Intrapleural activation, processing, efficacy, and duration of protection of single-chain urokinase in evolving tetracycline-induced pleural injury in rabbits

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Idell S, Allen T, Chen S, Koenig K, Mazar A, Azghani A. Intrapleural activation, processing, efficacy, and duration of protection of single-chain urokinase in evolving tetracycline-induced pleural injury in rabbits. Am J Physiol Lung Cell Mol Physiol 292: L25–L32, 2007. First published September 15, 2006; doi:10.1152/ajplung.00118.2006.—Intrapleural fibrinolysins have been used to treat pleural loculations. However, the efficacy of clinically available agents has recently been questioned, providing a rationale for investigation of new interventions. Single-chain urokinase plasminogen activator resists inhibition by serpins, and repeated, daily intrapleural administration of this agent prevents intrapleural loculation more effectively than complexes of this proenzyme with its receptor (Idell S, Mazar A, Cines D, Kuo A, Parry G, Gawlak S, Juarez J, Koenig K, Azghani A, Hadden W, McLarty J, Miller E. Am J Respir Crit Care Med 166: 920–926, 2002). Understanding of the protective mechanism and intrapleural processing remains unclear. We speculated that single-chain urokinase could induce sustained local fibrinolysis and protection by selective administration either before, during, or following loculation after pleural injury induced by tetracycline in rabbits. Enzymography, immunoassays, histology, immunohistochemistry, morphometry, and morphometry were used to test the efficacy, duration of protective effect, and processing of single-chain urokinase. Intrapleural single chain urokinase prevented loculation at 72 h after injury (P < 0.01) if given either before or after adhesion formation and was converted to two-chain high-molecular-weight urokinase, which remained active for at least 24 h within pleural fluids. The effect was dose dependent, and established locations at 72 h after tetracycline-induced injury were reversed at 96 h by single-dose treatment. Single-chain urokinase bound and saturated intrapleural plasminogen activator inhibitor (PAI)-1-like activity and urokinase-related immunoreactivity of the mesothelium was comparable in treatment or vehicle-control groups. Adhesions occurred by 2 wk after treatment with recurrence of excess local PAI activity. Single-chain urokinase induces sustained local fibrinolysis and reversibly prevents pleural loculation for up to 48 h after intrapleural administration after tetracycline-induced injury.

pleurodesis; pleuritis; loculation; fibrinolysis

ABERRANT FIBRIN TURNOVER and intrapleural fibrin deposition are strongly implicated in the pathogenesis of pleural loculation and fibrosis (1, 7, 14, 16). Adhesions between the visceral and parietal pleural surfaces begin to form after 24 h following acute pleural injury induced by intrapleural administration of tetracycline (TCN) in rabbits and are primarily composed of fibrin (14). These fibrinous adhesions rapidly organize to form extensive loculations by 3 days after intrapleural TCN, and collagen is detectable within the adhesions at this interval (14, 18). The central role of fibrin in the pathogenesis of pleural organization is the predicate on which intrapleural fibrinolysins have been used to treat pleural loculations since the 1940s (22, 23).

The role of fibrinolysins in the treatment of pleural loculations is undergoing renewed scrutiny. The clinical efficacy of currently available agents has been questioned. On the one hand, the literature contains several reports and relatively small clinical trials that suggest that intrapleural fibrinolysins are beneficial and safe in selected patients with complicated parapneumonic effusions, empyema, or hemothoraces with intrapleural loculation (2–4, 6, 19). Conversely, a recent report of the findings from a large, randomized, controlled multicenter trial demonstrated that the intrapleural administration of streptokinase does not improve mortality, the rate of surgery, or the length of the hospital stay among patients with pleural infection (17). Like streptokinase, other currently available fibrinolysins, such as urokinase and tissue plasminogen activator, are rapidly inactivated or inhibited by proteases, plasminogen activator inhibitors (PAI), particularly PAI-1, or antiplasmins that commonly occur in exudative pleural effusions (12). The search for better, potentially more effective agents is therefore timely.

