated by the uPA/uPAR system could be the same pathways that induce their own expression (23). We therefore explored the possibility that expression of uPA/uPAR in epithelial cells leads to a nested signaling loop and/or activation of additional mechanisms that could contribute to enhanced uPAR expression. We used Beas2B epithelial cells as a lung epithelial cell model system to initiate these studies. We treated these cells with uPA (the high molecular weight, two-chain form) for varying periods (0-24 h) of time. uPAR expression at the cell surface was assessed by Western blotting using an anti-uPAR antibody. Results of these experiments (Fig. 2a), demonstrate that uPA induces uPAR in Beas2B cells in a time-dependent manner and that the induction starts between 3-6 h after addition of uPA. Maximal induction of uPAR by uPA was achieved around 12 h and the elevated level is maintained for at least 24 h. uPA also induced uPAR expression in primary bronchial epithelial cells but not in uPAR over-producing H157 or A549 cells (Fig. 2b). We also confirmed expression of increased functional uPAR in uPA-treated Beas2B cells by ligand blotting assay, demonstrating receptor binding consistent with the results of Western blotting experiments (data not shown).

To see if the high molecular weight uPA (HMW uPA) preparation we used in this study contains lipopolysaccharides (LPS), we used the Limulus Amebocyte lysate ELISA method. We found that this HMW uPA preparation contains very negligible amounts (about 1 pg/ml) of LPS. We next treated Beas2B cells with the same concentration; 1 pg/ml as well as a ten-fold increment; 10 pg/ml of LPS and measured uPAR expression by Western blotting as we described above. We found that these concentrations of LPS
failed to induce uPAR expression, indicating that the induction of uPAR by uPA could not be attributable to LPS contamination.

**Induction of uPAR by endogenous uPA**

We next prepared stable uPA-overexpressing Beas2B cells and vector-treated controls by transfecting these cells with the eukaryotic expression vector PRc\CMV2 containing uPA cDNA or PRc\CMV2 cDNA using lipofection. We analyzed the uPA expression of the stable cell lines by Western blotting. As shown in Fig. 3a, Beas2B cells transfected with uPA cDNA expressed a relatively large amount of uPA in both the conditioned media and at the cell surface, in comparison to vector-transfected or non-transfected controls. We then measured the uPAR expression by these cells by Western blotting using a uPAR monoclonal antibody and confirmed that Beas2B cells transfected with uPA cDNA produced large amounts of uPAR at the cell surface. Vector transfected or non-transfected control did not demonstrate increased uPAR expression, suggesting that induction of increased uPAR expression was attributable to stimulation effected by increased endogenous uPA (Fig. 3b).

**Binding of 125I-uPA**

We next wanted to determine the specificity of uPA binding to the surface of uPA-stimulated Beas2B cells. Accordingly, we treated PBS or uPA stimulated Beas2B cells with 0-61 nM of 125I-uPA for 2 h at 4°C with or without a 400-fold molar excess of unlabeled uPA. The specific binding was calculated from the difference between total and nonspecific counts (30). The data in Fig. 4 shows that exogenously added uPA bound
progressively with increasing concentration. Unstimulated Beas2B cells demonstrated functional uPAR however, uPA stimulation enhanced $^{125}$I-uPA binding at least two to three fold (Fig. 4a). Binding of $^{125}$I-uPA was competed for by increasing concentrations of unlabeled uPA and approximately 60% was accounted for specific binding (Fig. 4b). This observation is consistent with our previous finding that 30-35% of $^{125}$I-uPA binding is non-specific in PMA treated mesothelial cells (25, 27). Because the binding experiments in this study were performed at 4°C, it is unlikely that the magnitude of the nonspecific binding is attributable solely to internalization of uPA-uPAR-PAI-1 complexes. To determine if uPA enhances plasminogen activation through induction of functional uPAR, unstimulated or uPA treated cells were preincubated with uPA for 2 h at 4°C for 2 h, washed and incubated with plasminogen (6 µg) for 20 min at 37°C. The rate of plasminogen activation was obtained by measuring the liberated plasmin. uPA induced plasmin generation in a concentration dependent manner (Fig. 4c).

uPAR mRNA expression by uPA in Beas2B lung epithelial cells

Having determined that uPA mediates time-dependent uPAR expression at the lung epithelial cell surface, we wanted to confirm that the increased cell surface uPAR is attributable to an increased level of uPAR mRNA. We next measured the levels of uPAR mRNA in uPA-treated Beas2B epithelial cells by Northern blotting using a uPAR cDNA probe and densitometric scanning. As shown in Fig. 5, uPA induces uPAR mRNA and the induction is observed as early as 3 h after the treatment. Maximum accumulation of uPAR mRNA is achieved between 12-24 h after the treatment. These data confirm the induction of uPAR expression by uPA as determined by Western blot. The level of uPAR
mRNA was quantitated by densitometric scanning and normalized against β-actin loading controls. As shown in Fig. 5, resting Beas2B cells express small amounts of uPAR mRNA. However, uPAR mRNA levels increase about 20-fold by 24 h after uPA treatment.

