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The time course of resolution of adhesions during fibrinolytic therapy in tetracycline-induced pleural injury in rabbits

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1The Department of Cellular and Molecular Biology and the Texas Lung Injury Institute, The University of Texas Health Science Center at Tyler (UTHSCT), Tyler, Texas; 2The Department of Biology at the University of Texas at Tyler, Tyler, Texas; 3UTHSCT Vivarium, The University of Texas Health Science Center at Tyler, Tyler, Texas; 4The Department of Radiation Oncology, The University of Texas Health Science Center at Tyler, Tyler, Texas

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Komissarov AA, Florova G, Azghani AO, Buchanan A, Bradley WM, Schaefer C, Koenig K, Idell S. The time course of resolution of adhesions during fibrinolytic therapy in tetracycline-induced pleural injury in rabbits. Am J Physiol Lung Cell Mol Physiol 309: L562–L572, 2015. First published July 10, 2015; doi:10.1152/ajplung.00136.2015.—The time required for the effective clearance of pleural adhesions/organization after intrapleural fibrinolytic therapy (IPFT) is unknown. Chest ultrasonography and computed tomography (CT) were used to assess the efficacy of IPFT in a rabbit model of tetracycline-induced pleural injury, treated with single-chain (sc) urokinase plasminogen activators (scuPAs) or tissue PAs (sctPAs). IPFT with sctPA (0.145 mg/kg; n = 10) and scuPA (0.5 mg/kg; n = 12) was monitored by serial ultrasonography alone (n = 12) or alongside CT scanning (n = 10). IPFT efficacy was assessed with gross lung injury scores (GLIS) and ultrasonography scores (USS). Pleural fluids withdrawn at 0–240 min and 24 h after IPFT were assayed for PA and fibrinolytic activities, α-macroglobulin/ fibrinolysin complexes, and active PA inhibitor 1 (PAI-1). scuPA and sctPA generated comparable steady-state fibrinolytic activities by 20 min. PA activity in the scuPA group decreased slower than the sctPA group (kobs = 0.016 and 0.042 min⁻¹). Significant amounts of bioactive uPA/α-macroglobulin (but not tPA; P < 0.05) complexes accumulated at 0–40 min after IPFT. Despite the differences in intrapleural processing, IPFT with either fibrinolysin was effective (GLIS ≤ 10) in animals imaged with ultrasonography only. USS correlated well with postmortem GLIS (r² = 0.85) and confirmed relatively slow intrapleural fibrinolysis after IPFT, which coincided with effective clearance of adhesions/organization at 4–8 h. CT scanning was associated with less effective (GLIS > 10) IPFT and higher levels of active PAI-1 at 24 h following therapy. We concluded that intrapleural fibrinolysis in tetracycline-induced pleural injury in rabbits is relatively slow (4–8 h). In CT-scanned animals, elevated PAI-1 activity (possibly radiation induced) reduced the efficacy of IPFT, buttressing the major impact of active PAI-1 on IPFT outcomes.

Intrapleural fibrinolytic therapy (IPFT) activates the endogenous fibrinolytic system, resolving intrapleural adhesions and complex fibrinous deposits that sequester pockets of inflammation loculations, thus improving drainage and clinical outcome, in part by decreasing surgical interventions (10). There are multiple reports of successful (88–100% efficacy) IPFT in adult (1, 7, 8, 16, 17, 29, 42, 47, 48, 66, 67, 72) and in pediatric (2, 3, 6, 11, 20, 40, 41, 58, 59, 61, 63, 67) patients with empyema although outcomes in adult patients are inconsistent in these trials. Thus IPFT represents a less invasive and costly alternative to video-assisted thoracic surgery or other surgical interventions, which demonstrate comparable efficacy (16, 44, 45, 58, 60, 71) in pediatric practice. IPFT also represents a preferred choice in high-risk (1, 22) and otherwise inoperable patients (10, 43, 49, 56, 57, 69) or those with empyema/ localization who refuse surgery. The reasons for the inconsistent results of IPFT in adult patients remain unclear but likely reflect the empiric approaches that are currently used. Current dosing of IPFT differs by up to two orders of magnitude, variable dwell times (1–4 h) of chest tube clamping after IPFT, and dosing schedules (1–8 treatments, every 4–24 h) (1, 4–7, 15, 16, 28, 46, 50, 53, 58, 61, 65, 66). The empiricism in large part is attributable to the lack of formal toxicological and dose-escalation studies of presently used IPFT agents in humans and our relatively limited understanding of the molecular mechanisms governing the efficacy of IPFT.

The tetracycline (TCN)-induced model of pleural injury in rabbits is characterized by extensive pleural adhesions and exudative pleural fluids (PFs) that are reliably induced within 24 h of injury with relatively little variability in individual rabbits (23, 24, 27). Although TCN-induced injury recapitulates the features of pleural injury in humans, there are certain limitations, which preclude a direct correlation with infectious injury in empyema. The rabbit model offers fundamental advantages for the evaluation of IPFT with human tissue plasminogen (PLG) activators (tPAs) and urokinase PAs (uPAs). Unlike mouse fibrin, the structure of rabbit fibrin is similar to that in humans (52). Mouse PA inhibitor 1 (PAI-1) notably differs from human PAI-1 (12), and human uPA possesses poor affinity to the murine uPA receptor (30). The TCN-induced model of pleural injury has therefore been suc-

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cessfully used to evaluate the intrapleural processing of single-chain uPA (scuPA) (35) and to improve the efficacy of IPFT by targeting active PAI-1 (14).