We previously reported that repeated, daily doses of intrapleural single-chain urokinase-type plasminogen activator (scuPA) attenuates pleural localizations induced by TCN in rabbits more effectively than administration of this agent complexed to its recombinant receptor and that neither of these interventions induced local or systemic bleeding (13). This study was designed to determine the ability of these interventions to protect against the development of intrapleural localizations after induction of pleural injury. Neither the scope and duration of protection nor the mechanism by which scuPA is processed within the pleural compartment were explored in our prior report, representing potentially important knowledge-based gaps we sought to address in this study. We therefore...

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used selective single-dose administration of intrapleural scuPA to elucidate the activation and processing mechanisms that occur in vivo as well as their aggregate effects on the outcomes of altered local fibrinolytic capacity and adhesion formation. We inferred that single-dose intrapleural scuPA used before or during detectable adhesion formation could induce protracted fibrinolytic capacity and protection against loculation. This inference is based on several lines of evidence. First, scuPA can become an active fibrinolysin when it binds to fibrin or to its receptor, uPAR, which is expressed by pleural mesothelial cells (20). Second, receptor-bound or free scuPA resists inactivation by PAI-1 compared with two-chain urokinase-type plasminogen activator (tcuPA) and exhibits fibrin selectivity (9, 10). Last, scuPA provides better fibrinolytic capacity than “active” tcuPA in pleural fluids in vitro (13). These observations collectively support the possibility that the protective effects of intrapleural scuPA would be durable, a possibility that we tested in the present study.

METHODS

Animals. Female New Zealand White rabbits purchased from Myrtle’s Rabbity (Thompson Station) were used in this study. The rabbits weighed 3.5–4 kg at the time the experiments were initiated. A total of 70 rabbits was utilized in these experiments. Thirty-three rabbits were allocated to the following groups in which localization was assessed at 72 h after intrapleural TCN: 1) animals treated with single-dose intrapleural PBS vehicle for scuPA at 48 h after intrapleural TCN (PBS 48 h; n = 9); 2) animals treated with a single dose of scuPA at 48 h after intrapleural TCN (scuPA 48 h; n = 10); 3) animals treated with intrapleural PBS at 24 h after intrapleural TCN (PBS 24 h, n = 4); and 4) animals treated with intrapleural scuPA at 24 h after intrapleural TCN (scuPA 24 h, n = 10). The interventions were performed in several independent experiments in which up to eight animals were randomized to receive either PBS or scuPA at the designated intervals. Based on observations in initial experimental runs, four animals were randomized to the PBS 24-h group to conserve animals committed to these experiments while efficiently assessing differences in outcome. Six animals were used in dose-response experiments. Twelve rabbits were treated at 72 h, an interval at which intrapleural localizations were established, with either PBS vehicle (n = 6) or scuPA (n = 6), and protection was assessed at 96 h.

An additional 19 rabbits were utilized in experiments in which localization was interrogated at 1 or 2 wk after initiation of pleural injury by TCN: 1) animals treated with PBS vehicle at 48 h after intrapleural TCN and killed at 1 wk after TCN administration (PBS 1 wk, n = 5); 2) animals treated with scuPA at 48 h after intrapleural TCN and killed at 1 wk after TCN (scuPA 1 wk, n = 5); and 3) animals treated with PBS (PBS 2 wk, n = 4, 1 animal died early and was not included in analyses) or scuPA (scuPA 2 wk, n = 5) and killed 2 wk after intrapleural TCN. In previous studies, we showed that the administration of the PBS vehicle did not induce pleural injury compared with naïve rabbits and that PBS-treated TCN controls exhibited identical localization patterns vs. rabbits treated with TCN alone (13), allowing us to conserve animals dedicated to these experiments. All experiments performed in this project were approved by the Animal Review Committee of The University of Texas Health Center at Tyler.

Induction of pleural injury, postmortem pleural fluid collection, assessment of intrapleural adhesions, total red and white blood cells, white cell differential counts, pleural fluid protein, lactate dehydrogenase, pleural fluid recalcification times, and D-dimer assays. Pleural injury was induced by intrapleural administration of TCN into the right pleural space, and analyses were performed as we previously described (13). Pleural fluid D-dimer assays were performed using a rapid latex agglutination slide test (Diagnostics Stago, Asnieres-sur-Seine, France) and ELISA kit (American Diagnostica, Greenwich, CT).