**Transcriptional activation of uPA: Run-on transcription experiments.**

In order to determine whether uPA enhances uPAR gene transcription, we treated Beas2B cells with uPA for 6 and 12 h, isolated nuclei and $^{32}$P-labeled RNA was hybridized with uPAR cDNA immobilized on a nitrocellulose membrane. The results of nuclear run-on transcription analyses demonstrated that uPA did not induce transcriptional activation of the uPAR gene (n=3 data not shown).

**The effect of uPA concentration on uPAR expression**

We next treated Beas2B cells with varying amounts (0-3 µg/ml) of two-chain uPA for 24 h and then measured cell surface uPAR expression by Western blot assay. Fig. 6 shows that uPA induced uPAR in a concentration-dependent manner. Induction is apparent with as low as 10 ng/ml uPA. Maximal uPAR expression was observed with 500 ng/ml uPA, beyond which there was a steady decline in uPAR expression. The steady decrease in uPAR expression at higher uPA concentration could be due to degradation of uPAR by uPA (31). These data suggest that the induction of uPAR by uPA is specific and concentration-dependent.

**Effects of phosphatase and phosphotyrosine kinase inhibitors on uPA-mediated induction of uPAR**
To determine whether uPA-mediated uPAR expression involves cellular signaling, we pretreated Beas2B cells with herbimycin A (2 µM) and genistein (6 µg/ml), protein tyrosine kinase inhibitors, separately or in combination with uPA. As shown in Fig. 7, neither herbimycin A nor genistein alone induced uPAR expression (Fig. 7, lanes 3 and 5). However, when Beas2B cells were treated with uPA, pretreatment with these inhibitors reversed uPA mediated uPAR expression (Fig. 7, lanes 4 and 6). Pretreatment of cells with vanadate (10 µM) (a tyrosine phosphatase inhibitor), on the other hand, did not block uPA-induced uPAR expression, but appeared to enhance the uPA-mediated effect. These data suggests protein phosphorylation may be involved in the process.

The role of uPAR in uPA-mediated uPAR expression

We next sought to determine if uPA interacts with cell surface uPAR to induce the receptor. To investigate this possibility, we pre-treated Beas2B cells with anti-uPAR antibody for 2 h, then treated with uPA for 24 h. As shown in Fig. 8a, uPAR antibody (2 µg/ml) alone failed to induce cell surface uPAR expression and pretreatment of Beas2B cells with this antibody failed to block uPA-induced uPAR expression (Fig. 8a). To confirm that uPA-mediated uPAR expression does not involve uPAR, we next treated Beas2B cells with A5 (1 µg/ml) compound (a receptor agonist that blocks association of uPA with uPAR) (32) alone or in combination with uPA. As shown in Fig. 8a, A5 alone minimally induced uPAR expression, but in combination with uPA, did not inhibit expression of uPAR at the cell surface. Using a third independent approach, we removed uPAR from cells by treating with PI-PLC (10 units/ml), then tested to see if uPA would stimulate uPAR expression. It is known that uPAR is a GPI-linked protein and PI-PLC
completely removes GPI-linked proteins, including uPAR from the cell surface. Under these conditions, we still observed uPAR induction by uPA (Fig 8a). In a separate experiment, we found that PI-PLC completely cleaved uPAR from the cell surface by Western blotting of membrane fractions (data not shown), providing further evidence that the induction of uPAR by uPA is not mediated by their association at the cell surface.

