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REGULAR ARTICLE

Thrombin—thrombomodulin inhibits prourokinase-mediated pleural mesothelial cell-dependent fibrinolysis

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Abstract Fibrin deposition is a hallmark of pleural inflammation and loculation but understanding of mechanisms by which mesothelial cells regulate intrapleural fibrinolysins remains incomplete. We speculated that pleural mesothelial cells regulate local fibrinolytic capacity via processing of single chain urokinase type plasminogen activator (scuPA). Pretreatment of human pleural mesothelial (MeT-5A) cells with TGF-β or thrombin, either alone or in combination, inhibited urokinase (uPA)-mediated fibrinolysis by MeT-5A. Thrombin, unlike TGF-β, inhibited fibrinolysis without induction of PAI-1, suggesting that thrombin-mediated cleavage of scuPA inhibits the fibrinolytic capacity of MeT-5A cells. Thrombin cleaves both purified scuPA as well as that secreted by MeT-5A cells and cell surface thrombomodulin accelerates thrombin-mediated cleavage of scuPA to inhibit cellular fibrinolytic activity. Molecular dynamics analyses demonstrated that thrombin-cleaved scuPA (uPAt) do not acquire a catalytically active conformation and that secondary plasminogen binding sites of uPA implicated in plasminogen activation are distorted in uPAt, explaining, at least in part, why uPAt is a poor enzyme. uPAt was detectable in transudative and exudative pleural effusions from patients. Intrapleural administration of scuPA generated increased levels of uPAt in PF of rabbits with pleural injury and loculation induced by tetracycline *in vivo*. This pathway is operative in diverse forms of pleural injury, restricts the urokinase-dependent fibrinolytic capacity of

Abbreviations: uPA, urokinase type plasminogen activator; t-PA, tissue type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; pro-uPA and scuPA, single chain uPA; uPAt, thrombin-cleaved scuPA; PLG, plasminogen; cAsp194, an example of chymotrypsinogen numbering of amino acid residues of serine proteases; TBS, Tris-HCl buffered saline, pH 7.4; PBS, phosphate buffered saline, pH 7.2; HBS, HEPES buffered saline, pH 7.2; PF, pleural fluid.

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pleural mesothelial cells and contributes to local control of fibrinolytic activity via processing of endogenous or exogenous scuPA within the pleural compartment.

Introduction

Fibrin deposition in the pleural space is a hallmark of inflammatory pleural disease [1,2]. Fibrin is deposited as a result of excessive activation of coagulation and insufficient fibrinolysis. Under physiological conditions, coagulation and fibrinolysis are precisely regulated and molecular links between these systems permit timely removal of ongoing or acutely induced fibrin deposits [3,4]. The major fibrinolytic protease — plasmin, is generated by activation of plasminogen (PLG) by both tissue type PLG activator (tPA) as well as by urokinase (uPA) [5–7]. Plasmin cleaves both tPA and uPA, transforming them from single chain forms to more active two-chain polypeptides [7,8]. As a result, urokinase and plasmin enhance generation of each other through a positive feedback mechanism. Once formed, plasmin cleaves fibrin, generating soluble degradation products, and exposing carboxy-terminal lysine residues (CO_2–Lys). PLG kringle domains 1 and 4 contain lysine-binding sites, which mediate further binding to fibrin, leading to enhanced plasmin generation and fibrin removal. Binding can be blocked by lysine analogues, such as epsilon aminocaproic acid (ε-ACA) as well as by the thrombin-activatable fibrinolysis inhibitor (TAFI) [9]. When activated by thrombin–thrombomodulin (TM), TAFI removes CO_2–Lys residues, thereby attenuating plasmin generation, stabilizing fibrin thrombi, and establishing a regulatory connection between coagulation and fibrinolysis. Fibrin dissolution is also regulated by inhibitors of PLG activation, such as PLG activator inhibitor-1 (PAI-1), which is a major inhibitor of uPA and tPA *in vivo* and is produced by many cell types upon exposure to TGF-β, TNF-α and other cytokines [10,11]. Cells synthesize uPA as a single-chain precursor form (pro-uPA or scuPA) that can be converted to the two-chain uPA (tcuPA) after cleavage at Lys158–Ile159 by plasmin [8] or other activators [12]. Thrombin cleaves scuPA at Arg156–Phe157, two residues preceding the activation cleavage site, to generate a less active form of tcuPA called thrombin-cleaved tcuPA (uPAt) [13] The mechanism by which these two amino acid residues prevent conversion of zymogen scuPA into proteolytically active tcuPA is not known.

Pericellular proteolysis, including degradation of provisional fibrin matrix by serine- and metalloproteinases, has been implicated as a common feature of the pathogenesis of several diseases, including lung and pleural injury [10,14–16]. During the last decade, evidence for the involvement of the uPA system in pleural injury and repair has steadily increased [2,16,17]. Supporting this view, intrapleural administration of uPA or heparin reduces pleural adhesions and fibrosis [17]. While cleavage of scuPA is a putative mechanism by which fibrin is protected from early fibrinolysis, the role of this pathway in the regulation of intrapleural fibrinolysis has not been investigated. Thrombin-cleaved uPA has previously been detected in plasma of patients with sepsis [18] and in synovial fluids of patients with rheumatoid arthritis [18], leading us to infer that thrombin–TM-mediated cleavage of scuPA contributes to intrapleural regulation of fibrinolysis during evolving pleurodesis or other pleural diseases. We also inferred that this mechanism may be involved in cleavage of exogenous scuPA, which prevents intrapleural loculations and lung trapping induced by tetracycline and is being evaluated as a candidate for future clinical use [16].

To address these gaps in current knowledge of the regulation of fibrinolysis in the pleural space, we established a new model of cell-dependent fibrinolysis to determine how thrombin regulates the fibrinolytic capacity of pleural mesothelial cells *in vitro*. We provide new evidence that thrombin-dependent cleavage of scuPA contributes to the regulation of local fibrinolysis in diverse forms of pleural disease and in the processing of exogenous scuPA administered in a therapeutic context. Lastly, we performed molecular dynamics experiments to elucidate the molecular mechanism by which thrombin cleavage prevents the conversion of scuPA to an active fibrinolysin.