Interventional agent: scuPA. scuPA was provided as a generous gift by Dr. Jack Henkin, Abbott Laboratories, Chicago, IL. We injected 0.5 mg/kg or 0.25 mg/kg in dose-response experiments of scuPA into the pleural space of the rabbits at either 24, 48, or 72 h post-TCN-induced lung injury. Control animals received intrapleural PBS, pH 7.4, the scuPA vehicle, at the same intervals.

Western blotting, fibrin enzymography, and reverse fibrin enzymography. Pleural fluid samples were initially microfuged to remove cells and other particulates after which the samples were incubated with A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C to remove excess plasma proteins. The supernatants were next immunoprecipitated by incubation with a monoclonal antibody against human uPA for 18 h at 4°C (#3689, American Diagnostica). The uPA-antibody complexes were next precipitated by incubation for 2 h at 4°C in the presence of the A/G agarose beads. The samples were then subjected to SDS-PAGE (12%) to separate the proteins, after which they were transferred to a nitrocellulose membrane for Western blotting. A goat-derived polyclonal antibody against human uPA (a gift from Dr. Jack Henkin) was used to probe the membranes, and an enhanced chemiluminescence system (Perkin Elmer, Boston, MA) was used to detect the signals. The autoradiographic images were analyzed using the Gel-Doc System (Bio-Rad, Hercules, CA).

Briefly, in fibrin enzymography, pleural fluid samples were immunoprecipitated with a monoclonal antibody to uPA as described above and then subjected to 10% polyacrylamide gel electrophoresis (SDS-PAGE), after which the gel was blotted dry and placed on top of a freshly prepared fibrin indicator gel. Zones of fibrinolytic activity were identified as bands in which fibrin was lysed by constituents of the pleural fluids. The overlay system was allowed to develop at 37°C in a moist chamber, and the developing zones of fibrinolytic activity were sequentially photographed. In antibody neutralization experiments, the fibrin detector gel was additionally impregnated with 25 μg/ml antibodies to uPA (#3689, American Diagnostica), tissue plasminogen activator (tPA; #373, American Diagnostica), or control isotypic IgG antibody (IgG1; Sigma, St. Louis, MO). Reverse fibrin enzymography was similarly performed, except that the fibrin indicator gel was additionally impregnated with 0.075 IU/ml of two-chain urokinase. Zones of resistance to fibrinolysis developed as opaque bands that appeared in the cleared, lysed indicator gel and were photographed in the same manner.

Histological, immunohistochemical, and morphometric analyses. Histological analyses of hematoxylin- and eosin-stained tissue were performed as we previously described (21). Immunohistochemical analyses were performed using monoclonal antibodies to human uPA, uPAR, and PAI-1 (#3689, 3936, and 3785, respectively, American Diagnostica), which cross-reacted with the rabbit proteins and were used at a concentration of 10 μg/ml. Controls included immunostaining with mouse IgG (IgG1; Sigma, #9269, Sigma). We used the Innogenex IHC Kit (San Ramon, CA) in which diaminobenzidine substrate and horseradish peroxidase-streptavidin-biotinylated complexes are employed to detect the selected mouse antigens in rabbit tissues. Morphometric analyses were performed using tissues from each group of animals killed at 72 h after induction of pleural injury by TCN (n = a total of 4 groups including 3 animals/group treated with either PBS or scuPA at 24 and 48 h). In the morphometric analyses, 10 high-power (×40) fields were examined using pleural tissues in each case. In each field, the total number of mesothelial cells was counted, followed by the total number of immunopositive mesothelial cells in that same field. The number of immunopositive mesothelial cells counted from all 10 high-power fields was divided by the total number of mesothelial cells from all 10 fields, yielding an average percentage of immunopositive mesothelial cells for each antigen in each case. Analyses between groups were done by comparing expression of mesothelial cell uPA, uPAR, and PAI-1 in the ipsilateral TCN-injured
visceral pleural tissues and their expression in the contralateral, unchallenged, histologically normal lung tissues.

Statistics. We used the Mann-Whitney or two-sample Wilcoxon rank sum test to determine significance of the difference between the two treatment groups, and we report two-sided P values based on t approximations. The Kruskal-Wallis test, a nonparametric one-way analysis of variance, was used in selected instances to compare more than two groups. If the Kruskal-Wallis test was significant at the 0.05 level, further multiple comparisons based on the Bonferroni method were performed. For comparison of the pleural fluid parameters from PBS, scuPA treatment, and contralateral (NL) groups, each comparison was done using the Mann-Whitney test and a two-sided significance level of 0.0167. Box plots are utilized to display the data, as we previously reported (13).