Previously, we showed that the fibrinolytic activity in the first 1.5 h after IPFT does not determine outcome and hypothesized that the time of successful fibrinolysis in TCN-induced pleural injury in rabbits is longer than 2 h (14, 35). We also found that the intrapleural level of active PAI-1 dramatically affects the half-life of fibrinolysins and outcome of IPFT and that in vivo PAI-1 neutralization improves IPFT outcomes and decreases the required effective dose of a fibrinolysin (14). In the present study, we used chest ultrasonography (US) and computed tomography (CT) imaging modalities to define the time course for effective intrapleural fibrinolysis after IPFT by using known minimal effective doses (MED) of sc tissue PA (sctPA) and scuPA in our well-characterized TCN-induced pleural injury in rabbits (14, 24, 31, 35). We found that the time needed for effective fibrinolysis for sctPA and scuPA were similar and protracted over several hours. Moreover, serial CT scanning in the TCN-induced model of pleural injury results in a local increase in the level of active PAI-1, which decreases the efficacy of IPFT.

MATERIALS AND METHODS

Proteins and reagents. The scuPA used in this study was a generous gift from Dr. Jack Henkin, Abbott Laboratories (Chicago, IL). The activity standard for human two-chain (tc) uPA (100,000 IU/mg) was from Sekisui Diagnostics (Sanford, CT). Human recombinant sctPA (Activase) was from Genentech (San Francisco, CA). Wild-type recombinant human PAI-1, human fibrinogen, and FITC fibrinogen (3 mol fluorescein/mol of fibrinogen) were from Molecular Innovations (Novi, MI). Fluorogenic uPA and tPA substrates (Pefachlor uPA and tPA, respectively) were purchased from Centerchem (Norwalk, CT). Fluorogenic plasmin substrate, PLG, and plasmin were from Haematologic Technologies (HTI, Essex Junction, VT). Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). All in vitro and ex vivo experiments were carried out at pH 7.4 in either 50 mM phosphate or Hepes/NaOH buffers, with or without BSA (1 mg/ml).

Rabbit model of TCN-induced pleural injury. All experiments involving animals were approved by the Institutional Animal Care and Utilization Committee of The University of Texas Health Science Center at Tyler and conformed to all applicable NIH guidelines. Female New Zealand white rabbits, weighing 3.0–3.6 kg, were used (n = 27). Pleural injury was induced by intrapleural administration of a single dose of TCN, as previously reported (14, 24). Single intrapleural doses of either scuPA (Abbott Laboratories) or tPA (Activase, Genentech), known to clear pleural organization (MEDs 0.5 mg/kg and 0.145 mg/kg, respectively) in the model (in n = 12 and 10 rabbits, respectively), or a vehicle control (n = 3) were administered 48 h after induction of pleural injury by TCN (26). Two animals were excluded from the scuPA/CT group; one had a large pneumohorax induced at the time of TCN administration precluding efficient collection, whereas TCN administration was unsuccessful in a different animal assigned to the US/CT group. The control vehicle animals received TCN-induced pleural injury but were not imaged. Treatments were administered using a catheter (18 gauge, 1.25 inches in length), which was cleared using 0.5 ml PBS. Anesthesia and postoperative pain medication were administered, as previously reported (31). During each preoperative and postoperative period, rabbits were carefully monitored for signs of overt pain or distress to ensure animal stability and comfort. Euthesol (0.25 ml/kg), administered intravenously, followed by exsanguination via the renal arteries, was used for euthanasia performed 24 h following the intrapleural interventions.

US was performed for all rabbits at 0, 2, 8, and 24 h (n = 22), as previously described (14). Additional US imaging was performed at 4 h (n = 16; scuPA/US n = 3, sctPA/US/CT n = 6, scuPA/US n = 3, sctPA/US/CT n = 4) and at 12 h (n = 12; scuPA/US n = 4, scuPA/US/CT n = 3, scuPA/US n = 3, scuPA/US/CT n = 2). Aliquots of PF were collected at 0 and 24 h from all animals (n = 22; scuPA/US n = 6; scuPA/US/CT n = 6; scuPA/US n = 6, sctPA/US/CT n = 4), at 10, 20, and 40 min (n = 16; scuPA/US n = 5, scuPA/US/CT n = 3, scuPA/US n = 6, scuPA/US/CT n = 2), and at 4 h (n = 5; scuPA/US/CT n = 3, scuPA/US/CT n = 2). Citrated cell-free fluids were immediately aliquoted and stored at −80°C (35). Four animals treated with intrapleural scuPA and six animals treated with scuPA were imaged with four sequential CT scans. CT was performed at baseline (before TCN injury), at an injured baseline (48 h after TCN and immediately before IPFT), and at 24 h after IPFT. Additional CT imaging was done after pleural effusions were evacuated using a 20-ml plastic syringe at 24 h. At 24 h after administration of the intrapleural interventions, the rabbits were euthanized, and gross outcomes of IPFT were visualized and scored using the gross lung injury score (GLIS) as previously described (14, 24, 31, 35). CT outcome assessments. The progression of pleural injury was imaged by serial chest diagnostic and cine US in gently restrained, awake animals at 0, 2, 4, 8, 12, and 24 h after intrapleural delivery of either fibrinolysin. B-mode US was performed using the Logic e system (GE Healthcare, Milwaukee, WI), equipped with R5.2x software and a multi-frequency transducer model 12LS-R (3.0–10.0 MHz) at a working frequency of 10 MHz (14). The right pleural space was scanned using the same mid-chest transverse and longitudinal planes in each prone animal. All sonographic imaging was performed by the same operator. A US score (USS) was developed based on the scoring of still images derived from the cine motion studies with atelectasis/consolidated lung associated with diffuse intrapleural density/fluid collections equivalent to a score of 50; lung attached to the heart or chest wall by fibrin strands and/or collections of solid intrapleural material scored 25; detectable but reduced visualization of retained collections scored 10; residual single strands or small webs scored 5; complete clearance, with no pleural adhesions, scored 0.