Materials and methods

Reagents

Polyclonal goat anti-human urokinase (uPA), anti-TM, anti-PAI-1 (mono- and poly- clonal) antibodies, rabbit lung thrombomodulin, and plasmin were purchased from American Diagnostica (Greenwich, CT). Anti-TM antibody clone 1009 was purchased from DakoCytomation (Mississauga, Ontario, Canada). Mouse anti-human TAFI monoclonal antibody clone 3H375 was from USBiological (Swampcott, MA). Chromogenic substrate S-2251 was from DiaPharma (Grochester, OH). Human α-thrombin was purchased from Enzyme

Research Laboratories (South Bend, IN). The scuPA and uPA were from Abbott Laboratories (Chicago, IL). Human fibrinogen (FGN) was from Sigma (St. Louis, MO). Human plasminogen (PLG) was isolated from plasma (Stewart Regional Blood Center, Tyler, TX) using affinity chromatography on lysine-sepharose 4B beads [19]. The PLG preparation was homogenous and appeared on a Coomassie-stained gel as a doublet with apparent molecular weights corresponding to Glu- and Lys- forms of PLG, confirming that the preparation was highly purified and consisted of these two forms. TGF-β was from R&D Systems (Minneapolis, MN). FGN and scuPA were labeled with sodium iodine-125 (PerkinElmer Life Sciences, Boston, MA) using iodogen method as recommended by the manufacturer (Pierce, Rockford, IL).

Cell culture conditions

MeT-5A human pleural mesothelial cells were purchased from ATCC (Manassas, VA) and propagated in accord with ATCC recommendations. In preliminary experiments in which we used fibrin enzymography, we found that MeT5A cells infected with Mycoplasma expressed readily detectable uPA antigen and activity. Mycoplasma positive MeT-5A cells were therefore utilized as a model of uPA-overexpressing cells in selected experiments. Naïve MeT5A cells, which expressed relatively smaller amounts of uPA, were used as comparator controls. Primary rabbit pleural mesothelial cells (RPMC) were isolated and cultured as previously described [16].

Cell-dependent fibrinolysis assay

MeT-5A cells were grown to 90% confluence in 96-well plates and serum-starved for 18 h in F12K or RPMI-1640 medium. Treatments with TGF-β (5 ng/ml), thrombin (1 NIH U/ml, about 8 nM) and antibodies (3 μg/ml) were conducted in the F12K medium (0.2 ml/well). Conditioned medium (170 μl) from control and treated wells was replaced with Tris-HCl buffered saline, pH 7.4 (TBS) containing FGN (0.17 mg/ml), PLG (10 μg/ml), and Ca^{2+} (17 mM) and mixed with 30 μl of conditioned medium, used as a source of scuPA. Immediately after the addition of thrombin (0.1 U/ml), fibrin formation and fibrinolysis were detected as changes in the absorbance at 405 nm (A_{405}) every 15 min for 16 h at 37 °C in a microplate spectrophotometer Spectramax Plus-384, Molecular Devices (Sunnyvale, CA). Since the concentration of thrombin affects the structure of deposited fibrin [20], and possibly A_{405}, control experiments were performed to define the conditions in which pretreatment with thrombin did not appreciably change A_{405}. We found that an increment of the thrombin concentration from 0.1 U/ml to 1 U/ml increased A_{405} of deposited fibrin from 0.3 OD to 0.4 OD in the presence of 1 mM Ca^{2+}. However, addition of excess of Ca^{2+} (17 mM) increased the turbidity of the fibrin gel in both control and thrombin pretreatment wells to about 1.0 OD, rendering changes in gel turbidity due to changes in thrombin concentration negligible. These experiments confirm that changes in fibrin turbidity in our experimental conditions are indicative of cell-dependent fibrinolysis. PAI-1 expression was induced by treatment of cells with TGF-β as described in the legend of Fig. 1 in order to validate the assay and verify that the assay is sensitive to changes in major *in vivo* inhibitors of fibrinolysis. To test the role of cell surface TM, anti-TM antibody clone 1009 known to block thrombin binding to TM was added as indicated in figure legend. To check whether trace amounts of TAFI could influence fibrinolysis in this system, anti-TAFI antibody (10 μg/ml) was added to some wells; along with isotype matched mouse IgG.

To determine whether uPAt formation could affect fibrinolysis in plasma milieu, we quantified ^{125}I-labeled fibrin degradation products released from clot formed on top of MeT-5A cells grown in 12-well plate. The scuPA (0.5 μg/ml, 0.1 ml/well) was incubated with the cells in the presence of thrombin (0.1 U/ml, 1.0 U/ml, or 10.0 U/ml) for 20 min at 37 °C. Recalcified normal human pooled plasma (0.1 ml/well) spiked with ^{125}I-fibrinogen (250,000 cpm/well) was added to each well at the

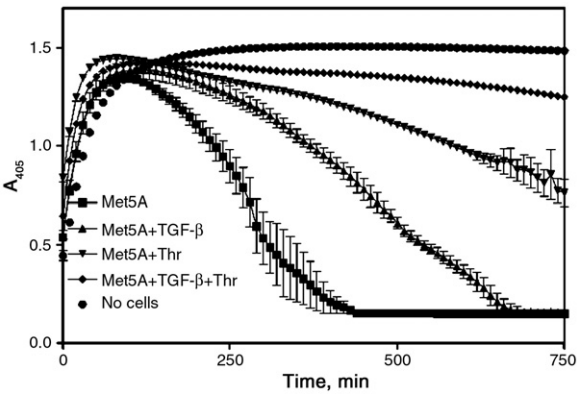

Figure 1 Effect of TGF-β and thrombin on MeT-5A cell-dependent fibrinolysis. Serum-starved MeT-5A cells grown in 96-well plates were pretreated with TGF-β (5ng/ml), thrombin (Thr, 1 U/ml), or both agonists for 4 h. Fibrin deposition was induced by addition of 0.17 ml of a solution containing fibrinogen (1.7 mg/ml), plasminogen (0.01 mg/ml) and Ca^{2+} (17 mM), followed by the addition of thrombin (0.1 U/ml). Data from a typical experiment with triplicate determinations are presented. Three independent experiments were conducted and the illustrated figure is representative of the findings of each independent experiment.

end of the incubation, and allowed to form a clot for 10 min. The clot was overlaid with 1.0 ml of F-12 medium and radioactivity of 50 μl aliquots taken from the wells every 1-hour starting from the moment of addition of medium was determined.

