ORIGINAL RESEARCH

Targeting of Plasminogen Activator Inhibitor 1 Improves Fibrinolytic Therapy for Tetracycline-Induced Pleural Injury in Rabbits

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Abstract

Endogenous active plasminogen activator inhibitor 1 (PAI-1) was targeted *in vivo* with monoclonal antibodies (mAbs) that redirect its reaction with proteinases to the substrate branch. mAbs were used as an adjunct to prourokinase (single-chain [sc] urokinase [uPA]) intrapleural fibrinolytic therapy (IPFT) of tetracycline-induced pleural injury in rabbits. Outcomes of scuPA IPFT (0.25 or 0.0625 mg/kg) with 0.5 mg/kg of mouse IgG or mAbs (MA-33H1F7 and MA-8H9D4) were assessed at 24 hours. Pleural fluid (PF) was collected at 0, 10, 20, and 40 minutes and 24 hours after IPFT and analyzed for plasminogen activating (PA), uPA, fibrinolytic activities, levels of total plasmin/plasminogen, α-macroglobulin (αM), mAbs/IgG antigens, free active uPA, and αM/uPA complexes. Anti–PAI-1 mAbs, but not mouse IgG, delivered with an eightfold reduction in the minimal effective dose of scuPA (from 0.5 to 0.0625 mg/kg), improved the outcome of IPFT ($P < 0.05$). mAbs and IgG were detectable in PFs at 24 hours. Compared with identical doses of scuPA alone or with IgG, treatment with scuPA and anti–PAI-1 mAbs generated higher PF uPA amidolytic and PA activities, faster formation of αM/uPA complexes, and slower uPA inactivation. However, PAI-1 targeting did not significantly affect intrapleural fibrinolytic activity or levels of total plasmin/plasminogen and αM antigens. Targeting PAI-1 did not induce bleeding, and rendered otherwise ineffective doses of scuPA able to improve outcomes in tetracycline-induced pleural injury. PAI-1–neutralizing mAbs improved IPFT by increasing the durability of intrapleural PA activity. These results suggest a novel, well-tolerated IPFT strategy that is tractable for clinical development.

Keywords: plasminogen activator inhibitor 1; fibrinolytic therapy; animal model; prourokinase; monoclonal antibodies

Clinical Relevance

Organizing pleural injury remains an important clinical problem for which fibrinolytic therapy has been used with variable results for children and adults. This study demonstrates, for the first time, that the targeting of active plasminogen activator inhibitor 1 enhances the ability of relatively low doses of intrapleural single-chain urokinase to clear pleural effusions after induction of organizing injury. This work defines a new, well-tolerated approach for intrapleural fibrinolytic therapy that is promising and tractable for clinical trial testing.

The results of Multicenter Intrapleural Sepsis Trials 1 and 2 demonstrated that intrapleural fibrinolytic therapy (IPFT) with either streptokinase, or tissue-type plasminogen activator (tPA) alone were ineffective (1, 2). In contrast, there is a growing body of clinical reports demonstrating the successful use of IPFT, including tPA, in empyema (3–5). It is likely that the disparate results of IPFT trials, which are largely successful in children (2, 6) and variably effective in adults (6, 7), relate to the lack of formal toxicological and dose-escalation studies, resulting in empiric dosing. A further impediment to the field is an incomplete understanding of the mechanisms governing intrapleural fibrinolysis and

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plasminogen activator processing. It is clear, however, that plasminogen activator inhibitor 1 (PAI-1), the major endogenous inhibitor of tPA and urokinase (uPA) (8, 9), affects the outcome of pleural injury. An increased level of PAI-1 is a biomarker in a variety of serious pulmonary conditions, including acute respiratory distress syndrome and acute lung injury (10, 11), empyema, and other forms of organizing pleural injury (7, 12–14). Increments of PAI-1 antigen have been reported to occur in inflammatory pleural effusions and empyema (15, 16). However, the contribution of PAI-1 activity to the severity of loculation and poor outcomes of IPFT in a preclinical model of tetracycline (TCN)-induced pleural injury in rabbits were only recognized recently (17, 18). The mechanism of intrapleural processing of single-chain uPA (scuPA) (18) predicts the critical role of a combination of high levels (10–20 nM) of endogenous active PAI-1 and fast PAI-1–independent inactivation of uPA in the outcome of intrapleural fibrinolysis. During IPFT, the initial excess of uPA quenches active PAI-1, and the activation of endogenous plasminogen results in intrapleural fibrinolysis. However, fast PAI-1–independent inactivation of uPA (0.018 ± 0.005 min^{-1} [18]) results in the progressive loss of intrapleural plasminogen activating (PA) activity. Such serpin-independent rapid inactivation likely contributed to the ineffectiveness of streptokinase IPFT in Multicenter Intrapleural Sepsis Trial 1 (1). Therefore, intrapleural fibrinolysis that is maintained by activation of endogenous plasminogen operates as long as the total level of plasminogen activators is higher than PAI-1's antiproteinase activity (18). Thus, we postulate that neutralization of endogenous PAI-1, resulting in an increased half-life of intrapleural PA activity, should increase the efficacy of IPFT. Moreover, scuPA has been shown to generate high levels of durable PAI-1–resistant α-macroglobulin (αM)/uPA complexes, which provide durable, low-level intrapleural PA activity (18). We therefore reasoned that targeting endogenous active PAI-1 would enhance scuPA-based IPFT. Although a number of low–molecular weight inhibitors of PAI-1 are available, monoclonal antibodies (mAbs) remain the most effective and specific for PAI-1 neutralization (19). Among several mechanisms of PAI-1 modulation by mAbs, redirection of the PAI-1 reaction from the inhibitory to the substrate branch (Figure 1) is one of the best studied and most effective (20–23). MA-33H1F7 and MA-8H9D4 additively redirect more than 95% of the reaction of human PAI-1 with uPA or tPA to the substrate branch the PAI-1 mechanism (20). Moreover, vitronectin, which binds PAI-1 with nanomolar affinity, potentiates PAI-1 neutralization by mAbs (21–23). In this study, MA-33H1F7 and MA-8H9D4 were selected after *in vitro* and *ex vivo* testing against rabbit PAI-1, and used as