**The effect of uPA enzymatic activity on induction of uPAR**

Having confirmed that uPA-mediated uPAR induction is not mediated through interaction with uPAR, we next wanted to determine if enzymatic activity of uPA is required for uPA-mediated uPAR induction. To address this possibility, we tested the ability of an anti-uPA monoclonal antibody to inhibit uPA expression. As shown in Fig. 8b, an anti-uPA antibody (2 µg/ml) did not induce uPAR expression whereas inactivation of uPA catalytic activity by this antibody reversed the effect. Similarly plasminogen activator inhibitor-1; PAI-1 (4 µg/ml) (Fig. 8b) inhibited uPA mediated uPAR induction, indicating that uPA activity is required. uPA antibody or PAI-1 are large molecules and could therefore be inhibiting uPA-mediated functions by interfering with its interaction at distal sites rather than by inactivating enzymatic activity. We therefore pretreated uPA with B-428 (0.02 mM) (an active site inhibitor of uPA) (33) and found that inactivation of uPA activity by B-428 inhibited uPA-mediated uPAR expression (Fig. 8b). Likewise, chloromethyl ketone inactivated uPA (27) failed to induce uPAR expression (n=2 data not shown).
The effect of ATF or LMW uPA on induction of uPAR

We next treated Beas2B cells with the aminoterminal fragment of uPA (ATF) or the active low molecular weight (LMW) fragment of uPA to confirm that the uPA effect is not mediated by receptor occupancy. As shown in Fig. 8c, ATF (1 µg/ml) alone did not induce uPAR nor did pretreatment alter uPA-mediated uPAR expression. The uPA LMW fragment (1 µg/ml), conversely, induced uPAR at the cell surface.

The effect of proteases and protease inhibitors on uPA-mediated uPAR expression

Since we found that uPAR is not involved in uPA-mediated uPAR expression, we inferred that proteases might have been induced by uPA to stimulate uPAR expression. To address this possibility, we treated Beas2B cells with uPA in the presence of aprotinin, a broad-spectrum protease inhibitor that inactivates trypsin-like activity. As shown in Fig. 9, neither aprotinin (1 µg/ml) alone nor in combination with uPA inhibited uPAR induction. We also investigated the possibility that uPA induces uPAR expression through plasmin, the end product of uPA-mediated plasminogen activation. Neither plasmin (1 µg/ml) nor plasmin and uPA altered uPAR expression (Fig. 9). We also treated Beas2B cells with α-thrombin (1 µg/ml) another serine protease alone or in combination with uPA. Thrombin alone did not induce uPAR expression; however, it reversed the uPA effect when the cells were treated with both proteases in combination (Fig. 9).
Discussion

Local generation of plasmin by uPA is a central mechanism by which cells degrade extracellular matrices to relocate from one anatomical location to another. uPA/plasmin mediated proteolysis is critical for cellular migration and tissue remodeling following either lung injury or the propagation and metastasis of lung neoplasms (4,7). uPAR is essential for uPA-dependent pericellular proteolysis and is localized at the leading edge of migrating cells (13). The interaction between uPA and uPAR at the cancer cell surface appears to influence neoplastic growth and metastasis by mediating effects on tissue remodeling, tumor cell invasion, cellular adhesion and proliferation (1,33,34). Tumor cell invasion is also facilitated by saturation of uPAR with either exogenously supplemented uPA or overexpressed endogenous uPA (34,35). In addition, the binding of uPA to uPAR mediates cell proliferation in several cell-types including nonmalignant and malignant epithelial cells and mesothelioma cells (25,26).

Epithelial carcinomas originating in the lung and other tissues including breast, ovary, prostrate and kidney all express increased amounts of uPA and its receptor (34-40). This observation suggested the possibility that interaction between these molecules might account for the relative overexpression of the receptor. Since uPAR expression is implicated in the pathogenesis of lung cancer as well as lung injury and fibrosis (2,5,8,10,21), we sought to elucidate how uPAR is regulated in lung epithelial cells derived from neoplasms or nonmalignant epithelium. We chose Beas2B cells as an in
vitro lung epithelial cell model system to determine if uPAR expression is regulated by uPA and found that this was the case.

Regulation of uPAR expression in different cell populations involves both transcriptional and posttranscriptional mechanisms. In previous studies, we found that a posttranscriptional pathway influences levels of uPAR mRNA in lung cancer and malignant mesothelioma cells (26) as well as rabbit mesothelial cells and fibroblasts (41). Cytokines that occur in both the tumor microenvironment and in lung inflammation and repair increase uPAR expression at the cell surface through this mechanism. Similar findings were previously reported in PMA and TGF-β–treated U937 cells (14). In this study, we found that uPA-mediated expression of uPAR did not involve transcriptional activation of the uPAR gene, implicating regulation at alternative levels.