Chromogenic assay of PLG activation

PLG activation was measured by plasmin generation using plasmin-specific chromogenic substrate S-2251. The rate of S-2251 cleavage was measured in a Spectramax Plus-384 and plasmin concentration was determined using the calibration curves created with known concentrations of purified plasmin. To determine cell-dependent PLG activation, serum starved MeT-5A cells grown in 24-well plates were incubated with medium containing PLG (10 μg/ml) in the presence or absence of ε-aminocaproic acid (ε-ACA, 10 mM) for 2 h. Conditioned medium (80 μl) was mixed with 20 μl of S-2251 (3 mM) solution and changes in A_{405} were detected. To determine the PLG activator activity of thrombin cleaved scuPA (uPAt), the scuPA (0.004 mg/ml) was incubated in the presence of 0.0, 0.5, or 4.0 NIH U/ml of thrombin, PLG (0.01 mg/ml) and S-2251 (3 mM) in HEPES, pH7.2 buffered saline and A_{405} was determined continuously at 2 min intervals.

Determination of TM, uPA and PAI-1 antigens

These antigens were determined by Western blotting of the cell lysates and conditioned medium of control and treated MeT-5A cells. Briefly, cell lysate proteins (20 μg/lane) or conditioned medium (50 μl/lane) in SDS-sample buffer, pH 6.8 were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were developed using primary anti-TM, anti-uPA, and anti-PAI-1 antibody followed by incubation with a corresponding secondary antibody conjugated to horseradish peroxidase and visualization with the Western Lightning chemiluminescent reagent (PerkinElmer, Boston, MA). Control experiments employing known amounts of uPA, PAI-1, and preformed uPA-PAI-1 complex were conducted to test detection limits of anti-PAI-1 or anti-uPA antibodies for purified proteins as compared to their inhibitory complex. TM in the pleural tissue was detected by staining the paraffin embedded tissue sections with Antibody clone 1009 (DakoCytomation, Canada). Briefly, tissue sections were rehydrated for 5 min and blocked with normal mouse serum for 30 min at 37 °C. Diluted primary antibody was applied and incubated overnight at 4 °C. The slides were rinsed with PBS and developed according to the protocol of the Super Sensitive Immunodetection System (BioGenex, San Ramon, CA).

Cleavage of scuPA by thrombin–TM in purified system

Thrombin (1 NIH U/ml) or thrombin–TM (1 U/ml, defined as amount of TM that complexes 95% of 1 NIH U/ml of bovine thrombin) complex were added to scuPA (0.1 mg/ml) in F12K medium and incubated at 37 °C. Aliquots taken at selected time periods were transferred to SDS-sample buffer and analyzed by SDS–PAGE under reducing conditions. The Coomassie R-stained bands were quantified by densitometry. Incubation of scuPA for 18 h in these conditions allowed for complete cleavage of scuPA, as judged by SDS–PAGE in the presence of dithiothreitol (DTT, 5 mM) followed by Coomassie staining. There were no bands other than the two chains detected using these gels. To exclude the possibility that additional proteolysis not detected by Coomassie staining had been taken place, ^{125}I-scuPA was cleaved with higher concentration of thrombin (20 U/ml) under conditions otherwise identical to those described above for unlabeled scuPA. The resulting fragments were separated by SDS–PAGE in reducing (DTT, 5 mM) and nonreducing conditions and visualized by autoradiography.

Determination of uPAt in patient and rabbit PF

PF from patients were collected as described previously [2], under a protocol approved by the Human Subjects IRB of The University of Texas Health Center at Tyler. The study included PF from the patients with congestive heart failure (CHF), pneumonia (PN), and lung cancer (CA/lung). PF from rabbits harvested after 72 h of intrapleural administration of tetracycline (TCN) were processed as previously described [16]. PF from rabbits treated with intrapleural scuPA (0.5 mg/kg) at 24 h (scuPA-24) and at 48 h (scuPA-48) after TCN administration, or treated with vehicle [phosphate buffered saline, pH 7.2 (PBS)] at the same time intervals (PBS-24 and PBS-48, respectively) were tested for the presence of uPAt. To determine uPAt in these samples, 96-well plates (MaxiSorp™, Nalge Nunc, Denmark) were coated with an anti-uPA polyclonal antibody (Abbott Laboratories, Chicago, IL) (20 μg/ml in PBS) by incubation for 18 h at 4 °C. The plates were washed with PBS and blocked in PBS-BSA (1%)-Tween-20 (0.05%) for 1 h at 4 °C. After washing 50 μl of the PF were added to the wells containing 50 μl of HEPES (20 mM) buffered saline, pH 7.4 (HBS), and incubated for 18 h at 4 °C. The plates were washed three times with HBS and wells intended for uPAt determination were treated with Cathepsin C [20 nM in a phosphate (50 mM) buffer pH 6.0, supplemented with NaCl (100 mM), EDTA

(2 mM), and L-cysteine (10 mM)] for 30 min at 37 °C to reactivate uPAt [21]. The plate was next washed and incubated with PLG (10 μg/ml, 100 μl/well) for 2 h at 37 °C. uPA concentrations in PF were determined using a calibration curve based on the correspondence of known uPA concentrations to generated plasmin. The uPAt concentrations were calculated using a calibration curve created with known concentrations of uPAt after subtraction of the basal uPA-related signal from the cathepsin C-generated signal in the same sample.

Molecular dynamics (MD) simulations

The structure of uPAt was generated by addition of N-terminal FK-dipeptide to the catalytic domain of uPA [22] (Protein Data Bank entry 1C5W.pdb) using Swiss-Pdb Viewer [23]. The VMD program [24] was used to add hydrogen atoms and then solvate the initial structure in a box of TIP3P water model to ensure a 15 Å thick water layer to cover the protein. The resulting systems contained ~ 48,500 atoms. Sodium ions were added to neutralize the total charge of the system. The NAMD2 package [25] with the CHARMM22 force field was used for simulations. The NPT ensemble (pressure 1.025×10^5 Pa (Nose–Hoover Langevin piston method), temperature 300 °K (Langevin dynamics method)) and periodic boundary conditions with flexible cells were used. The SHAKE algorithm was applied for all bonds involving hydrogen atoms and all the simulations were performed with integration time step of 2 fs [26]. The system was energy minimized to slowly relax and reduce bad contacts. The system was then heated to 300 °K for 0.3 ns and equilibrated for 1.7 ns. The equilibrated structure was used as input for the MD production run, which was carried out for 2 ns. Every frame from the trajectory was aligned with a least square fit to the initial conformation, and root mean square deviation (RMSD) was calculated. Salt bridges were assigned when two oppositely charged residues were within 4 Å distance.