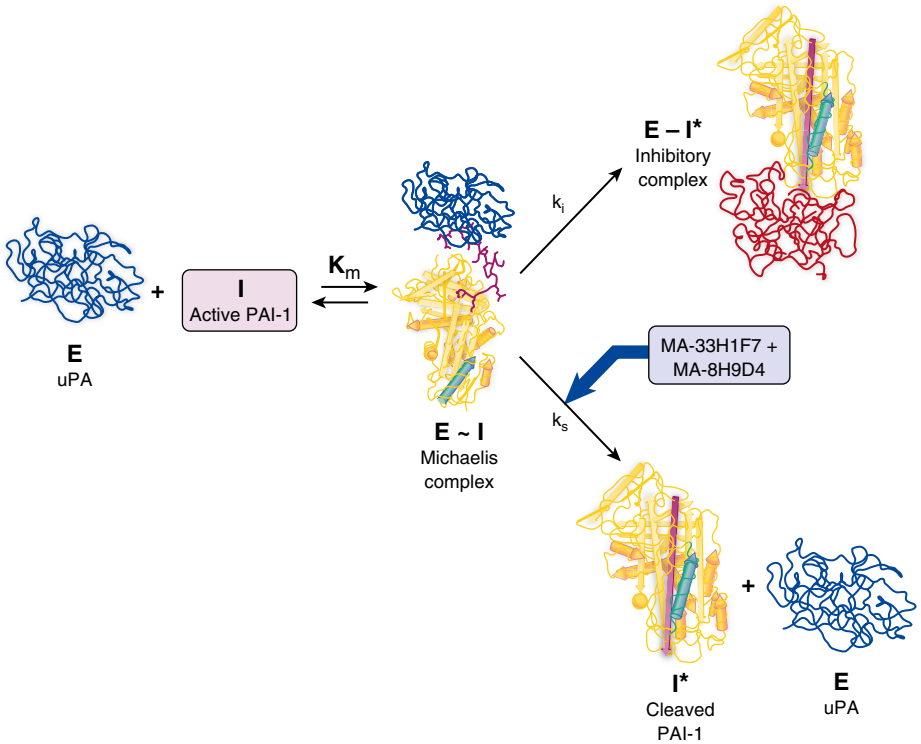

Figure 1. Protection of urokinase (uPA) (E) from inactivation by endogenous plasminogen activator inhibitor (PAI)-1 (I) by monoclonal antibody (mAb)-mediated redirection of the mechanism from the inhibitory (k_i) to the substrate (k_s) branch. An enzyme (*blue*) forms a Michaelis complex (E ~ I) with PAI-1 (*yellow*), where the serpin's reactive center loop (*pink*) is inserted into the active site of the enzyme. The inhibitory branch (k_i) yields a stoichiometric inhibitory complex (E–I) where the serpin inactivates uPA (*red*). E–I results from a "North-to-South Pole" translocation of the acylated enzyme during fast insertion of cleaved reactive center loop (*pink strand*) into β-sheet A of the serpin molecule behind α-helix F (*cyan*). Interaction of MA-33H1F7 and MA-8H9D4 (*blue arrow*) with their epitopes at the α-helix F and "South" end of PAI-1, respectively, additively redirects (20) the reaction to the substrate branch (k_i), resulting in an irreversibly inactivated, cleaved PAI-1 (I*) and active, intact fibrinolysin (E). K_m, Michaelis constant.

adjuncts in combination with scuPA IPFT. As a result, the minimal effective dose of scuPA was decreased eightfold: from 0.5 mg/kg (24) to 0.0625 mg/kg.

Materials and Methods

Animal Model

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Tyler (Tyler, TX). The TCN-induced pleural injury model was used as previously described (24, 25). New Zealand, white, pathogen-free, female rabbits (weight = 3.0–3.6 kg) acquired from Charles River (Wilmington, MA) were used in this study. After 48 hours, rabbits with pleural injury were treated with either scuPA or vehicle control PBS (sterile PBS), as previously reported (18, 24). scuPA alone, scuPA/anti–PAI-1 mAbs, scuPA/IgG, or vehicle control was administered intrapleurally using an 18-gauge, 1.25-inch long Excel Safelet Catheter (Los Angeles, CA), which was then cleared using 0.5 ml PBS. Aliquots (0.5 ml) of pleural fluid (PF) were collected at 10, 20, 40, and/or 80 minutes after IPFT. Samples were immediately centrifuged and snap frozen (17, 18). Citrated, cell-free samples were then stored at −80°C and analyzed. Anesthesia, postoperative pain medication, and animal care were provided as previously reported (17). Rabbits were carefully monitored for signs of pain, discomfort, or distress to ensure animal stability and well-being. Animals were killed with 0.25 ml/kg Euthesol, administered intravenously, followed by exsanguination via the aorta.