CT imaging. CT imaging was done before induction of pleural injury, at 48 h after TCN but before IPFT administration, and at 72 h after removal of all PF amenable to aspiration after TCN but before IPFT. Additional CT imaging was performed at 0, 2, 8, and 24 h (n = 22, as previously described (14, 24, 31, 35). CT images were obtained using a GE Discovery 750HD 64-slice CT scanner (General Electric Healthcare, Little Chalfont, UK), which reduces breathing artifact, with a technique of 80 kV, 320 mA, 2-mm slice thickness, and a lung window reconstruction algorithm. Images were imported into the treatment-planning software Eclipse (Varian Medical Systems, Palo Alto, CA) in which the lungs were outlined and lung volumes were recorded. Morphometric determinations of pleural thickness and the depth of underlying pulmonary nits were assessed as previously described (70).

Metrics of pleural injury. GLIS were determined at necropsy, 24 h after IPFT for each animal, as described (31, 35). The autopsy of each animal was carried out by the same surgeon, who did not see the results of the US until this study was being prepared for submission. Two to three different scientists performed GLIS scoring evaluation simultaneously with the surgeon averaging all of the data. Pleural injury at 24-h autopsy was also photographed as described previously (31). IPFT was considered successful with a GLIS < 10. Multiple visceral-parietal interconnected fibrin webs and sheets or “too numerous to count” strands correlated with a GLIS = 50. In morphometric analyses, rabbit lung tissue slides stained with hematoxylin and eosin were examined using a Nikon Eclipse Ti Microscope. Morphometric analyses of pleural thickness were obtained in 30 fields per animal studied by ×20 magnification. The thickness of the pleura was measured from the basement membrane to the external border of the visceral pleura on the ipsilateral side of the pleural injury.

uPA and tPA amidolytic activity assay. The amidolytic activities of uPA and tPA in PFs were measured and analyzed as described.
elsewhere (37). Briefly, 50 μl of 0.2 mM fluorogenic substrate (Pefabflour uPA or tPA, respectively; Centerchem) in DPBS with 1 mg/ml BSA was added to samples of PF (0.05–2.00 ml) in 50 μl of the same buffer in white, 96-well, flat-bottom plates from Costar (Corning, NY). An increase in the fluorescence emission with time was monitored using a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Lincolnshire, IL).

Formation of PAα-macroglobulin complexes. The observed first-order rate constants (k_on) for the intrapleural formation of α-macroglobulin (αM)/PA were determined from measurements of the amidolytic activities of uPA and tPA in PF in the presence of 20–50-fold excess of exogenous human PAI-1, as described elsewhere (35). The amidolytic activity in samples of PFs was measured before and after supplementation with recombinant human PAI-1, with values corresponding to the activities of αM/PA and total PA, respectively. A single exponential equation was fit to the time-dependences of the concentration of active free PA ([free PA] = [total PA] – [αM/PA]) or αM/PA using SigmaPlot 12.0.

PAI-1 activity assay. Active PAI-1 in the PFs was determined by titrating the active inhibitor with solutions of uPA of known concentration, after which residual uPA amidolytic or PA activity was measured as previously described (36, 37).

PLG activation assay. PA activity was measured as we previously reported (36). Briefly, PF (0.1–2.0 μl) was added to the mixture of Glu-PLG (100–250 nM) and fluorogenic plasmin substrate (SN-5) (0.2–0.5 mM) in 0.05 M phosphate buffer, pH 7.4, with 1 mg/ml BSA.

Measurements of fibrinolytic activity in PFs. Fibrinolytic activity in PFs was measured using a FITC-fibrin assay as described previously (14, 36). Briefly, 100 μl of an equimolar mixture of unlabeled and FITC (3:1) fibrinogen (Molecular Innovations) (0.4 mg/ml) in 0.05 M Hepes/NaOH (pH 7.4, 20 mM NaCl, 5 mM CaCl2, room temperature) was transferred to black, 96-well flat-bottom plates from Costar. Human thrombin (10 μl, 10 nM) was added to each well and mixed. Plates were then dried overnight at room temperature in the dark. Formed FITC-fibrin films were washed three times with 0.3 ml of cold Hepes/NaOH buffer and stored at −20°C. Endogenous fibrinolytic activity in PFs was proportional to the rate of the increase in fluorescence emission of fluorescein at 512 nm (excitation at 490 nm) because of quenching of fluorescein residues during degradation of FITC-fibrin by PFs.

Data analysis and statistics. Levels of statistical significance were determined using Kruskal-Wallis one-way ANOVA on ranks and pairwise multiple-comparison procedures (Holm-Sidak method and Tukey’s test). Data analysis was performed using SigmaPlot, v. 11, as previously described (14, 25).