RESULTS

Analysis of intrapleural adhesion formation. In initial experiments, intrapleural adhesion formation was assessed in all animals at 72 h after intrapleural administration of TCN. Extensive intrapleural adhesion formation and loculations that were too numerous to count were identified in all rabbits treated with PBS vehicle at 24 h after intrapleural TCN (n = 4). Conversely, only rare adhesions were found at 72 h in rabbits that received one dose of intrapleural scuPA at 24 h (median 0, range 0–6 adhesions; n = 10 rabbits; P = 0.0122).

Adhesions were likewise too numerous to count at 72 h after TCN challenge in all rabbits receiving the PBS control vehicle at 48 h after TCN (n = 9), whereas those that received one dose of intrapleural scuPA at the same interval developed rare adhesions (Fig. 1, median 2, range 0–10 adhesions; n = 10 rabbits; P = 0.0111). These findings confirm that a single dose of intrapleural scuPA can effectively prevent intrapleural loculation when given at 24 h after TCN administration (18) before detectable visceral–parietal adhesion formation. Intrapleural single-dose scuPA is likewise effective if administered at 48 h after induction of pleural injury by TCN, during initiation and progression of adhesion formation. The effect was dose dependent, as intrapleural administration of one-half of the effective dose (0.25 mg/kg) at 48 h after intrapleural TCN failed to provide any protection. Extensive intrapleural loculations (n = 6 rabbits) were identical to those found in TCN vehicle-treated animals. To test the ability of single, full (0.5 mg/kg) dose scuPA to reverse established loculations, the agent or scuPA vehicle was administered by intrapleural injection at 72 h after induction of injury by TCN. Virtually complete reversal of loculation was observed in scuPA-treated rabbits (n = 6, single adhesions in 2 animals, 2 in another), whereas loculations that were too numerous to count were found in all the vehicle-treated rabbits at 96 h (n = 6).

One week after administration of TCN, protection was markedly attenuated in scuPA-treated vs. PBS-treated animals (n = 5 animals/group) with no adhesions noted in one scuPA-treated animal and few adhesions noted in two others. By 2 wk after induction of pleural injury by TCN, pleural loculations were too numerous to count in both the PBS- (n = 4) and scuPA-treated animals (n = 5), indicating that the protective effect noted at 72 h had lapsed and was reversible.

Effects of scuPA on formation of pleural effusions. We first sought to determine if alterations in pleural fluid volume occurred in association with the salutary morphological changes induced by single-dose scuPA. Pleural effusions were observed in the right (ipsilateral) hemithoraces of all rabbits following TCN-induced injury. In 14/33 of the animals, all of which were challenged with TCN in the right pleural space, small, serous contralateral sympathetic effusions were noted at 72 h, but there was no evidence of contralateral loculation in any of these animals. In PBS 24-h animals, large pleural effusions were found at the 72-h end point (median 25 ml, range 10–25 ml, n = 4). The size of these effusions did not significantly differ from those of scuPA 24-h animals (median 23 ml, range 2.5–49 ml, n = 10, P = 0.89). There was likewise no difference in the volume of PBS 48-h animals (median 29
ml, range 16–40 ml, n = 9) vs. scuPA 48-h-treated rabbits (median 48 ml, range 4–82 ml, n = 10, P = 0.10). Pleural fluids harvested at 72 h were sanguinous in all cases, but pleural fluid red blood cell counts did not significantly differ in PBS 24-h (median 218,000/ml, n = 4) vs. scuPA 24-h (median 172,750/ml, n = 10, P = 0.63) or PBS 48-h (median 125,000/ml, n = 9) vs. scuPA 48-h rabbits (median 109,500/ml, n = 10, P = 0.97). Sanguinous effusions of comparable size were identified in PBS (n = 4) and scuPA-treated rabbits (n = 5) at 1 wk after TCN challenge (medians 25 and 49 ml, respectively), whereas pleural effusions fully resolved in two PBS-treated animals (median 16 ml, n = 5) and three scuPA-treated animals (median 0 ml, n = 5) by 2 wk after intrapleural TCN.