Ultrasonography

B-mode ultrasonography of the chest was performed as described elsewhere (18) using Logiq e system (GE Healthcare, Milwaukee, WI), equipped with R5.2.x software and a multifrequency transducer model 12L-RS (3–10.0 MHz) at a working frequency of 10 MHz.

Metrics of Pleural Injury

Gross loculation injury scores (GLIS) were calculated for each animal as described previously (17, 18). IPFT was considered successful for GLIS less than 10. Multiple visceral–parietal interconnected fibrin webs and sheets or "too numerous to count" strands correlated with a GLIS of 50.

uPA Amidolytic Activity Assay

Measurements of the amidolytic activity of uPA in rabbit PFs were performed and analyzed as previously described (26).

Plasminogen Activation Assay

Plasminogen activation assay were performed as previously described (27).

PAI-1 Activity Assay

Levels of active rabbit PAI-1 in the PFs were determined either by ELISA (Molecular Innovations, Novi, MI) or titrating PAI-1 with uPA of a known concentration (26).

Measurements of Total αM in Rabbit PFs

Total rabbit αM was determined in PFs by a commercially available ELISA (ICL, Portland, OR).

Measurements of Total Plasmin/Plasminogen in Rabbit PFs

Rabbit plasmin/plasminogen in PFs was measured by ELISA (ICL, Portland, OR).

Western Blot Analysis

Rabbit PFs were subjected to nonreduced SDS-PAGE (4–12% gradient NuPage gel; Invitrogen, Grand Island, NY), and analyzed via Western blot (26).

Fibrinolytic Activity in PFs

An FITC-fibrin film assay (*see* the online supplement) (28, 29) was used in these analyses. SigmaPlot 12.0 (SPSS Inc., San Jose, CA) was used to calculate the values of area under the curve (AUC) for fibrinolytic activity analyses.

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 Turkey test). Data analysis was performed using SigmaPlot 12.0 for Windows, as previously described (25). Correlation coefficients (r) calculated from curve fittings were used as a parameter of the exactness of the fit (r^2>0.90 for all the kinetic data).

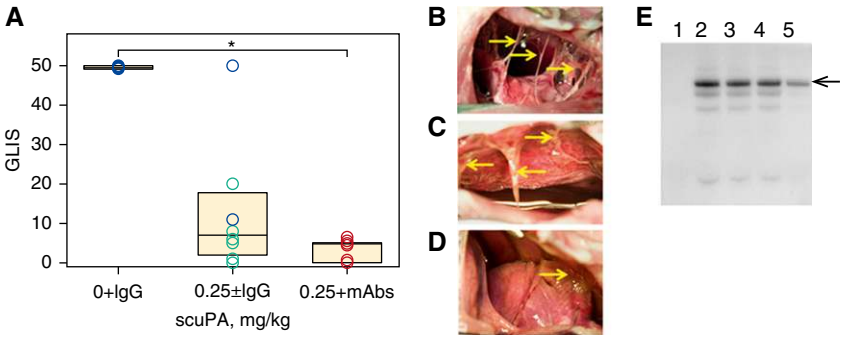

Figure 2. Anti–PAI-1 mAbs (0.5 mg/kg) improve the outcome of intrapleural fibrinolytic therapy (IPFT) with 0.25 mg/kg single-chain uPA (scuPA). Animals were killed 24 hours after administration of IPFT (72 h after initiation of tetracycline [TCN]-induced pleural injury), and the level of injury was assessed and documented as described in the Materials and Methods and previously (18, 24). (*A*) A box plot of the gross loculation injury score (GLIS) (17) 24 hours after injection of (from *left* to *right*): a vehicle with 0.5 mg/kg mouse IgG (n = 6; *dark blue*); scuPA alone (0.25 mg/kg; n = 6; *green*); scuPA (0.25 mg/kg) together with mouse IgG (0.5 mg/kg) (*light blue*; n = 2); and scuPA (0.25 mg/kg) with anti–PAI-1 mAbs (0.5 mg/kg each) (n = 6; *red*). GLIS for the clear and injured pleural space (strands too numerous to count) were 0 and 50, respectively (17, 18). GLIS less than 10 was considered successful IPFT. Data in (*A*) are presented as box plots (showing interquartile ranges). Results for Kruskal-Wallis ANOVA tests on ranks showed a statistically significant difference (P = 0.007) in the median values among the treatment groups (*asterisk*). Photographs of representative intrapleural fibrin deposition and fibrin strand formation (*arrows*) at 24 hours after administering mouse IgG (0.5 mg/kg) alone (*B*), with scuPA (0.25 mg/kg) (*C*), and scuPA (0.25 mg/kg) with anti–PAI-1 mAbs (0.5 mg/kg) (*D*). The values of GLIS are 50, 11, and 5, respectively. (*E*) A Western blot analysis of intrapleural mAbs at 0, 10, 20, 40 minutes, and 24 hours after IPFT (*lanes 1–5*, respectively). The Western blot was probed with anti-mouse polyclonal antibodies conjugated with HRP, as described in Materials and Methods and in the online supplement. The relative mobility corresponding to the full mAb (150 kD) is shown with an *arrow* on the *right*. A representative Western blot depicting anti–PAI-1 mAbs (n = 6 independent experiments for mAbs and IgG).