RESULTS

Outcomes of IPFT in TCN-induced pleural injury in rabbits are adversely affected by imaging with multiple chest CT scans. Animals with TCN-induced pleural injury were imaged with either chest US or US/CT, as described in MATERIALS AND METHODS. Surprisingly, GLIS levels indicative of effective IPFT (GLIS ≤ 10) were found in rabbits imaged with US alone, whereas scores indicative of ineffective IPFT (10 < GLIS ≤ 50) were found in rabbits imaged with both US and serial chest CT scanning. The distributions of GLIS for the three groups of animals are shown in Fig. 1 (controls without treatment with PA (n = 3); animals treated with MED of fibrinolyisins and imaged with US only (n = 12) and with US and CT (n = 10)). Animals imaged with US/CT and treated with scuPA demonstrated a trend toward higher GLIS, and there was a statistically significant increase in GLIS (P < 0.05) indicative of unsuccessful IPFT (GLIS > 10) in the scuPA group (Fig. 1). As expected, the GLIS in the TCN-injured, imaging vehicle control group animals (n = 3) was maximal (indicating adhesions that were too numerous to count in each animal, GLIS = 50) (Fig. 1). Therefore, addition of serial chest CT imaging to chest US is associated with a decrease in the efficacy of IPFT in TCN-induced pleural injury and adversely impacts outcomes in animals treated with otherwise effective intrapleural doses of PAs. In all animals, injury resolution after IPFT was assessed using chest US (14), when sequentially applied over 24 h, which detected adhesions, lung consolidation/atelectasis, and pleural effusions (Fig. 2). The pleural thickness was below the limit of detection for US and CT in TCN-induced pleural injury in rabbits. By morphometric analyses (data not shown), no significant differences in the extent of pleural thickening were observed between the tPA- or scuPA-treated groups. The contralateral uninjured lungs did not typically demonstrate pleural thickening or pneumonia although small amounts of PF were generally present in the contralateral left hemithorax attributable to the partial communication between the hemithoraces that is usually found in rabbits. We also tested the in vivo USS at 24 h and postmortem visual-based assessments (GLIS) for correlation (Fig. 3). The USS at 24 h correlated well (r² = 0.85; Fig. 3) with GLIS, indicating that the USS comparably reflects the severity and extent of pleural injury in the same animals at 24 h after IPFT and immediately before euthanasia.

USS at 0–24 h after IPFT reports slow fibrinolysis with MEDs of scuPA and scuPA confirms adverse outcomes in animals imaged with serial CT. USS was next used to evaluate and assess the severity of the pleural injury before and at 2, 4, 8, 12, and 24 h after IPFT. USS were determined for each intervention with US imaging alone and in combination with CT and plotted against time (Fig. 4, A and B, respectively). Reduction of USS was detectable at 2–4 h after intrapleural delivery of either fibrinolysis and approached the level of effective IPFT (US ≤ 10) by 4–8 h in 11/12 animals imaged
with US alone (Fig. 4A). However, the USS at 2–24 h also demonstrated a trend toward worsening injury in animals imaged with both US and CT, with most animals in this group showing ineffective responses (GLIS > 10) by 12 h (Fig. 4B). In contrast to the group scanned with US alone, there were no animals with either USS/H11005 or GLIS/H11005 in the CT group at 24 h (Fig. 4). Moreover, five US/CT animals had USS/H11022 (and comparable GLIS scores; Figs. 1 and 3) at 24 h, likewise indicative of unsuccessful IPFT. Together, these findings indicate that initial detection of pleural organization resolution by US was relatively protracted and only detectable hours after either tPA- or scuPA-based IPFT. Moreover, the changes in USS observed with MED scuPA and tPA (Fig. 4A) noninvasively define the time of effective fibrinolysis (TEF; the minimal time required to achieve successful clearance of pleural adhesions; USS ≤ 10). The TEFs ranged from 4–8 h (Fig. 4A), irrespective of the fibrinolysin used. Notably, multiple CT scans affected the outcomes of IPFT with MEDs of both fibrinolysins (Fig. 4B), strongly suggesting that intrapleural fibrinolysis was impaired.

As anticipated, CT assessment demonstrated clear evidence of pleural injury, with readily apparent collections of PF associated with complex densities that were difficult to distinguish from parenchymal injury or atelectasis of the underlying lungs (Fig. 5). CT images clearly demonstrate changes in the affected lung and pleural space after induction of the injury and injured lung expansion after successful IPFT, which becomes evident upon PF drainage (Fig. 5, A–C). Moreover, CT image-

Fig. 3. A strong linear correlation between GLIS and US scores (USS). USS were determined at 24 h after IPFT, using the definitions described in MATERIALS AND METHODS and Fig. 2 and plotted against GLIS (Fig. 1). The values of GLIS and USS for animals treated with scuPA, scuPA, and vehicle controls are shown as and , respectively. Open and closed symbols correspond to imaging with US alone and combined with CT, respectively. A solid line represents the best fit of a linear equation, USS = 0.92GLIS − 0.25, to the data (r² = 0.85). The dotted line (GLIS = 10) and the right axis represent the ranges of GLIS for successful (GLIS < 10) and unsuccessful (10 < GLIS ≤ 50) IPFT.