It has also previously been reported that the specific binding capacity for uPA and cell surface uPAR expression is increased by PMA, EGF, LPS, TGF-β and TNF-α in various cell lines (28, 41). To extend our understanding of the regulation of uPAR by epithelial cells, we analyzed the effect of uPA on uPAR and uPAR mRNA expression. uPA strongly and rapidly induces cell surface uPAR expression, an effect that can be traced back to a rapid antecedent increase in the cellular level of uPAR mRNA. PMA, TGF-β and TNF-α increase the rate of transcription and stability of uPAR mRNA whereas EGF increases transcription alone (14). We found that cytokine-induced uPAR was posttranscriptionally regulated in these cells by a mechanism known to influence uPAR mRNA stability at this level (16, 26). The increased uPAR mRNA stability in lung
tumor and pleural mesothelioma cells correlates with the increased uPAR mRNA and cell surface uPAR. It may be that uPA-mediated induction of uPAR involves posttranscriptional pathways, a possibility that will require future, detailed investigation.

We previously showed that the binding of uPA to its receptor was mitogenic for MS-1 and MeT5A cells and that proliferation of these cells could be blocked by antisense oligonucleotides directed against uPAR (25). Our present study demonstrates that uPA-mediated uPAR expression is concentration-dependent. This pathway represents a potentially versatile regulatory system in which uPA in the tumor stroma or lung microenvironment could favor amplification of its receptor. This molecular mechanism may be crucial to our understanding of the participation of the uPA/uPAR system in pathological conditions such as cancer or organizing alveolitis. uPA expression under such circumstances could lead to excessive pericellular proteolysis and a change in the behavior of epithelial cells, predicated upon increased expression of uPAR.

The mechanism by which uPA induces uPAR appears to be proteolysis – dependent and uPAR-independent. The regulatory mechanism further involves activation of tyrosine kinases. The sustained effect of uPA on both uPAR protein and mRNA levels suggests that the specific message sent by tyrosine kinase likely prolongs the half-life of uPAR mRNA. uPA also induces synthesis of growth factors or cytokines which, in turn, may induce a late increase in the level of uPAR mRNA. These possible mechanisms also warrant further investigation.
Studies performed in other cell types have demonstrated the involvement of uPAR in uPA-mediated signaling. Along this line, activation of FAK and MAP kinases in cultured endothelial cells have been reported. Similarly, activation of a 38 kDa tyrosine phosphorylated uPAR-associated protein has been identified in U937 cells (42). In both cases, signaling by uPA was abolished when the cells were treated with PI-PLC and other studies also support the association of uPAR with PKC and cytokeratin (43). However, in our study, pretreatment of Beas2B cells with PI-PLC for 2 h followed by uPA treatment for 24 h did not inhibit uPAR expression. The explanation could be that prolonged exposure to uPA (24 h) reverses the effect of removal of PI-PLC. uPAR also appears to associate with β2-integrins and members of the Src family of kinases (44,45). In vascular smooth muscle cells, uPAR was associated with JAK1, Tyk2 and Src kinases (46) whereas in kidney epithelial tumor cells, uPAR associates with gp130 and JAK1 (47).

The mechanism by which uPA induces uPAR in Beas2B cells is at present unknown. Clearly the mechanism is not plasmin-dependent because exposure of cells to aprotinin or treatment with plasmin did not, respectively, alter uPAR expression. One possibility is that uPA may proteolytically activate a transmembrane protein to initiate signaling. It has been reported that uPA interacts with a novel, high-affinity binding protein in platelet membranes (48). Similarly, in melanoma cells uPA appears to bind to an unidentified membrane protein to initiate signal transduction. Whatever the receptor for uPA-mediated uPAR induction proves to be, the magnitude of induction is greater than that effected by PMA or TGF-β, which are among the most potent inducers of cellular uPAR expression (27).
In summary, we confirmed that uPA stimulates cell surface expression of uPAR by cultured Beas2B and primary lung epithelial cells. This newly recognized pathway represents another mechanism by which uPAR-dependent responses of the lung epithelium may be regulated in the context of lung injury and repair or in the transformation, growth and spread of lung neoplasms.
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References


Figure Legends

Figure 1. Expression of urokinase receptors in normal and carcinoma-derived lung cells. Membrane proteins (100 µg) isolated from cells of epithelial lineage (a) (lanes 1-7, SAEC, Beas2B, H157, H460, A549, H1395 and H146), lung vascular-derived (b) (lane 1-2, PASMC and HPMVEC) and pleural (c) (lanes 1-5, Met5A, Ren, MS-1, M9K and M33K) were separated on 8% SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were subjected to Western blotting using a urokinase receptor monoclonal antibody.