Results

PAI-1–Neutralizing mAbs Improve the Outcome of scuPA-Based IPFT in TCN-Induced Pleural Injury

Preliminary *in vitro* experiments (*see* Figures E1A and E1B in the online supplement) demonstrated the additivity of the neutralizing effects of MA-33H1F7 and MA-8H9D4 (30) on the reaction between recombinant rabbit PAI-1 and human uPA. Although the affinity of MA-33H1F7 to rabbit PAI-1 was reported to be decreased due to a single amino acid substitution in the epitope (31), results of *ex vivo* experiments (Figure E1C) have shown directly that mAbs added to the PFs of rabbits with TCN-induced pleural injury protect exogenous uPA from inactivation. Half (0.25 mg/kg) of the effective dose of scuPA (24) was initially selected to test whether or not adjunctive PAI-1–neutralizing mAbs (0.5 mg/kg) affect the outcome of IPFT of TCN-induced pleural injury in rabbits. Rabbits treated with intrapleural mouse IgG (0.5 mg/kg) with and without scuPA (0.25 mg/kg) were used as controls. Pleural injury outcomes were assessed at 24 hours after IPFT. GLIS values (Figure 2A) indicate an increase in the efficacy of the IPFT in the presence of MA-33H1F7 and MA-8H9D4. In contrast, intrapleural treatment with mouse isotypic IgG did not improve IPFT outcomes and/or GLIS versus vehicle alone (18) and 0.25 mg/kg scuPA (Figures 2A–2C). Chest ultrasonography before killing of the animals (data not shown) supported the visual assessment of the pleural injury at 24 hours after IPFT (Figures 2B–2D). mAbs and IgG were detected in PFs throughout the experimental time course (Figure 2E), and there was no increase in bleeding complications in any of the animals that received IPFT consisting of scuPA with mAbs (Figure E2). Therefore, intrapleural neutralization of PAI-1 improved the therapeutic outcome (Figure 2A), but did not affect local hemostasis, and was otherwise well tolerated.

Anti–PAI-1 mAbs Protect Intrapleural uPA and PA Activity and Promote the Formation of Endogenous αM/uPA Complexes

To test the effects of anti–PAI-1 mAbs on the processing of intrapleural scuPA, samples of PF withdrawn during IPFT (0–80 min and 24 h) were analyzed as previously described (18). Intrapleural levels of free uPA (free two chain [tc] uPA; Figure 3A), αM/uPA complexes (Figure 3C), and PA activity (Figure E3A) were determined. The observed first-order rate constant (k_{obs}) for the second, slow phase of intrapleural free uPA inactivation in the presence of mAbs (k_{obs} = 0.008 ± 0.002 min^{-1}; Figure 3A) was twofold lower than that for IPFT with scuPA alone (Figure 3B) (18), and was similar to k_{obs} for the inactivation of the PA activity (0.007 ± 0.002 min^{-1}; Figure E3A). Levels of intrapleural free uPA and PA activities in animals receiving 0.25 mg/kg scuPA and anti–PAI-1 mAbs (Figures 3A and E3A, respectively) were higher than those observed with the same dose (Figure 3B) or the effective dose (0.5 mg/kg) (Figure E3B) (18) of scuPA alone. The intrapleural concentration of αM/uPA in the presence of mAbs (Figure 3C) was found to be