Fig. 4. Time course of the clearance of pleural adhesions after IPFT by USS. Changes in the USS with time following IPFT with MEDs of either scuPA (n = 6, ) and scuPA (n = 6, ) with US imaging only (A) and in combination with serial CT scanning (scuPA, n = 6, , and scuPA, n = 4, ) (B). 48 h after induction of pleural injury with TCN, animals were treated with 0.5 mg/kg scuPA (n = 12) or 0.145 mg/kg tPA (n = 10), and the level of injury was assessed using US, as described in MATERIALS AND METHODS, and as previously reported (14). The dotted line (USS = 10) and the right axis represent the ranges of USS for successful (USS < 10) and unsuccessful (10 < USS ≤ 50) IPFT. The data are presented as box plots using the same format as described in the legend of Fig. 1.
based reconstruction of lung volumes (Fig. 5B) visualizes and supports these conclusions. However, a box plot of the reconstructed lung volumes (Fig. 5C) demonstrates insignificant changes in lung volume after treatment, whereas lung volumes after injury were reduced vs. baseline levels ($P < 0.001$). The inability of CT to discern changes in lung volume after IPFT related to the presence of large ($>30$ ml) pneumothoraces, as confirmed by detection of anticipated increments in CT-derived lung volume after complete drainage of the injured right hemithorax. Although GLIS and USS more readily detect the outcomes of IPFT than CT imaging, they do not explain the underlying causes for the disparate responses to IPFT in US alone, US/CT-imaged animals. To address this issue, intrapleural PA processing was evaluated in each group and compared to understand the basis for the relatively poorer outcomes of IPFT with combined US/CT imaging.

**Serial chest CT scanning does not affect changes in intrapleural PA or fibrinolytic activities during the first 4 h after IPFT.** Changes in intrapleural PA (Fig. 6) and fibrinolytic (Fig. 7, A and B) activities following administration of IPFT were evaluated by analyzing aliquots of PF, as previously described (14, 35). PF PA activity progressively decreased over 40 min with approximate first-order rate constants ($k_{obs}$) 0.016 and 0.042 min$^{-1}$ in the scuPA- and tPA-treated animals in both the US and US/CT group (Fig. 6). Levels of PA activity in tPA-treated animals were significantly ($P < 0.05$) less at 10–40 min than those in the scuPA-IPFT group (Fig. 6), reflecting both the lower specific PA activity of tPA and the larger scuPA dose that was delivered. There was no significant difference in the change of PA activity between animals scanned with US alone or with US/CT. By 4 h after scuPA or tPA IPFT, the PA activity was significantly decreased vs. those observed at 10 min, $P < 0.01$. There were no significant differences in the levels of PA activity at 240 min between the scuPA- and tPA-treated animals. However, despite 90–95% of the initially administered intrapleural PA activity being lost, it was detectable in both groups of animals at 4 h, providing complete activation of the PLG and providing maximal fibrinolytic activity. These results directly support our hypothesis (14, 35) that intrapleural fibrinolysis in TCN-induced pleural injury is relatively protracted after administration of IPFT.

The changes of fibrinolytic activity in PFs at 10–40 min after intrapleural delivery of scuPA or tPA are shown in Fig. 7. Unlike PA activity, which was higher for the scuPA-treated group at 10–80 min (Fig. 6), intrapleural fibrinolytic activity was similar in the PFs of scuPA- and tPA-treated animals (Fig. 7) from 20 min onward, indicating that PF fibrinolysis after IPFT is tightly regulated by the endogenous levels of PLG and its inhibitors. Notably, intrapleural fibrinolytic activity in scuPA-treated animals was significantly increased ($P < 0.05$) compared with the scuPA animals at 10 min (Fig. 7, A and B). Within the US or US/CT groups, there was no significant difference in the steady-state level of PF fibrinolytic activity up to 240 min after IPFT (not shown). The rate of inactivation of tPA and tcuPA by PAI-1 is diffusion limited ($k_{ass} > 10^6$).
proenzyme (37). Thus slow (of an equilibrium that favors the inactive conformation of the
activation of endogenous PLG by scuPA is delayed (35) because
and PLG activation occur instantly. In contrast, the acti-
fibrinolytic activity by almost 2 orders of magnitude.
accumulated PLG with uPA (or tPA; not shown) results in a higher burst of
for PFs withdrawn at 10, 20, and 40 min after IPFT with scuPA (A).
was significantly lower than that expected from baseline PF analysis (C). Fibrino-
level, which is the same for both fibrinolysins but
significantly lower than that expected from baseline PF analysis (C). Fibrino-
approaches a steady-state level, which is the same for both fibrinolysins but
stead-state level, which is almost an order of magnitude less
was expected based on the ex vivo experiments (Fig. 7C). The
The observed difference in fibrinolytic activity induced by
scuPA and tPA at 10 min likely reflects the delayed activation of
PLG (and further inactivation of plasmin in PF) by scuPA in the
in the presence of high levels of endogenous PAI-1 (35, 37). In
contrast to the treated groups, both fibrinolytic and PA activities
were suppressed at 0–24 h in the control (untreated) group
(37). Thus, at any point in time, total uPA amidolytic activity is a sum of free and
and imaged by either US/CT (closed symbols) or US alone (open
sy) of an excess of exogenous PAI-1 reflects the level of
M-complexed enzyme. The activity measured in the presence
of an excess of exogenous PAI-1 reflects the level of αM/uPA.
The time-dependent changes of intrapleural αM/uPA reflect an exponential increase to a maximum level (Fig. 9,
insert), [αM/
uPA]max = [αM/uPA]max × [1-exp(-kobs × time)]; r² = 0.95, where [αM/uPA]r, [αM/uPA]max, and kobs are molar
centrations of intrapleural αM/uPA at time t and at saturation and a pseudo first-order rate constant for the αM/uPA formation,
respectively. The values of [αM/uPA]max and kobs were 160 ± 30 nM and 0.022 ± 0.008 min⁻¹, respectively (Fig. 8, inset).
In sharp contrast to scuPA treatment (Fig. 8, inset), there was little accumulation of PAI-1-resistant bioactive complexes in
PFs of the sctPA-treated animals collected at 0–40 min.
M/uPA complexes, which have an intrapleural lifetime that
exceeds that of uPA by almost an order of magnitude (35),
were readily detectable in PFs of animals treated with scuPA
24 h after IPFT (Fig. 9). There was no free PA in these PFs,
M⁻¹.s⁻¹) (21, 68); as a result, endogenous PAI-1 neutraliza-
tion and endogenous PLG by scuPA is delayed (35) because
of an equilibrium that favors the inactive conformation of the
proenzyme (37). Thus slow (k on = 0.072 min⁻¹) (37) forma-
tion of the active species of scuPA limits intrapleural PAI-1
neutralization and promotes a delay in subsequent activation of
the endogenous PLG. Indeed, fibrinolytic activity in the PFs of
scuPA-treated animals was higher at 10 min compared with
tsctPA-treated animals (Fig. 7, A and B; P < 0.05) before
reaching steady-state levels at 20 min. Notably, baseline PFs,
where both PA and fibrinolytic activities are suppressed (Fig.
7C), contain significant amounts of accumulated PLG. Indeed,
the activation of endogenous PLG in baseline PFs by supple-
mentation with exogenous PA, which mimics IPFT, resulted in
an increase of nearly two orders of magnitude in the fibrino-
lytic activity from the limit of detection level (Fig. 7C).
However, the fibrinolytic activity generated by bolus adminis-
tration of IPFT (Fig. 7C) was rapidly decreased in vivo by the
first 10–20 min after IPFT (Fig. 7, A and B) and approached the
steady-state level, which is almost an order of magnitude less
was expected based on the ex vivo experiments (Fig. 7C). The
Obs: accumulation of high levels of αM/uPA (○) and putative
M/uPA (○) molecular-cage-type complexes detected in aliquots of pleural
fluid. The concentration of the αM/uPA complexes was estimated from the
residual amidolytic activity after incubation (10 min, 4°C) of a sample of PF
with 100–150 nM of exogenous active human recombinant PAI-1, as previ-
ously described (37).