Biochemical analysis and white blood cell counts. Pleural fluid total and differential white blood cell counts as well as total protein content, lactate dehydrogenase (LDH), recalcification times, and D-dimer levels were determined as selective indexes of pleural inflammation and local fibrin turnover. These analyses were performed in all animals in each group except that one differential cell count clotted in a 48-h scuPA-treated animal (n = 9 animals included in this group). There were no significant changes between the pleural fluid total white cell counts in the PBS 24-h (n = 4) vs. PBS 48-h (n = 9, P = 0.17), PBS 24-h (n = 4) and scuPA 24-h (n = 10, P = 0.45), or PBS 48-h (n = 9) and scuPA 48-h animals (n = 10, P = 0.13). There were likewise no significant differences between the 24- and 48-h PBS and scuPA-treated groups on differential white blood cell analyses, total protein, or 72-h pleural fluid recalcification times. scuPA treatment did not cause a significant change in levels of pleural fluid total protein concentrations, which were comparable in all groups (medians: PBS 24 h, 40 mg/ml; scuPA 24, 39 mg/ml; PBS 48h, 37 mg/ml; and scuPA 48 h, 36 mg/ml). Pleural fluid LDH did not differ in PBS 24-h (n = 4) vs. scuPA 24-h (n = 10; P = 0.63) animals, but was significantly lower in scuPA 48-h animals (median: 126.5 U/ml, n = 10) vs. the levels found in PBS 48-h control animals (median: 197.0 U/ml, n = 9, P = 0.03).

D-dimer concentrations were elevated in the pleural fluids of scuPA-treated rabbits vs. the control animals treated with PBS at either 24 h (median scuPA 53, range 16–121 ng/ml vs. PBS 17, range 0–35 ng/ml, P = 0.05) or 48 h (scuPA 102, range 12–212 ng/ml vs. PBS 11, range 0–70 ng/ml, P = 0.01; Fig. 2). These observations document that a single dose of scuPA generates detectable increments in pleural fluid fibrinolytic activity and that a specific byproduct of that activity persists in pleural fluids for up to 48 h after intrapleural administration. There were no significant differences in pleural fluid D-dimer concentrations between scuPA- and PBS-treated animals at 1 or 2 wk after TCN challenge (medians at 1 wk: 13.5 and 4 ng/ml, respectively; medians at 2 wk: 13.0 in PBS-treated animals, whereas D-dimer levels were below the assay detection threshold in the 2 pleural fluids from the scuPA-treated rabbits at this time).

Characterization of fibrinolytic activity and fate of scuPA in pleural fluids. All pleural fluids harvested at 72 h from the 24- and 48-h animal groups were tested under nonreducing or reduced conditions, and representative findings are illustrated in Fig. 3, A and B. By Western blotting under nonreducing conditions, we found that a 50-kDa uPA-related moiety that comigrated with the scuPA standard was increased in several of the pleural fluids from 48-h scuPA-treated animals vs. PBS controls (Fig. 3B). In addition, bands of 110 and 23 kDa were detected under nonreducing conditions (Fig. 3A). Under reducing conditions, the appearance of a uPA-related band of ~25 kDa was also detectable in the scuPA-treated animals at the 24- and 48-h intervals (Fig. 3B). Material that migrated in close proximity to the 50-kDa scuPA standard as well as the 110-kDa band was also observed under reducing conditions (Fig. 3B). These bands appeared to be increased in some of the pleural fluids of the rabbits treated with scuPA at 24 or 48 h but were not observed when the primary anti-uPA antibody was omitted from the blotting procedure (data not shown), suggesting that a proportion of the administered scuPA was detectable intact at these intervals. Nonspecific contributions to the signals observed by Western blot under nonreducing conditions at ~100 and 48 kDa in pleural fluids from both the scuPA- and PBS-treated animals are consistent with F(ab’)2 and F(ab’)
fragments that are detected by the chemiluminescence detection system used in this study and can be observed in control blots where the primary anti-uPA antibody is excluded (data not shown). These fragments have previously been detected by Western blotting using similar detection systems in other biological fluids, including plasma (Mazar, unpublished observations). The band observed at \( \sim 200 \) kDa under nonreduced conditions is a nonspecific band detected by the anti-uPA antibody and has not been identified.