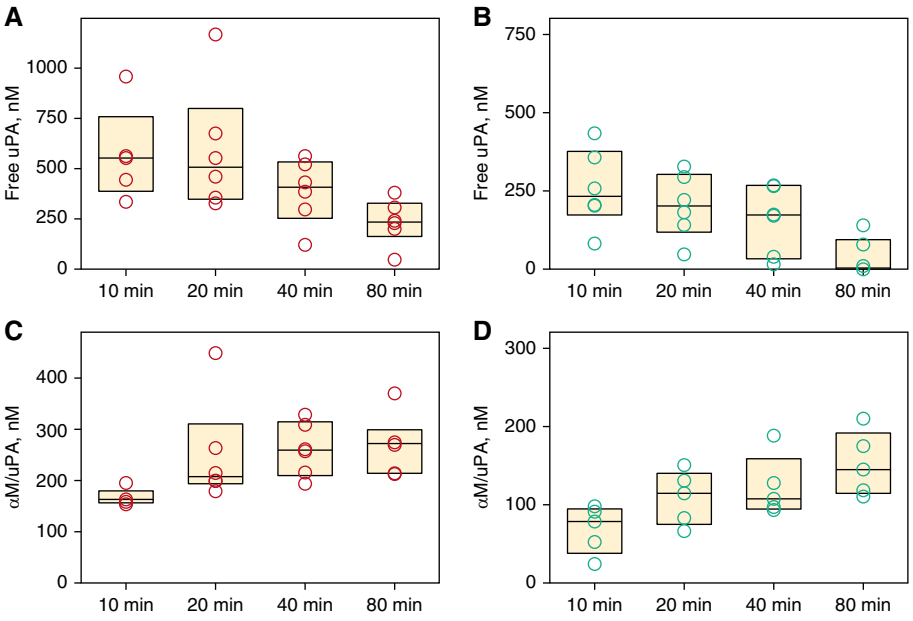

Figure 3. Anti–PAI-1 mAbs protect free intrapleural uPA activity (*A*), and potentiate fast formation of the α-macroglobulin (αM)/uPA complexes (*C*). Aliquots of pleural fluid (PF) were withdrawn during IPFT, snap frozen, and analyzed, as described previously (18). Changes in the level of the free intrapleural uPA activity with time during IPFT with 0.25 mg/kg scuPA in the presence of anti–PAI-1 mAbs (*A*), or alone (*B*). The intrapleural αM/uPA during IPFT with 0.25 mg/kg scuPA increased and approached the maximal level faster with anti–PAI-1 mAbs (*C*) than those with scuPA alone (*D*). The maximal level of intrapleural αM/uPA with anti–PAI-1 mAbs approximated that observed previously with the effective dose of scuPA alone (0.5 mg/kg; 260 ± 70 nM) (18).

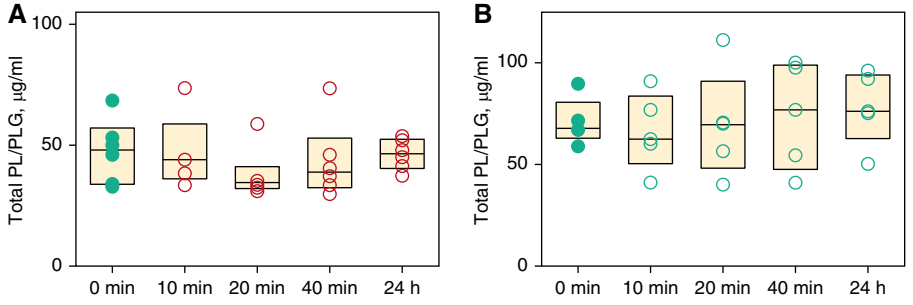

Figure 4. Time changes in total plasmin/plasminogen antigen in the PFs of rabbits during IPFT. Levels of total rabbit plasmin/plasminogen (PL/PLG) antigen were measured in samples of PFs using ELISA, as described in Materials and Methods. Concentration of PL/PLG in PF withdrawn before (zero point, *filled symbols*) and at 10, 20, 40, minutes, and 24 hours after IPFT (*A*) with 0.25 mg/kg scuPA plus anti–PAI-1 mAbs (*red*; *n* = 6), and (*B*) 0.25 mg/kg scuPA alone (*green*; *n* = 5).

similar to that observed previously for the effective dose of scuPA alone (260 ± 70 nM [18]), but higher than that observed for 0.25 mg/kg scuPA alone (Figure 3D). Moreover, the maximal level of αM/uPA in the presence of mAbs occurred 1.2–1.5 times faster (k_{obs} = 0.011 ± 0.003 min^{-1}) than that with the same dose of scuPA alone (Figure 3D) (18). To evaluate the effect of IPFT on intrapleural fibrinolysis, the level of plasmin/plasminogen antigen, including plasmin complexed with the serpins, α_2-antiplasmin and PAI-1, was determined in PF samples (Figure 4). Although there was variation in the PF plasmin/plasminogen antigen before the IPFT (Figures 4A and 4B; 0 min) and small changes during IPFT, there was no significant difference between 0.25 mg/kg scuPA plus mAbs (Figure 4A) and scuPA alone (Figure 4B) at 10 minutes to 24 hours. Similar levels of plasmin/plasminogen were observed in samples (n = 3) from the mouse IgG plus vehicle control group (data not shown). The lack of an increase in the level of total plasmin/plasminogen in the presence of anti–PAI-1 mAbs supports the conclusion that PAI-1 targeting IPFT does not induce extravascular leakage.

Anti–PAI-1 mAbs Notably Improve the Outcome of IPFT with 0.0625 mg/kg scuPA

Because anti–PAI-1 mAbs (Figure 1) increased the efficacy of 0.25 mg/kg scuPA (Figure 2A), protected uPA from inactivation (Figure 3A), potentiated intrapleural formation of αM/uPA (Figure 3C), and caused no bleeding complications (Figure 4, Figure E2), the dose of scuPA was further decreased fourfold. The dose of 0.0625 mg/kg represents a consistently and completely ineffective scuPA dose based on previous IPFT dose–response characterization (18), and is eightfold less than the established minimal effective dose of scuPA (0.5 mg/kg [24]). Rabbits with TCN-induced pleural injury were treated with intrapleural injections of scuPA (0.0625 mg/kg) and mAbs (0.5 mg/kg) (n = 5). Samples of PF were collected, and the efficacy of IPFT was evaluated by GLIS at 24 hours after treatment (Figure 5A). Controls included scuPA (0.0625 mg/kg) with mouse IgG (0.5 mg/kg; n = 6). The severity of the pleural injury and formation of fibrin strands was verified before IPFT using chest ultrasonography (Figures 5B and 5C,