Protein assays

Total protein was determined in duplicate using the BCA Protein Assay (Pierce, Rockford, IL) or based on spectrophotometric absorbance at 280 nm.

Data analysis

The experiments were repeated as indicated in each figure. Statistical assessments were performed using nonparametric analyses using the Kruskal–Wallis test with GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

Pretreatment of the MeT-5A cells with thrombin inhibits cell-dependent fibrinolysis

Incubation of FGN (1.7 mg/ml) and PLG (0.01 mg/ml) with the uPA expressing Met-5A cell monolayer in the presence of thrombin and Ca^{2+} resulted in time-dependent fibrin deposition followed by its dissolution (Fig. 1). To validate the assay and to see whether this method is sensitive to increase of PAI-1 expression by the cells, MeT-5A cells were treated with TGF-β (5 ng/ml for 4 h). This treatment reduced the rate of fibrin dissolution, as did pretreatment of the cells with thrombin (1 NIH U/ml). Thrombin and TGF-β in combination additively

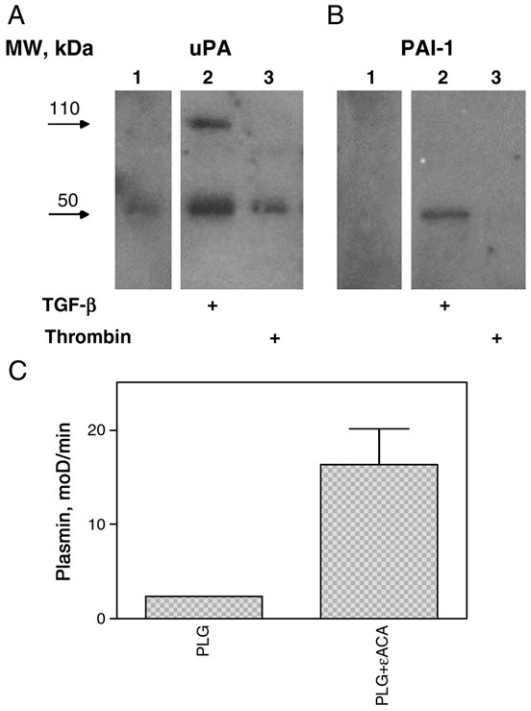

Figure 2 Expression of uPA (A) and PAI-1 (B) by untreated, TGF-β treated, or thrombin treated Met-5A cells. Lane 1: untreated cells incubated in similar conditions as treated cells; 2: TGF-β (5 ng/ml) treated cells; 3: thrombin (1 NIH U/ml) treated cells. The uPA and PAI-1 antigens were detected in 50 μl of conditioned medium of uPA overexpressing cells by Western blotting. The data are representative of the findings of three independent experiments. Panel C. MeT-5A cell-dependent activation of PLG (10 μg/ml) in the presence of ε-ACA (10 mM). Serum-starved scuPA expressing cells were incubated with PLG and with or without ε-ACA for 2 h at 37 °C and generated plasmin was detected by cleavage of the chromogenic substrate S-2251. Aggregate data of two independent experiments with duplicate determinations are shown.

reduced fibrin dissolution by MeT-5A cells (Fig. 1), suggesting synergistic effect or two independent complementary mechanisms of action. To reveal the molecular mechanism by which thrombin inhibits cell-dependent fibrinolysis, we compared PAI-1 and uPA expression by TGF-β or thrombin treated cells.

Inhibition of fibrinolysis by thrombin was PAI-1 independent

The uPA was readily detected in the conditioned medium of serum-starved untreated, TGF-β, or thrombin-treated MeT-5A cells (Fig. 2A). We could not detect t-PA in these samples by Western blotting (data not shown), suggesting that uPA was mainly responsible for the fibrinolytic activity of MeT-5A cells. Consistent with these results, ε-ACA, which inhibits t-PA-dependent PLG activation, and stimulates uPA-dependent plasmin generation [27], markedly increased plasmin generation (Fig. 2C). These observations document the dominant role of uPA in PLG activation in this system.

Western blotting of the conditioned medium of TGF-β treated cells using an anti-uPA antibody revealed 50 kDa and 110 kDa bands corresponding to uPA and uPA-PAI-1 complexes, respectively (Fig. 2A, lane 2). Western blotting with an anti-PAI-1 antibody detected PAI-1 in the conditioned medium of TGF-β treated cells (Fig. 2B, lane 2) suggesting that formation of uPA-PAI-1 inhibitory complex was responsible for the inhibition of fibrinolytic activity in this case. To determine why the uPA-PAI-1