![Graph](image-url)

**Fig. 7.** Changes in the fibrinolytic activity in PFs of animals treated with scuPA (A, n = 8) and sctPA (B, n = 8) at 0–40 min. Intrapleural fibrinolytic activity approaches a steady-state level, which is the same for both fibrinolysins but
significantly lower than that expected from baseline PF analysis (C). Fibrino-
lytic activity in PFs withdrawn at 10, 20, and 40 min after IPFT with scuPA (A)
or sctPA (B) and imaged by either US/CT (closed symbols) or US alone (open
symbols) was determined using a FITC-fibrin film assay, as described previ-
ously (36).

**Fig. 8.** Intrapleural concentration of α-macroglobulin (αM)/uPA complexes at 24 h after scuPA IPFT with (○) or without (○) CT imaging (n = 6 for each
group). Insert: accumulation of high levels of αM/uPA (○) and putative
M/uPA (○) molecular-cage-type complexes detected in aliquots of pleural
fluid. The concentration of the αM/uPA complexes was estimated from the
residual amidolytic activity after incubation (10 min, 4°C) of a sample of PF
with 100–150 nM of exogenous active human recombinant PAI-1, as previ-
ously described (37).
and the molecular-cage-type complexes with αM were detected only in PFs of animals treated with scuPA (Fig. 8). The intrapleural concentration of αM/uPA was almost three orders of magnitude (P < 0.05) less than the concentration of uPA at 10 min after injection. In contrast to uPA, the level of tPA activity resistant to PAI-1 at 24 h (not shown) was at the limit of detection. Increments of αM/uPA in scuPA IPFT-treated animals were independently confirmed in PFs of scuPA- vs. sctPA-treated animals by enzymographic analyses (not shown). There was no statistical difference in levels of αM/ uPA complexes between scuPA groups scanned with US alone and with US/CT.

CT imaging results in an increased level of active PAI-1 at 24 h after IPFT. Both PA (not shown) and fibrinolytic (Fig. 9A; • and ▼) activities were suppressed at 24 h after administration of scuPA or sctPA, as we previously reported (14), indicating that additional dwell time was unlikely to increase the efficacy of IPFT (further decrease GLIS) for any treatment. Complete inactivation of intrapleural PA results in rapid termination of fibrinolytic attributable to a lack of freshly synthesized plasmin and the accumulation of PLG. Indeed, supplementation of PFs collected at 24 h after IPFT with tcuPA (Fig. 9A; ○ and ▼) resulted in a significant increase in the fibrinolytic activity, as observed with baseline PF samples (Fig. 7C) (14). Elevated active PAI-1 plays a critical role in the inactivation of low-level PA activity generated endogenously and attributable to the slow degradation of the molecular-cage-type complexes with αM (14, 35). Thus, the higher the level of active PAI-1, the sooner the PA activity is completely inactivated and fibrinolysis terminated. Notably, concentrations of active PAI-1 in PF at 24 h were statistically increased in the US/CT group (Fig. 9B). Therefore, CT scanning resulted in increased intrapleural expression of active PAI-1 and its accumulation to a higher level at 24 h, which likely contributed to the adverse outcomes of IPFT in the scuPA- or tPA-treated US/CT groups.