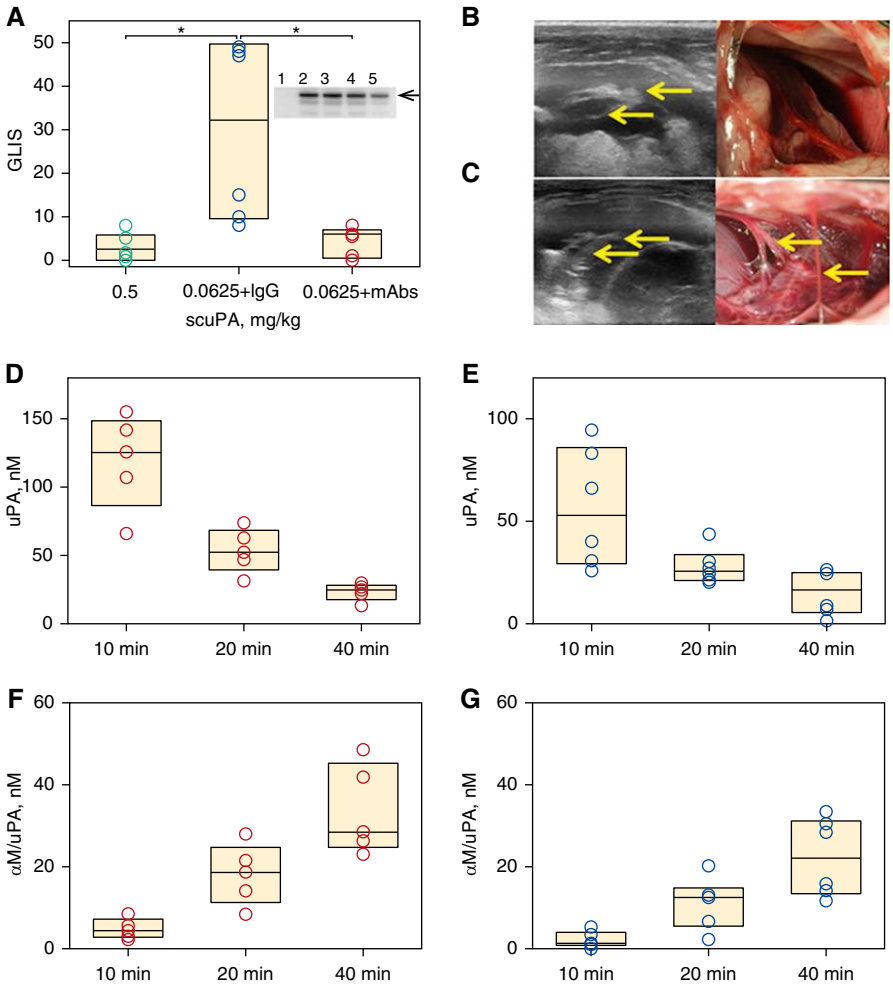

Figure 5. Anti–PAI-1 mAbs (0.5 mg/kg) significantly improve the outcome of IPFT with one-eighth of the effective dose of scuPA. Animals were killed 24 hours after initiating IPFT (72 h after TCN-induced pleural injury), and the level of injury was assessed as described in the Materials and Methods as well as previously (18, 24). (*A*) Box plots of the GLIS (17, 18) 24 hours after injection of (from *left* to *right*): a positive control (18) 0.5 mg/kg scuPA (n = 6); 0.0625 mg/kg scuPA with 0.5 mg/kg mouse IgG (n = 6); and 0.0625 mg/kg scuPA with 0.5 mg/kg anti–PAI-1 mAbs (n = 5). GLIS was calculated as described in Figure 2A. Data in (*A*) are presented as box plots (showing interquartile ranges); results for Kruskal-Wallis ANOVA tests on ranks presented a statistically significant difference (P = 0.006) in the median values among the treatment groups (*asterisks*). *Inset:* intrapleural mAbs at 0, 10, 20, 40 minutes and 24 hours after IPFT; *lanes 1–5*, respectively, were visualized using Western blot analysis as described in Figure 2E. The *arrow* indicates relative mobility for 150-kD proteins. (*B*) The accumulation of PFs and adhesion formation (*arrows*) was evaluated by ultrasonography (18) just before treatment (48 h after TCN injury induction; *left*). A gross image of representative successful IPFT with pleural space clearance (GLIS = 0) 24 hours after injection of scuPA (0.0625 mg/kg) with mAbs (0.5 mg/kg) (*right*). (*C*) PF accumulation and adhesion formation evaluated by ultrasonography (18) just before treatment (48 h after TCN injury induction; *left*). A gross image of unsuccessful IPFT (GLIS = 50) 24 hours after injection of scuPA (0.0625 mg/kg) with mouse IgG (0.5 mg/kg) (*right*). The intrapleural fibrin strands are indicated by *arrows*. Changes in the level of the free intrapleural uPA activity, with time, during IPFT with 0.0625 mg/kg scuPA in the presence of anti–PAI-1 mAbs (*D*), or IgG (*E*). An increase in the intrapleural concentration of αM/uPA during IPFT with 0.0625 mg/kg scuPA with anti–PAI-1 mAbs (*F*), and with mouse IgG (*G*). Concentrations of free uPA and αM/uPA complexes were significantly (P < 0.05) higher in the presence of anti–PAI-1 mAbs at 10 and 20 minutes, and 10 minutes after IPFT, respectively.