Fibrin enzymography was used to detect zones of fibrinolytic activity in all of the pleural fluid samples and to thereby facilitate interpretation of the Western analyses. By fibrin enzymography, small amounts of incremental uPA-related activity in pleural fluids were observed in some 24-h scuPA-treated rabbits vs. 24-h PBS-treated rabbits. Zones of fibrinolysis that comigrated with the 50-kDa tcuPA standard were characteristically increased in the scuPA 48-h-treated rabbits vs. the PBS 48-h-treated rabbits (Fig. 4). In the scuPA 48-h animals, increased zones of activity were also observed at \( \sim 110 \) kDa, representing uPA-PAI complexes that can be detected using this technique (21). This zone is consistent with the 110-kDa band observed by Western blot analyses (Fig. 3) and indicates the formation of PAI-uPA complexes, detectable by enzymography. The zones of fibrinolytic activity were confirmed to be related to uPA by neutralization of the lytic bands with an antibody to uPA but not with a neutralizing antibody to tPA or a mouse nonspecific IgG (Fig. 5). These observations confirm that pleural fluids of scuPA-treated animals retain increased fibrinolytic activity related to uPA for at least 24 h after local administration and that a portion of the scuPA was converted to the two-chain catalytically active, two-chain high-molecular-weight form that was detectable by enzymography. The absence of detectable fibrinolytic zones at 30 kDa indicates that scuPA was not converted to the catalytically active low-molecular-weight form of uPA and that the low-molecular-weight fragments observed by Western blot analyses were inactive. The 25-kDa bands that appear under reducing conditions on Western blotting of the pleural fluids of animals receiving uPA therefore likely represent the B-chain of the active tcuPA that is formed from scuPA. The A-chain of tcuPA cannot be observed by Western blotting since the antibody used to detect uPA is B-chain specific.

Effects of scuPA on expression of plasminogen activator inhibitory activity in pleural fluids. All pleural fluid samples from the 24-h, 48-h, 1-wk, and 2-wk post-TCN animals were next analyzed by reverse fibrin enzymography to assess expression of PAI activity. In pleural fluids harvested at 72 h after induction of injury from PBS-treated animals at 24 and 48 h after TCN, zones of resistance to fibrinolytic activity that migrated in proximity to the human PAI-1 standard were readily detected (Fig. 6). In all of the pleural fluid samples from rabbits treated with scuPA at 24 or 48 h, this zone was not detectable at the same development intervals, likely relating to increased complex formation with tcuPA formed from the administered scuPA (Fig. 3). The rabbit pleural zone of PAI activity migrated in close proximity with human PAI-1 elaborated by HepG2 cells and inhibited urokinase activity as did PAI-1-elaborated human pleural mesothelial cells (15). By reverse enzymography, we found that we could attenuate the rabbit PAI-like zone with a monoclonal to human PAI-1 (Idell, Fig. 3. Western blot analyses of uPA-reactive material in pleural fluids of TCN-injured rabbits treated with either scuPA or control PBS vehicle. A: uPA-reactive material in pleural fluids under nonreducing conditions. Lane 1: scuPA standard. Lanes 2 and 3: randomly selected pleural fluid samples from rabbits treated with PBS at 24 h and harvested at 72 h. Lanes 4 and 5: pleural fluid samples from rabbits treated with a single dose of scuPA at the same interval. Lanes 6 and 7: randomly selected pleural fluids harvested at 72 h from rabbits treated with PBS at 48 h. Lanes 8 and 9: randomly selected pleural fluids harvested at 72 h from rabbits treated with a single dose of scuPA at 48 h. B: same format with samples analyzed under reducing conditions using 100 mM DTT (Bio-Rad, Hercules, CA). All pleural fluid samples from the 24-h PBS (n = 4), 24-h scuPA (n = 10), 48-h PBS (n = 9), and 48-h scuPA (n = 10) groups were analyzed, and representative data from duplicate, independent experiments are shown.
unpublished observation). The 50-kDa PAI-1-related zone of resistance to fibrinolysis recurred in all available pleural fluids at 2 wk after TCN in 48-h PBS- or scuPA-treated animals (Fig. 6B). The same zones of resistance to fibrinolysis were observed in all pleural fluid samples of animals treated with either PBS ($n=4$) or scuPA ($n=5$) at 48 h and then harvested 1 wk after administration of TCN (data not shown).