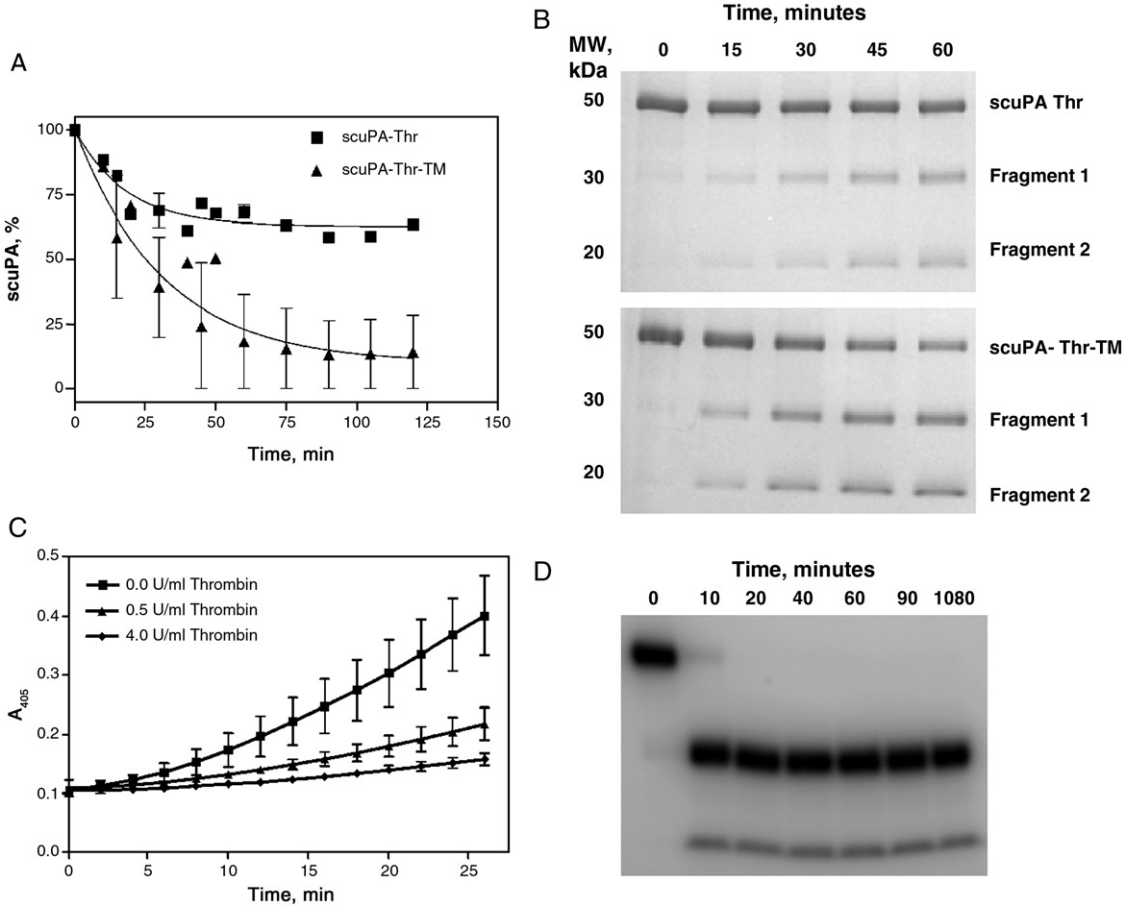

Figure 3 Cleavage of scuPA by thrombin or the thrombin–TM complex. Thrombin (1 NIH U/ml) (A) or complex of thrombin-rabbit TM (1 U/ml) (B) was added to scuPA (0.1 mg/ml) in F12K medium and incubated at 37 °C for the indicated time periods. Aliquots containing 2 μg of scuPA were taken at each time point and added to SDS-sample buffer containing 5 mM dithiothreitol. The samples were separated by SDS–PAGE on a 6–15% gradient gel. Protein bands were visualized by Coomassie R250 staining, quantified by densitometry and data plotted after normalization, taking band intensity at the beginning of the incubation period as 100%. Panel C shows time-dependent plasminogen activating activity of thrombin-cleaved scuPA as compared to intact scuPA. Aggregate data from two independent experiments are illustrated. Panel D demonstrates that there was no additional proteolysis of scuPA by thrombin–TM during prolonged incubation of scuPA (1 μg/ml) with 20 NIH U/ml of thrombin–TM.

inhibitory complex was not detected with anti-PAI-1 antibody, we performed comparative Western analysis of known amounts of PAI-1 and preformed PAI-1-uPA complex (50 ng/lane) using this antibody. We found that the antibody poorly detects the inhibitory complex, as compared to PAI-1 alone (data not shown). Therefore, the absence of 110 kDa band on this blot can be explained by concentration of inhibitory complex being present below the detection limit for this antibody. Pretreatment of MeT-5A cells with thrombin did not induce appreciable PAI-1 expression (Fig. 2B, lane 3), or uPA-PAI-1 inhibitory complex formation (Fig. 2A, lane 3). The thrombin concentration used in these experiments (1 NIH U/ml or about 8 nM) was chosen because we documented its marked effect on fibrinolysis in our assay. This concentration is pathophysiologically relevant as it could conceivably be achieved *in vivo* during activation of coagulation.

Cleavage of scuPA by thrombin–TM is responsible for inhibition of fibrinolysis

Thrombin cleaves scuPA and purified rabbit lung TM accelerates scuPA cleavage by thrombin (Fig. 3, panel A and B). The average times at which 50% of scuPA was cleaved in these experiments were 0.5 and 4 h for the thrombin–TM complex and thrombin, respectively. Thrombin-cleaved scuPA demonstrated minimal functional activity in a time-dependent plasmin generation assay (Fig. 3C). There was no additional proteolysis of scuPA by thrombin, other than formation of two-chain uPAt (Fig. 3D). Both, Mycoplasma positive (Fig. 4A) and negative (not shown) MeT-5A cells as well as mesothelial cells *in situ* on the human pleural lining (Fig. 4B) express TM by Western blotting and immunohistochemistry, respectively. To elucidate the role of cell surface TM in thrombin-mediated cleavage of scuPA, MeT-5A cells, which did not express prouPA were incubated with scuPA (15 ng/ml) in the presence or absence of an anti-TM antibody directed against EGF-like domains 4 to six of TM that blocks binding of thrombin to TM. Antibody treatment markedly reduced thrombin-dependent inhibition of fibrinolysis (Fig. 5A), confirming that cell surface TM contributes to the cleavage of scuPA by mesothelial cells. An anti-uPA polyclonal antibody completely inhibited fibrinolysis in this model, whereas anti-tPA or anti-PAI-1 (poly- and mono-clonal) antibodies had no effect (data not shown). Because anti-PAI-1 antibody was a mouse monoclonal antibody, it serves as an isotype-matched control for anti-TM antibody. Since anti-TM antibody could potentially inhibit TM-dependent TAFI activation, additional experiments