left panels, *yellow arrows*). The results of IPFT shown in Figure 5A demonstrate that PAI-1–neutralizing mAbs improve the efficacy of IPFT (GLIS $<$ 10; $P < 0.05$) with 0.0625 mg/kg scuPA, as compared with the same dose of scuPA with mouse IgG. Postmortem gross examination confirmed the efficacy of PAI-1–targeted IPFT (Figures 5B and 5C, *right panels*). Anti–PAI-1 mAbs do not induce an increase in red blood cells (Figure E4A), nor do they affect the level of plasmin/plasminogen antigen in PFs (Figures E4B and E4C). Thus, intrapleural neutralization of PAI-1 possesses minimal risk for local bleeding. Similar to 0.25 mg/kg scuPA (Figure 3), intrapleural free uPA, αM/uPA (Figures 5D and 5F), and PA activity (Figure E5A) in the presence of anti–PAI-1 mAbs were higher than those with mouse IgG (Figures 5E and 5G and Figure E5B, respectively) for the first 10–20 minutes after IPFT ($P < 0.05$; there was no statistical difference at 40 min). Nevertheless, these values (Figures 5D–5G and E5) were significantly ($P < 0.05$) lower than those observed during IPFT with 0.25 mg/kg scuPA with or without anti–PAI-1 mAbs or the known effective dose (0.5 mg/kg) (Figures 3 andE3) (18). Thus, anti–PAI-1 mAbs that remain in the pleural space up to 24 hours after IPFT (Figure 5A, *inset*) substantially protect low-grade uPA activity derived from 0.0625 mg/kg scuPA from inactivation by endogenous PAI-1 (Figure 1).

Although anti–PAI-1 mAbs potentiate fast formation of αM/uPA complexes, the level of total αM in PFs before the treatment (Figure E6, 0 min) did not significantly differ from those of both successful IPFT (GLIS $<$ 10; with mAbs; Figure E6A) and unsuccessful IPFT (GLIS $>$ 10 with IgG; Figure E6B). Therefore, only a fraction of intrapleural αM is active (Figures 3C, 3D, 5E, and 5G), and the formation as well as degradation of αM/uPA complexes did not affect the overall level of αM.

Effect of Anti–PAI-1 mAbs and the Dosage of scuPA on Intrapleural Fibrinolytic Activity at 0–80 min after Administration of IPFT

To determine whether PAI-1–targeted IPFT with 0.0625 mg/kg scuPA affects intrapleural fibrinolytic activity, PFs were withdrawn at 0–40 minutes and at 24 hours after IPFT and tested using an FITC–fibrin plate assay as previously described (29). Notably, there was no significant ($P > 0.05$) difference in the fibrinolytic activity (Figure 6A) at any time point or for AUCs between successful (GLIS $<$ 10; mAbs) and unsuccessful (GLIS $>$ 10; IgG) IPFT. Although the baseline (before IPFT) fibrinolytic activity was suppressed (close or equal to the limit of detection by FITC–fibrin assay), supplementation with

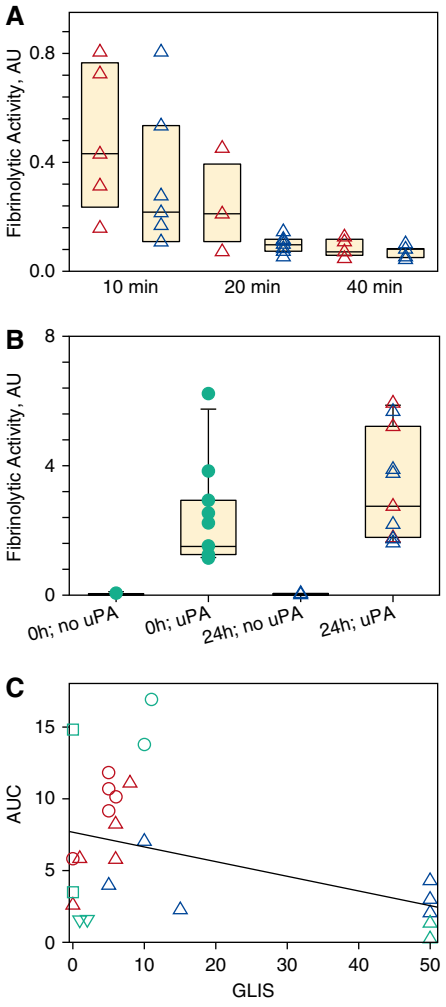

Figure 6. Changes in fibrinolytic activity in PFs during IPFT. (*A*) Fibrinolytic activity in PFs withdrawn at 10, 20, and 40 minutes after administration of 0.0625 mg/kg scuPA either with anti–PAI-1 mAbs (0.5 mg/kg; *red triangles*; $n = 5$) or mouse IgG (0.5 mg/kg; *blue triangles*; $n = 6$). Aliquots of PF withdrawn 10–40 minutes after intrapleural administration of the fibrinolysin were analyzed with FITC–fibrin film assay as described previously (29). (*B*) Aliquots of PF withdrawn before (zero point; 0 h) and at 24 hours after injection of the fibrinolysin were analyzed via FITC–fibrin film assay (29) with (uPA) or without (no uPA) addition of exogenous two-chain uPA (tcuPA; 5 nM). Intrapleural fibrinolytic activity is suppressed before and at 24 hours after IPFT (*bars*, 0 h; no uPA [$n = 10$]; and 24 h; no uPA [$n = 11$]). Activation of endogenous plasminogen with exogenous tcuPA results in a significant increase in the fibrinolytic activity in PFs withdrawn before (*box*, 0 h; uPA; $n = 11$) and 24 hours after (*box*, 24 h; uPA; $n = 11$) initiating IPFT. Shown at 24 hours are the values of fibrinolytic activity for animals treated with scuPA 0.0625 mg/kg with mAbs (*red triangles*; $n = 5$); scuPA 0.0625 mg/kg with IgG (*blue triangles*; $n = 6$). (*C*) Lack of correlation between the area under the curve (AUC) and outcome of IPFT (GLIS) for different treatments with scuPA. Values of AUC for the results of the experiments shown in Figures 6A and E7B were calculated using SigmaPlot 12.0 and plotted against corresponding GLIS. The *solid line* represents the best linear fit to the data (r^2=0.19). Although there was a significant difference between AUC for IPFT with mAbs and scuPA alone or scuPA with mouse IgG ($P < 0.05$), there was no difference between AUC for successful and unsuccessful IPFT (GLIS $<$ 10 and GLIS $>$ 10, respectively).