Figure 2. Time dependent uPAR expression by uPA in lung epithelial (Beas2B) and small airway epithelial (SAEC) cells. (a) Confluent Beas2B cells were treated with or without two-chain uPA (1 µg/ml) for 0, 3, 6, 12 and 24 h (lanes 1-5) or PBS 24 h (lane 6) at 37°C in basal medium containing 0.5% BSA and membrane proteins were isolated. The total membrane proteins were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membrane was immunoblotted with anti-uPAR antibody. The data illustrated is representative of at least three independent experiments and mean density of the individual bands is presented in the line graph. The p values are \( \leq 0.05 \) for the 3, 6 and 24 h treatment periods versus the 0 or 24 h PBS treatment time points. Relatively large error bars are attributable to the semiquantitative nature of the densitometric analyses and the difference between signal/background ratios in individual experiments. (b) Western blot for urokinase receptor of primary bronchial epithelial cells treated with PBS (lane 1) or uPA (lane 2) or identically treated H157 (lanes 3 and 4) or A549 cells (lanes 5 and 6) for 24 h.
**Figure 3.** Overexpression of endogenous uPA induces uPAR expression at the cell surface. a) Western blotting for uPA expression in uPA cDNA transfected Beas2B cells. Proteins from cell lysates (CL) and conditioned media (CM) of untreated Beas2B cells alone (lane 1) or Beas2B cells transfected with uPA cDNA in eukaryotic expression vector pRc/CMV (lane 2) or Beas2B cells transfected with expression vector pRc/CMV alone (lane 3). Proteins were separated on 8% SDS-PAGE, transferred to a nitrocellulose membrane and developed by Western blotting using anti-uPA monoclonal antibody. b) Western blotting for uPAR expression in uPA cDNA transfected cells. Membrane proteins from Beas2B cells treated as above were subjected to Western blotting using anti-uPAR monoclonal antibodies.

**Figure 4.** a) Saturation binding of $^{125}$I-uPA to Beas2B cells treated with or without uPA. Beas2B cells grown in multiwell plates were treated with PBS or uPA (1 µg/ml) for 24 h at 37°C. The receptor bound endogenous uPA was removed by glycine-HCl treatment and the cells were later treated with varying concentrations of $^{125}$I-uPA for 2 h at 4°C. Cell bound radioactivity was measured and specific binding was calculated based on nonspecific binding in the presence of 400-fold molar excess of unlabeled uPA. b) Competitive inhibition of $^{125}$I-uPA binding to uPA treated Beas2B cells by high molecular weight (HMW) uPA. Beas2B cells treated with uPA were incubated with varying amounts of unlabeled uPA for 2 h at 4°C, followed by 2 h with $^{125}$I-uPA. Bound radioactivity was measured and the percent binding was calculated from cell-associated radioactivity in the absence of unlabeled uPA. c) Effect of uPA concentration on uPA receptor mediated plasminogen activation in Beas2B cells. Beas2B cells treated with
or without varying concentration of uPA were preincubated with 2.5 nM uPA for 2 h at 4° C, washed, and assayed for activation of added plasminogen (6 µg). Background endogenous uPA activity of glycine-HCl-treated cells has been subtracted from the values shown.

Figure 5. Time dependent induction of uPAR mRNA by uPA. Beas2B cells were treated with two-chain uPA (1 µg/ml) for 0, 3, 6, 12 and 24 h (lanes 1-5). Total RNA (20 µg/lane) was isolated using TRI-reagent and separated on an agarose-formaldehyde gel and subjected to Northern blotting using 32P-labeled uPAR and β-actin cDNAs. The figure shown is representative of the results of at least three independent experiments and the bar graph illustrates the mean band densities from these experiments. The p values for the uPA treatments versus the 0 h treatment interval are 0.05. The relatively large error bars are attributable to the semiquantitative nature of the densitometric analyses and the difference between signal/background ratios in individual experiments.

Figure 6. Effect of uPA concentration on uPAR expression. Beas2B cells grown to confluence were treated with varying amount of uPA (0, 10, 50, 100, 250, 500, 750, 1000, 2000 and 3000 ng/ml lanes 1-10) for 24 h at 37° C in basal medium containing 0.5% BSA and membrane proteins were isolated. The total membrane proteins were separated on 8% SDS-polyacrylamide gel, transferred to nitrocellulose membrane. The membrane was immunoblotted with anti-uPAR antibody. The figure is representative of the results of three separate experiments and the bar graph illustrates the mean band densities of these experiments. Relatively large error bars are attributable to the
semiquantitative nature of the densitometric analyses and the difference between signal/background ratios in individual experiments. The p values for the uPA treatments versus the PBS control p ≤ 0.05.