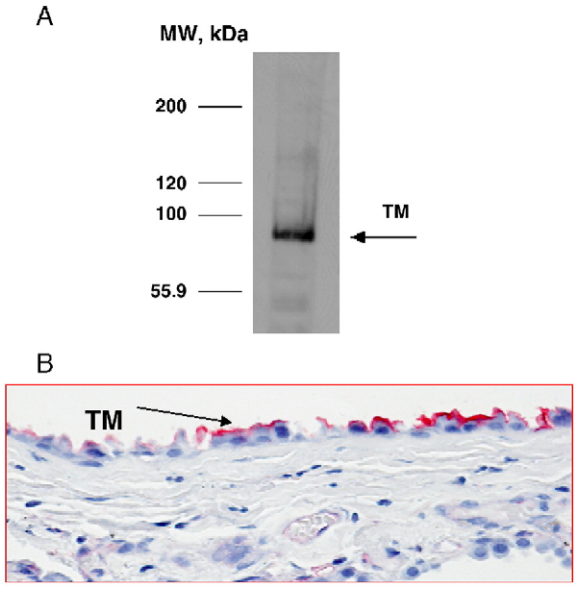

Figure 4 Detection of thrombomodulin in MeT-5A cells by Western blotting (A), and in human pleural tissue sections by immunohistochemistry (B). The arrow indicates TM antigen detected by red staining of reactive pleural mesothelial cells (original magnification – 400×). Representative findings of at least three independent experiments are illustrated.

were conducted to reveal the possible role of TAFI. Function blocking anti-TAFI monoclonal antibody (10 μg/ml), as well as isotype-matched mouse IgG had no detectable effect on fibrinolysis in this system (data not shown). These experiments document that the effect was not the nonspecific effect of immunoglobulins on the fibrinolysis. We observed the same effect of thrombin on uPA-dependent fibrinolysis when primary RPMC were incubated with scuPA (Fig. 5B), confirming that primary cells exhibit the same thrombin-mediated inhibition of scuPA-dependent fibrinolysis as the transformed MeT-5A cells. Thrombin cleavage of scuPA had detectable inhibitory effect on fibrinolysis in plasma milieu (Fig. 5C), in conditions resembling fibrinolysis during pleural injury.

A putative mechanism of restriction of uPAt activity by thrombin cleavage by MD analyses

Superimposition of structures after the equilibration stage of MD simulations revealed two distinct conformational clusters of novel NH_2-termini of uPAt. Therefore, two representative models of uPAt corresponding to these conformations were used to perform MD simulations. Time-dependent changes in RMSD between the final equilibrated structures of uPA, uPAt and their respective instantaneous structures during MD stage were small, 1.1 Å and 1.2 Å on

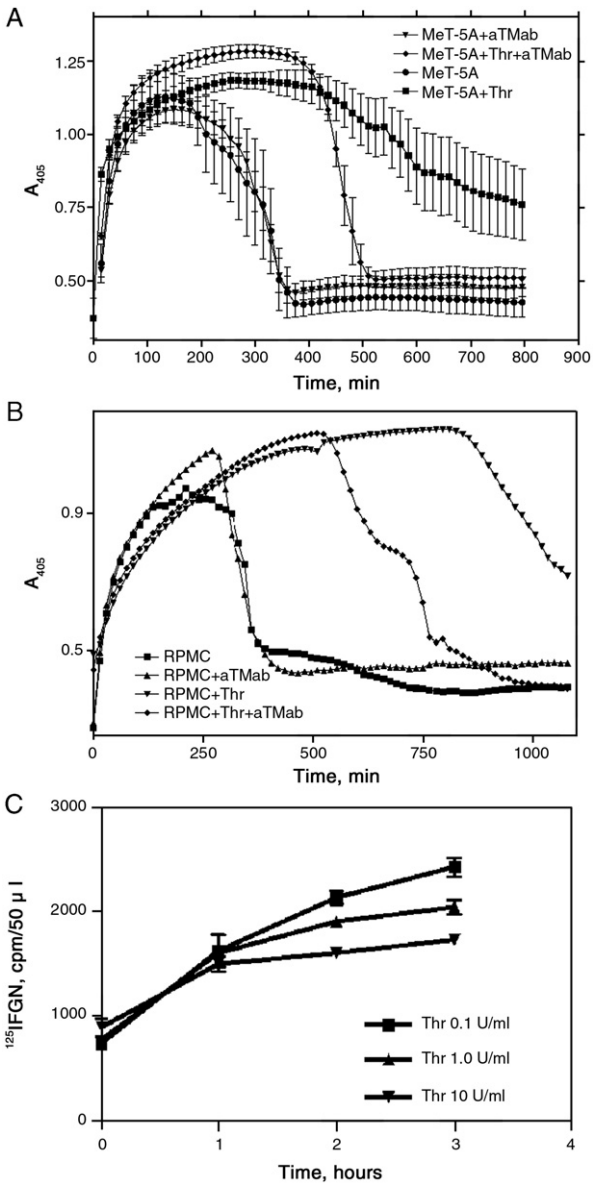

Figure 5 The role of TM in cell-dependent fibrinolysis (panels A and B). Cell-dependent fibrinolysis in plasma milieu (panel C). MeT-5A cells (A) or RPMC (B), which do not express uPA were incubated with scuPA (15 ng/ml) in the presence and absence of thrombin or anti-TM antibody. Fibrin deposition and fibrinolysis were determined under the same conditions as described in Fig. 1. The representative data of three independent experiments, each including triplicate determinations is illustrated. The average values only are provided in Panel B for better clarity. Panel C. ^{125}I-fibrin degradation products released from clot formed on top of MeT-5A cells grown in 12-well plate were determined at indicated time periods as described in methods section. Aggregate data from two independent experiments with duplicate determination of each value are illustrated.

average for backbone deviation of uPA and uPAt, respectively. These observations indicate that the structures of uPA and scuPA were equilibrated and stable during the MD simulations. All eight salt bridges identified in the original crystal structure of uPA were preserved in all the simulations except for the bridge between N-terminal isoleucine (cIle16) and Asp179 (cAsp194). The uPAt N-terminus was unable to form this bond, which is a crucial structural determinant of uPA activity. The N-terminus instead formed a salt bridge with cGlu23.