DISCUSSION

The results of the present study clearly demonstrate that the rate of fibrinolysis during IPFT with known effective doses of fibrinolysin is relatively slow (minimal TEF; TEF = 4–8 h; Fig. 4A). The potential burst of fibrinolytic activity immediately after IPFT attributable to activation of the endogenous PLG (Fig. 7C) is followed by a rapid (kobs = 0.23 min⁻¹) for scuPA and less for sctPA fall to approximately equivalent steady-state levels for both fibrinolysins (Fig. 7, A and B). This steady-state level of intrapleural fibrinolytic activity appears to reflect a dynamic equilibrium between inhibition of fibrinolysis and activation of replenished PLG. Both steady-state fibrinolytic (not shown) and PA (Fig. 6) activities were durable through 4 h and thus detected in the samples from animals imaged with US/CT (Fig. 4B). A significant increase in the fibrinolytic activity in baseline PFs after supplementation with PA (Fig. 7C) most likely reflects a potential burst of fibrinolysis in the short period of time after fibrinolysin injection (10–20 min for scuPA and <10 min for sctPA; Fig. 7, A and B). However, as extrapolated from Fig. 7, the approximate areas under the curve (AUCs) for initial fibrinolytic activity (0 to 10–20 min) were three to five times smaller than those for the steady-state part (10–20 to 240–480 min; Fig. 4A). In contrast, the AUCs for animals from the control group (not treated with PA) were minimal because the fibrinolytic activity was at the level of detection. Interestingly, the steady-state levels of accumulated PLG at the baseline (Fig. 7C) and at 24 h (Fig. 9, A and B) were on the same order of magnitude, indicating that 1) there are mechanisms that prevent an uncontrolled increase in the level of endogenous PLG, and 2) it is unlikely that CT dramatically affects intrapleural PLG expression. Moreover, we have previously demonstrated that the level of total PLG/plasmin antigen in PFs during IPFT also does not vary significantly (14, 35). The results shown in Figs. 4, 6, and 7 suggest that there was a similarity between the changes in fibrinolytic activity at 0–240 min for animals imaged with US only (effective IPFT; GLIS ≤ 10) and with US/CT (ineffective IPFT; 10 < GLIS ≤ 50). Thus the intrapleural expression of PLG is not likely to depend on the type or dose of fibrinolysin. Although changes in the expression of other genes could potentially affect degradation of adhesions after CT imaging, endogenous active PAI-1 may function as an all-or-nothing switch for intrapleural PLG activation and fibrinolysis. As soon as endogenous active PAI-1 neutralizes the intrapleural PA activity, the dynamic equilibrium between the activation of newly synthesized PLG and inactivation of plasmin, which determines the steady-state fibrinolytic activity (Fig. 7, A and B), moves toward the latter, and fibrinolysis stops. Previously, we demonstrated that changes in the level of active PAI-1 in PF
 affect the half-life of intrapleural PA activity and the outcomes of IPFT (14, 35) and that neutralization of PAI-1 results in an increase in the efficacy of IPFT (14). Thus using PAI-1 inhibitors such as monoclonal antibodies, which redirect the reaction from the inhibitory to the substrate branch of the PAI-1 mechanism (32–34, 39), would likely improve outcomes of IPFT with CT imaging in a manner similar to that observed without CT (14). On the other hand, even if an initial short (0–10 min) burst in the intrapleural fibrinolytic activity occurs, it may contribute to, but does not determine, the effective IPFT attributable to the long TEF (4–8 h) observed in this model. The observed low rate of intrapleural fibrinolysis raises the possibility that relatively shorter IPFT dwell times could slow intrapleural fibrinolysis through removal of both plasmin and fibrinolysin, which could adversely impact outcomes.

US was applied in a diagnostic imaging mode and was sensitive enough to consistently detect changes in discrete pleural adhesions/collections amenable to imaging over time (Fig. 4, A and B). Use of the TCN model in these studies is justified by similarity between rabbit and human proteins of the fibrinolytic system and structure of fibrin (52) and also the relatively consistent time course of outcomes we observed over several years in individual rabbits (14, 24, 27, 31, 35). The USS was predicated by observing changes in adhesion density, size, and distribution. GLIS (31, 35), determined at the time of euthanasia in all animals, corroborated the USS results. Monitoring intrapleural fibrinolysis with US directly demonstrated slow clearance of fibrin deposition (Fig. 4) and a decrease in the efficacy of IPFT in animals that were subjected to CT scanning (Fig. 4B).

Active PAI-1 is one of the key profibrogenic molecules as well as a biomarker and a target for IPFT in TCN-induced pleural injury in rabbits (14, 31, 35). PAI-1 activity at 24 h after IPFT was significantly higher for animals imaged with US/CT (Fig. 9; P < 0.05). Thus, although a number of other factors could have contributed to the response, increased intrapleural PAI-1 in animals with US/CT imaging predicated (14) coincided with worse IPFT outcomes. Notably, the pleural space and lung that was not subjected to TCN-induced injury did not develop fibrosis (not shown), indicating that CT exacerbates the effects of TCN rather than independently inducing pleural injury. The absorbed dose of radiation for each rabbit was 1.14 cGy per CT scan, which represents an effective dose of 1.37 mSv per scan to the lungs. Thus each rabbit received a total CT lung dose of 5–7 mSv because each underwent four CT scans. Because the last two CT scans were performed within an hour before euthanasia, we speculate that the two earlier CT scans (15–20 min before TCN injection and IPFT) could have major effects on IPFT and injury outcomes. These levels of radiation exposure typically do not induce pleuropulmonary injury, consistent with the lack of adverse effects on the contralateral lung. However, accumulating experimental and clinical evidence demonstrates that even the low doses of ionizing radiation used in CT produce free radicals and peroxides, which increase oxidative stress, and decrease the oxygen radical capacity, and an increase in γ-H2AX in blood indicating dsDNA breaks as soon as 1 h after CT (18, 19). Moreover, chemically compromised tissues such as the lungs of smokers, may similarly be more sensitive to radiation damage by chest CT (9). Thus we hypothesized that low-level CT radiation could affect production of reactive oxygen species at the injury site, induce local increase in PAI-1 expression, and exacerbate pleural injury. Nevertheless, despite the fact that TCN-induced pleural injury in rabbits recapitulates features of the human disease, it is premature to extrapolate from these results to clinical ramifications of CT imaging for patients with empyema. To the best of our knowledge, the effects of CT scanning, a technique widely used to image patients with empyema, on infectious pleural injury have not been investigated. Additional studies of the effect of CT imaging on pleural injury could prove to be of clinical utility.