Figure 7. Effect of tyrosine kinase and phosphatase inhibitors on uPA mediated uPAR expression. The cells grown to confluence were treated with or without herbimycin A (2 μM), genistein (6 μg/ml) and sodium orthovanadate (10 μM) for 2 h followed by uPA (1 μg/ml) for 24 h at 37°C in basal medium containing 0.5% BSA and membrane proteins were isolated. The total membrane proteins were separated on an 8% SDS-polyacrylamide gel, then transferred to a nitrocellulose membrane. The membrane was immunoblotted with anti-uPAR antibody. The cells treated with PBS (lane 1), uPA (lane 2), herbimycin A (lane 3), herbimycin A and uPA (lane 4), genestein (lane 5), genestein and uPA (lane 6), sodium orthovanadate (lane 7) and sodium orthovanadate and uPA (lane 8). The data illustrated are representative of the findings of three separate experiments.

Figure 8. a) Role of uPAR in uPA mediated uPAR induction. Beas2B cells were grown to confluence, then treated with or without anti-uPAR antibody (Rab, 2 μg/ml), A5 (1 μg/ml) compound and PI-PLC (10 units/ml) for 2 h followed by uPA (1μg/ml) for 24 h at 37°C in basal medium containing 0.5% BSA. Membrane proteins were then isolated. The total membrane proteins were separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane. The membrane was immunoblotted with an anti-uPAR antibody. Data representative of three independent experiments are shown. b)
**Effect of uPA inhibitors on uPA mediated uPAR expression.** Beas2B cells grown to confluence were treated with or without B-428 (0.02 mM), anti-uPA monoclonal antibody (2 µg/ml) or plasminogen activator inhibitor (PAI-1) (4 µg/ml) for 2 h followed by uPA (1 µg/ml) for 24 h at 37°C in basal medium containing 0.5% BSA and membrane proteins were isolated. The total membrane proteins were separated on an 8% SDS-polyacrylamide gel, then transferred to a nitrocellulose membrane. The membrane was immunoblotted with anti-uPAR antibody. Data representative of three independent experiments are illustrated. 

**c) Effect of different fragments of uPA on uPAR expression.** Beas2B cells grown to confluence were treated with or without amino-terminal (ATF) (1 µg/ml) and low molecular weight (LMW) (1 µg/ml) of uPA for 2 h followed by uPA (1 µg/ml) for 24 h at 37°C in basal medium containing 0.5% BSA. Membrane proteins were then isolated. The total membrane proteins were separated on an 8% SDS-polyacrylamide gel, then transferred to a nitrocellulose membrane. The membrane was immunoblotted with an anti-uPAR antibody. The data are representative of the findings of three separate experiments.

**Figure 9. Effect of proteases and protease inhibitors on uPA-mediated uPAR induction.** Beas2B cells were grown to confluence, then treated with or without aprotinin (1 µg/ml), plasmin (1 µg/ml) or α-thrombin (1 µg/ml) alone for 2 h followed by uPA (1µg/ml) for 24 h at 37°C in basal medium containing 0.5% BSA. Membrane proteins were then isolated. The total membrane proteins were separated on 8% SDS-polyacrylamide gels, then transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-uPAR antibody. The cells treated with PBS (lane 1), uPA (lane
2), aprotinin (lane 3), aprotinin and uPA (lane 4), plasmin (lane 5), plasmin and uPA (lane 6), thrombin (lane 7) and thrombin and uPA (lane 8). The figure shown is representative of three separate experiments and the bar graph illustrates the mean band densities from these experiments.
Figure 1

a  b  c

1  2  3  4  5  6  7  1  2

uPAR→
Figure 3

(a) 1 2 3

uPA (CM)

uPA (CL)

β-actin

(b) 1 2 3

uPAR
Figure 4
Figure 5

[Image of a gel showing uPAR mRNA and β-actin mRNA with a graph plotting uPAR/β-actin mRNA Ratio over time (h).]
Figure 6
Figure 7
Urokinase induces expression of its own receptor in Beas2B lung epithelial cells
Sreerama Shetty and Steven Idell

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