Comparison of the C_α atomic positions in uPA with positions of the same atoms in uPAt revealed a good fit for these structures except for the above-mentioned cAsp194 with surrounding residues and also for a cLys143–cLys156 loop (Fig. 6). Positions of the active site residues in both uPA and uPAt were comparable (Fig. 6), except for the considerable shift in the position of Ser356 (cSer195) leading to an approximate 1.7 Å shortening of a distance between the active site cSer195 and cHis57. These results suggest that thrombin-cleaved scuPA is unable to undergo conformational changes in the catalytic domain that are required for conversion of zymogen scuPA into an enzymatically active plasminogen activator and the proposed plasminogen interacting loops are distorted in uPAt.

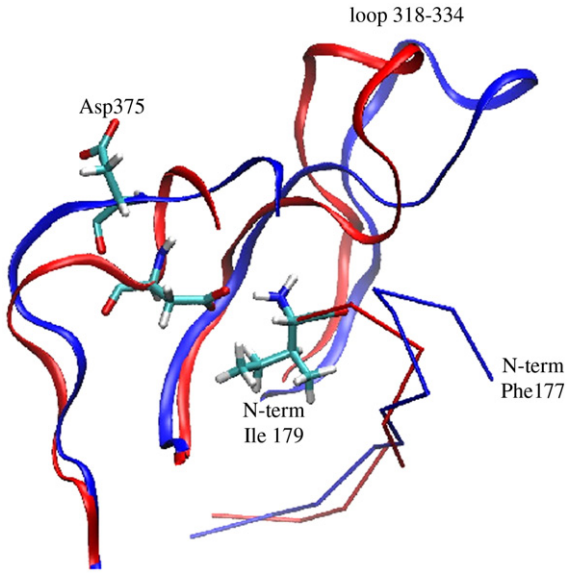

Figure 6 Superimposed ribbon structure representation of uPA (red ribbon) and thrombin cleaved scuPA (uPAt, blue ribbon) depicting the regions altered as a result of thrombin cleavage. Asp375 is a residue adjacent to active site cSer195, loop 318–334 is a flexible loop that plays a role in maintaining a zymogenic state and is implicated in plasminogen activation. The uPA NH_2-terminus – Ile179 (cIle16) and newly formed uPAt NH_2-terminus (Phe177) are shown.

Thrombin-cleaved scuPA can be detected in pleural fluids (PF) from patients and also in PF of rabbits with TCN-induced pleural injury treated with scuPA

To assess the pathohysiological relevance of the conversion of scuPA to uPAt by thrombin, uPA and uPAt concentrations were determined in clinical PF samples obtained from patients with congestive heart failure (CHF, $n=11$, lung cancer (CA/lung, $n=13$, parapneumonic effusions (PN, $n=9$) and in normal pooled human plasma (NPP). Increased amounts of uPA were found in the CA/lung pleural effusions as compared with CHF and PN ($P=0.0263$ for CA/Lung versus CHF) (Fig. 7A). uPAt could be detected in these samples, but there was no difference between patient groups in uPAt content (Fig. 7B).

To elucidate the possible role of thrombin in processing of exogenous scuPA, uPA and uPAt were determined in PF obtained from scuPA-treated rabbits with TCN induced pleural injury at 72 h after TCN challenge (Fig. 7, panels C and D). As we anticipated, PF from rabbits treated with scuPA 24 h before harvesting of PF (scuPA-48) contained higher amounts of uPA as compared with group treated 48 h before harvesting (scuPA-24). uPAt content was detectable and was likewise increased in both groups of rabbits that received intrapleural scuPA versus PBS ($P<0.0001$ by Kruskal–Wallis test) (Fig. 7D, $P>0.05$ for scuPA-24 samples, and $P<0.001$ for scuPA-48 samples by Dunn's test). These results confirm that uPA and uPAt detected in the PF of scuPA-treated animals were derived from human scuPA, administered by intrapleural injection and indicate that exogenous scuPA is, at least in part, converted to uPAt.

Discussion

We demonstrate that scuPA-mediated fibrinolytic activity of MeT-5A cells in culture is strongly inhibited by thrombin pretreatment of the cells. We could detect the effects of induction of PAI-1 expression in TGF-β-treated cells in our functional assay, which attests to its ability to interrogate pathophysiologically relevant effects. The observed effects were anticipated based upon the well documented overexpression of PAI-1 by a variety of TGF-β treated cells, including pleural mesothelial cells [1]. By contrast, thrombin treatment results in marked inhibition of fibrinolysis without overexpression of PAI-1 and formation of inhibitory complexes. An inhibitory antibody directed against thrombin binding site of TM protected against the decrement of uPA-mediated fibrinolytic activity in thrombin-treated MeT-5A cells. We therefore reasoned that