Whereas the MEDs of scuPA and sctPA cleared adhesions in a comparable manner in animals not imaged with CT (Fig. 1, A4), the intrapleural processing of these agents differed considerably. sctPA is sensitive to inhibition by active PAI-1 (64) but is relatively more fibrin specific (54) and partially protected from inhibition by PAI-1 when bound to fibrin (64). On the other hand, scuPA generates up to 250 nM of intrapleural bioactive αM/uPA that contributes to low-level, durable PA activity in PF over 24 h (14, 37). Molecular-cage-type (51) complexes between αM and endogenous or exogenous uPA have previously been found in both airway fluids and PFs from humans and rabbits (35, 37, 38). Whereas proteinases complexed with αMs interact with LMW molecules (substrates, inhibitors, ligands), they are sterically protected from high-molecular-weight (HMW) ligands (13, 55). Their activities toward HMW substrates and inhibitors, if they exist, are greatly suppressed (35, 62). Thus the loss of PA activity in scuPA-treated animals (Fig. 6) is accompanied by simultaneous accumulation of bioactive αM/uPA complexes (Fig. 8, inset), which have a considerably longer intrapleural half-life (35) and are found at nanomolar levels in the PFs at 24 h after IPFT (Fig. 8). Slow degradation of αM/uPA contributes to durable, low-level intrapleural PA activity (35). However, under conditions of elevated endogenous PAI-1, uPA, which formed because of slow intrapleural degradation of αM/uPA,
becomes inactivated. Indeed, in the CT-imaged group, in which PAI-1 levels were elevated (Fig. 9B), the efficacy of IPFT was notably decreased (Figs. 1 and 4B). The U-shape of dependence of USS on the time observed for two animals treated with scuPA (Fig. 4B) could reflect the blockade of αM/αuPA-derivatd PA by increased active PAI-1. However, intrapleural PAI-1 neutralization results in effective IPFT with a decrease in doses of fibrinolysins by almost an order of magnitude (14). These results support the critical role of active PAI-1 as a biomarker and a molecular target for IPFT in TCN-induced pleural injury. The lack of molecular-cage-type complexes with tPA could reflect the effect of tPA binding to intrapleural fibrin (64) on the reaction with aM. On the other hand, tPA bound to fibrin easily activates PLG, which incorporated into the fibrin structure, affecting the rate and efficacy of intrapleural fibrinolysis.

The proposed scheme of intrapleural fibrinolysis in rabbits with TCN-induced pleural injury during IPFT is shown in Fig. 10. Both PA and fibrinolytic activity are positive, whereas endogenous intrapleural PAI-1 (Fig. 10; gray dotted and black dashed lines represent active PAI-1 levels in animals imaged with and without CT, respectively) is neutralized. Activation of endogenous PLG results in durable fibrinolytic activity (Fig. 7, A and B) until the total PA activity exceeds the level of endogenous active PAI-1 (Fig. 10; to the left from intercepts of the MED sctPA or scuPA with dashed line). During the TEF (4–8 h) or later, fibrinolysis results in effective IPFT (Figs. 1 and 4A). However, under conditions of increased PAI-1 activity attributable to CT scanning (Fig. 9B), complete inactivation of PA activity occurs earlier (Fig. 10; intercepts of the MED sctPA or scuPA with the gray dotted line). As soon as PAI-1 inactivates residual PA, fibrinolysis stops; the balance between intrapleural fibrinolytic and profibrotic activities shifts toward the latter, and the efficacy of IPFT decreases (Figs. 1 and 4B).

Although a MED of scuPA and tPA cleared most of the organizing adhesions over 24 h (Figs. 1 and 4A), at 48 h and for up to 72 h (23) after TCN-induced injury, this study demonstrates, for the first time, that discernable clearance of pleural adhesions is relatively slow, based on serial US imaging (Fig. 4). This noninvasive approach obviated the need to perform additional dedicated experiments in control groups, allowing us to conserve animals. The results obtained support the proposed mechanisms of intrapleural processing of scuPA (35) and the critical contribution of endogenous active PAI-1 to the outcome of IPFT in this model (14, 31, 35). Our results further buttress the concept that targeting active PAI-1 is a promising avenue to develop novel therapeutic interventions for the treatment of organizing or loculated pleural injury. Although the TCN-induced pleural injury model possesses a number of features of organizing human disease (24), the effects of PAI-1 targeting in empyema are most clinically germane and have yet to be tested.

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DISCLOSURES

Dr. Idell is the unpaid Chief Scientific Officer of Lung Therapeutics, serves on its board of directors, and has an equity position in the company, which was created to develop and commercialize single-chain urokinase and other agents for use in lung and pleural disease. His work on single-chain urokinase and pleural injury has been supported by grants from the National Institutes of Health and philanthropy.

AUTHOR CONTRIBUTIONS


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