**Effects of scuPA on pleural inflammation and expression of components of the fibrinolytic system by pleural mesothelial cells in situ.** Histopathological examination confirmed our previously reported finding of acute pleural inflammation at 72 h after TCN challenge, associated with the presence of increased numbers of reactive mesothelial cells as well as formation of adhesions and fibrinous intrapleural neomatrix formation and neovascularization (14, 21). Apart from the paucity of intrapleural adhesions, there were no detectable differences between the tissue responses in the 24- or 48-h scuPA and 24- or 48-h PBS-treated groups ($n=3$ animals examined/group). By immunohistochemical analyses, uPA, uPAR, and PAI-1 were all variably detectable in pleural mesothelial cells and to an extent in subpleural tissues of animals from each group ($n=3$ animals/group). By morphometric analyses, there were no differences in expression of these antigens by pleural mesothelial cells between the PBS 24- or 48-h or scuPA-treated groups. Animals in which PAI-1 expression was increased also tended to exhibit increased expression of mesothelial cell PAI-1 at the surface of the contralateral lung. Considerable variation was noted in the expression of the antigens in the TCN and contralateral pleural tissues from each group.

**DISCUSSION**

In a recent double-blind, multicenter trial performed in the United Kingdom, Maskell and colleagues (17) showed that intrapleural streptokinase did not improve clinically relevant outcomes: mortality, rate of surgery, radiographic outcomes, or the length of hospital stay. On the basis of the findings of this study, it seems reasonable that routine use of fibrinolysins is not now indicated and that they may still be considered for use in patients who are poor candidates for surgical intervention (8). Less controversial is the notion that newer candidate fibrinolysins with potential advantageous properties should now be tested. Interventional approaches involving video-assisted thoracoscopy or surgery are now most commonly used at many institutions to treat pleural loculation, but the search for alternative, less invasive approaches provides a strong rationale for the work we now report.

Our findings confirm that single-dose intrapleural scuPA protects against formation of intrapleural adhesions in rabbits with TCN-induced pleural injury. The effect is apparent when single-dose scuPA is administered at 24 h after TCN challenge, an interval characterized by formation of pleural effusions but...
that precedes detectable intrapleural adhesions (14). The protective effect is equally apparent when scuPA is administered at 48 h during evolving pleural injury induced by TCN. The period between 48 and 72 h after intrapleural TCN administration is marked by initiation and propagation of intrapleural adhesions (14). We now show that the protective effects of intrapleural scuPA are dose dependent, persist for at least 48 h, and confer virtually full protection in a single dose administered during the initial exudative and organizational stages of evolving pleural injury induced by TCN. Along these lines, extensive adhesions that are established at 72 h post-TCN are resolved at 96 h by single-dose treatment with scuPA at 72 h, attesting to the therapeutic potential of this agent.

To elucidate the basis for these effects, we interrogated the pleural fluids and pleural tissues of rabbits treated at the 24- or 48-h intervals with intrapleural scuPA or its vehicle. By Western blotting and enzymography, we found that scuPA-derived products were conclusively increased in pleural fluids of animals treated at 24 and 48 h after intrapleural TCN. The results confirm that uPA-related moieties, including active high-molecular-weight tcuPA and uPA-PAI complexes, persisted and were readily detectable in pleural fluids during the phase of adhesions formation and loculation (48–72 h). Western blot analyses demonstrated the appearance of low-molecular-weight products of the catabolism of scuPA within the pleural fluids, and detectable increments were observed in pleural fluids of animals treated with scuPA vs. PBS vehicle at both 24 and 48 h. Zones of a 50-kDa antigen in the uPA-related antigen in PBS-treated fluids likely relate to cross-reactivity of the uPA antibody with the native rabbit scuPA protein (21). At the 48-h interval, increased appearance of a 23-kDa uPA-related band suggests degradation of the scuPA. Under reducing conditions, the increased appearance of a 25-kDa band in addition to the 23-kDa band supports conversion of part of the administered scuPA to tcuPA with the appearance of the uPA B-chain.