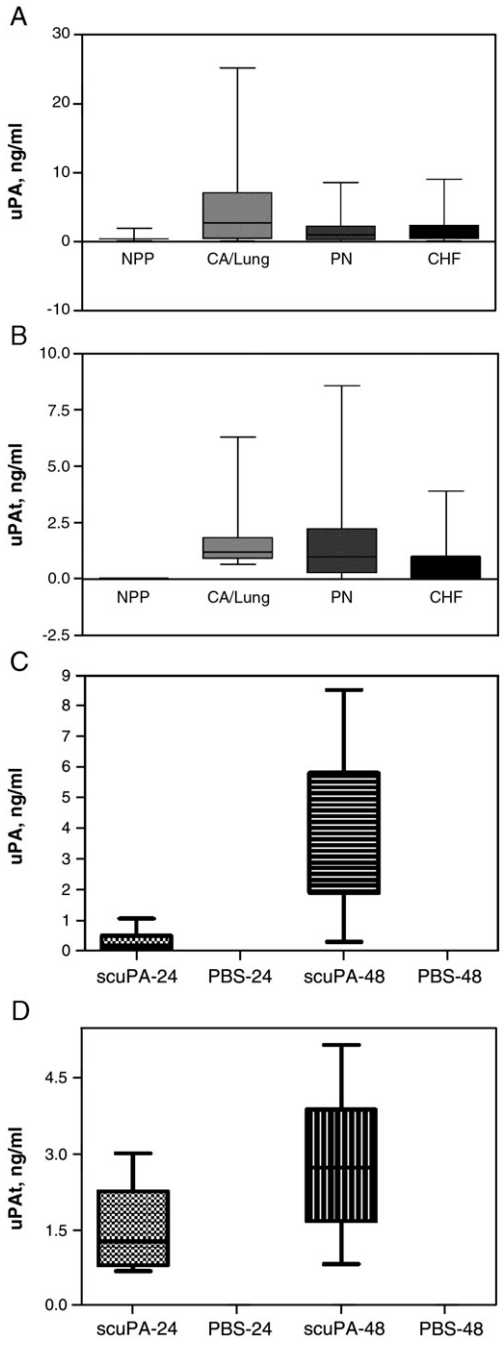

Figure 7 uPA and uPAt content of pleural effusions. (A) uPA and (B) uPAt in PF from patients with congestive heart failure (CHF, $n=11$), pneumonia (PN, $n=9$), and lung cancer (CA/lung, $n=13$). (C) uPA, and (D) uPAt content of PF from rabbits treated with either intrapleural scuPA or vehicle. Intrapleural scuPA or PBS was administered in a single dose at 48 h after TCN-induced pleural injury: scuPA-48 ($n=9$) and PBS-48 h ($n=9$), respectively. The scuPA-24 ($n=10$) and PBS-24 ($n=4$) groups received scuPA and PBS at 24 h after TCN challenge. PFs were harvested at 72 h after TCN challenge.

inhibition of mesothelial cell fibrinolysis by thrombin could, at least in part, be attributed to thrombin–TM-mediated cleavage of scuPA. We confirmed this hypothesis in experiments with purified proteins and in cell-based assays with MeT-5A and RPMC. We demonstrate, for the first time, that thrombin-mediated cleavage of scuPA can result in marked inhibition of cell-dependent fibrinolysis by pleural mesothelial cells. It is possible that anti-TM antibody could potentially inhibit activation of TAFI by thrombin and accelerate cellular fibrinolytic activity. We detected bands with apparent molecular weights corresponding to TAFI in conditioned medium from MeT-5A cells (data not shown). We think that it unlikely that TAFI activation significantly contributed to the observed effect of thrombin on cell-dependent fibrinolytic activity of pleural mesothelial cells. First, the cells were washed and the conditioned medium potentially containing TAFI was removed. Second, activated TAFI is unstable (half life about 15–20 min [9]). Third, mouse anti-human TAFI monoclonal antibody clone 3H375, known to inhibit TAFI activation as well activity of previously activated TAFI, has no effect on fibrinolysis in our experimental system. We therefore think it is very unlikely that trace amounts of TAFI could have significant impact on fibrinolysis in these long lasting experiments. Additionally, a comprehensive analysis of the contribution of TAFI to these effects is beyond the scope of this report.

Our MD simulation experiments designed to elucidate a putative molecular mechanism by which thrombin-cleaved scuPA is rendered catalytically less active revealed two major differences in the uPA and uPAt. First, the presence of N-terminal FK-dipeptide in the uPAt catalytic domain prevented formation of bonds in the cIle16-binding pocket with cAsp194 required for maintaining the catalytically active conformation of the active site. As a result, the position of cAsp194 in uPAt clearly differs from its position in active uPA as revealed by superimposition of these two structures (Fig. 6). Second, the loop comprised of residues c140–157 demonstrated a different conformation when compared to active uPA. Since this loop was shown to be important for maintenance of uPA activity [28], including its intrinsic activity [29], we speculate that the presence of FK dipeptide prevents conformational changes in this region that are otherwise required for maintenance of the active conformation of uPA. Moreover, the surface exposed autolysis loop c147–c150 and c97–c99 hairpin loop in uPA have been implicated in secondary (additional to active site) interactions with plasminogen [30]. Based on these observations we hypothesize that thrombin cleaved scuPA is a poor plasminogen activator as compared to plasmin cleaved scuPA. Taken into account that PLG activation in this system represents a positive feedback reaction, when uPA and plasmin reciprocally enhance their generation, the above hypothesis suggest that plasmin activation would be restricted in situations like that of pleural inflammation where scuPA can be cleaved by thrombin. Reduced generation of plasmin in the system containing thrombin (Fig. 4C) supports this hypothesis. It has previously been shown that thrombin can cleave scuPA both in purified system [13] and *in vivo*: in synovial and intravascular spaces [18]. Thrombin cleaved scuPA could be activated and reliably detected in the complex protein mixtures, including biological fluids, as demonstrated in these studies. Our study extends these observations by demonstrating that cleavage of scuPA by thrombin can markedly inhibit pleural mesothelial cell-dependent fibrinolysis. Moreover, detection of uPAt in PF (Fig. 7) demonstrates that thrombin-mediated cleavage of single chain uPA is pathophysiologically relevant. This pathway can contribute to regulation of fibrinolytic activity of blood plasma in cell culture conditions (Fig. 5C) and possibly, in pleural compartment *in vivo*. Detectable levels of uPAt found in pleural effusion in patients with metastatic lung cancer, parapneumonic effusions and congestive heart failure, suggest that processing of endogenous scuPA to uPAt occurs in a wide range of disease states associated with pleural effusions.

We also demonstrate that thrombin-mediated cleavage of scuPA occurs when exogenous scuPA is used to prevent pleural loculation. As an interventional agent, scuPA is especially promising as it remains active in PF for protracted periods of time, generates clear increments in local fibrinolytic activity in evolving pleural injury and protects against the development of pleural loculation [16]. Investigation of mechanisms by which scuPA is processed in this context assumes particular importance, as the effectiveness of currently available fibrinolysins has been challenged [31]. Our findings provide new information about the intrapleural processing of scuPA during evolving pleurodesis. These observations raise the intriguing possibility that the therapeutic efficacy of intrapleural scuPA could be improved by preventing its cleavage by thrombin–TM. It may be possible to reactivate the thrombin-cleaved scuPA by treatments similar to Cathepsin C.

Taken together, our observations demonstrate that excess intrapleural elaboration of thrombin can inhibit uPA-dependent fibrinolysis. This pathway appears to be operational in the setting of diverse forms of clinical pleural diseases characterized by formation of pleural effusions. Inhibition of local

fibrinolytic activity by thrombin-mediated cleavage of scuPA could potentiate excessive fibrin deposition in inflammatory pleural disease. This pathway may influence a broad range of pathophysiologic processes that rely on uPA-related enzymatic activity, including cell motility, proliferation and proteolysis, and may thereby influence inflammatory responses and remodeling in the pleural compartment after injury.

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