Fibrin enzymography was performed to confirm that uPA activity was increased in scuPA-treated animals and to elucidate uPA-related zones associated with fibrinolytic activity. We found a zone of fibrinolytic activity at 50 kDa that was characteristically increased in 48-h scuPA-treated animals, representing the high-molecular-weight tcuPA (Fig. 4). The identity of these zones of fibrinolytic activity was independently confirmed to relate to the presence of uPA moieties by antibody neutralization (Fig. 5). Although we cannot exclude the possibility that some of this activity relates to detection of small amounts of contaminating tcuPA in the scuPA preparation we used, it is more likely that at least some of the administered scuPA was converted to this form of uPA by plasmin generated within the pleural fluids during evolving injury. Together, the immunologic (Western) and functional (enzymographic) analyses suggest that part of the administered scuPA is progressively converted to tcuPA (50 kDa) or inactive low-molecular-weight fragments with a concomitant increase in PAI-1-tcuPA complexes.

The protective effects of single-dose scuPA observed at 72 h post-TCN challenge were not curative when the ipsilateral pleural effusions were allowed to remain in place for up to 2 wk. Protection was largely attenuated at 1 wk after scuPA treatment and fully dissipated by 2 wk after TCN challenge. By reverse enzymography, PAI-1-like activity recurred in pleural fluids at this interval. We found that this PAI exhibited properties suggesting that it was attributable to PAI-1. These observations demonstrate that the protective effect of single-dose scuPA is potent early during the course of TCN-induced pleural injury but can be overcome in part by local restoration of excess PAI-1-like activity within the pleural compartment as the effects of exogenously administered scuPA wane and loculation proceeds.

As we anticipated, administration of single-dose scuPA increased the levels of D-dimer in pleural fluids of rabbits treated with scuPA at 24 or 48 h and harvested at 72 h after TCN administration. Although this project was not designed as a comparative trial, these results are especially encouraging, as we previously reported that twice-daily dosing of the commercially available active, low-molecular-weight form of uPA over 3 days did not reliably induce increments of pleural fluid D-dimer concentrations at the same 72 h in pleural fluids using the same rabbit model of TCN-induced pleural injury (21). Although we previously found that D-dimer concentrations were increased in pleural fluids of rabbits that received daily doses of intrapleural scuPA at 24 and 48 h vs. PBS-treated
animals and those that received scuPA bound to its soluble recombinant receptor at these intervals (13), overlap between the groups was noted. We conclude that the increment of D-dimer concentrations in pleural fluids of rabbits treated with single-dose intrapleural scuPA attests to its potent ability to generate plasmin that in turn contributes to clearance of fibrinous adhesions induced by TCN.

There was no appreciable effect of intrapleural scuPA on the pleural inflammatory response, whereas fibrinous adhesion formation at the mesothelial surface was blocked. Because tcuPA can induce uPA, PAI-1, and uPAR in lung epithelial cells, as previously reviewed (11), we sought to determine whether similar effects derived from administration of scuPA at the pleural surface in vivo. No such effects were found. uPA immunoreactivity of the mesothelium was not increased in scuPA-treated vs. PBS-treated animals. This finding is likely attributable, in part, to dilution of scuPA within the pleural fluids and its dispersal over a relatively large surface area. The findings alternatively support the strong likelihood that scuPA, which exhibits fibrin selectivity, induced efficient intrapleural fibrinolysis by generating fibrin-associated plasmin, which is relatively protected from local inhibition (5).

In summary, these studies provide new information to support the concept that scuPA can be used in a single dose to prevent intrapleural loculation in this preclinical model. Our observations extend our previous work and show that protection is achieved in the TCN model of evolving pleuroedesis when scuPA is given in a single dose before, during, or after intrapleural adhesion formation. Our studies document that intrapleural single-dose scuPA provides nearly complete protection at 72 h after TCN challenge. The mechanism by which scuPA promotes increased intrapleural fibrinolytic activity involves generation of tcuPA with concurrent circumvention of local inhibition in vivo. Further testing of scuPA in alternative preclinical models of organizing intrapleural infection is a reasonable extension of the present findings, since its effects may vary under different conditions that predispose to the development of intrapleural loculation.

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