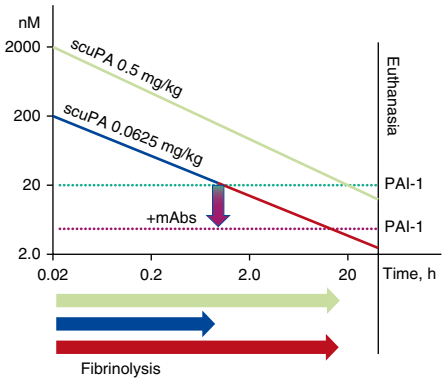

Figure 7. mAb-mediated inactivation of PAI-1 increases the half-life of low-level, durable intrapleural PA activity and results in successful fibrinolysis. The *blue line* represents PAI-1–independent inactivation of an ineffective dose (0.0625 mg/kg) of scuPA, which results in inhibition of intrapleural plasminogen activating (PA) activity (intercepting the *green dotted line* representing the upper level of the endogenous active PAI-1 [17, 18]). In the presence of PAI-1–neutralizing mAbs (Figure 1), the effective concentration of active PAI-1 decreases (*pink dotted line*). As a result, inactivation of intrapleural uPA during IPFT with 0.0625 mg/kg scuPA (interception of *red* and *pink dotted lines*) occurs later. Increasing the intrapleural scuPA dose to 0.5 mg/kg (*green line*) also increases the lifetime of intrapleural PA activity (18) and duration of fibrinolysis. The *green*, *blue*, and *red arrows* below the *time axis* represent time of fibrinolysis for treatments with 0.5 and 0.0625 mg/kg of scuPA alone and 0.0625 mg/kg scuPA with anti–PAI-1 mAbs, respectively. Fibrinolysis (*green* and *red arrows* below the *time axis*) maintained for a prolonged period of time results in successful IPFT (GLIS $<$ 10). Death at 24 hours after injection of the fibrinolysin is shown as a *vertical line*.

uPA induced an almost 100-fold increase, due to activation of accumulated endogenous plasminogen (Figure 6B, 0 h and 0 h; uPA). Remarkably, similar accumulation of plasminogen at 24 hours after IPFT was observed for every treatment used (Figures 6B and E7A, 24 h and 24 h; uPA). Thus, at some time between 2 and 24 hours after IPFT, intrapleural PA activity was neutralized, fibrinolysis was suppressed, and *de novo* synthesized plasminogen accumulated. Moreover, the intrapleural fibrinolytic activity in PFs of animals treated with 0.25 mg/kg scuPA and mAbs was similar to that observed for IPFT with 0.0625–0.5 mg/kg of scuPA alone (Figure E7B). Therefore, similar fibrinolytic activities observed at 0–80 minutes could result in both effective and ineffective IPFT. To determine whether or not the level of intrapleural fibrinolytic activity in the first 40–80 minutes affects the outcome of IPFT, the AUCs for the treatments, shown in Figure 6A and Figure E7B, were plotted against corresponding GLIS values (Figure 6C). There was no statistical difference between the AUC for successful (GLIS $<$ 10) and that for unsuccessful (GLIS $>$ 10) treatments (data not shown), and a poor linear correlation (r^2 = 0.19) between AUCs and GLIS. Therefore, although PF fibrinolytic activity in the first 20 minutes of IPFT contributes to intrapleural fibrinolysis, it does not correlate with the outcome of IPFT in TCN-induced pleural injury.

Discussion

The focus of this study was to evaluate the effect of neutralization of endogenous PAI-1 on the outcome of scuPA-based IPFT in TCN-induced pleural injury in rabbits. This model of pleural injury has been used for over two decades (24), and recapitulates the key pathologic and temporal features of human disease, including relatively high levels of active PAI-1 (17, 18). A profibrogenic state (high levels of active PAI-1 and α_2-antiplasmin together with accumulating endogenous plasminogen) before IPFT suppresses intrapleural fibrinolysis (Figures 6B and E7A) and promotes massive uncontrolled fibrin deposition (Figures 2A and 2B; GLIS = 50). MA-33H1F7 and MA-8H9D4, unlike MA-33B8 (32), do not inactivate PAI-1 without proteinase. Rather, they decrease the effective concentration of active PAI-1 by redirecting the PAI-1 mechanism to the substrate branch (Figure 1), increasing a lifetime of the intrapleural PA activity (Figure 7). Anti–PAI-1 mAbs not only protect uPA from immediate inactivation by intrapleural active PAI-1 and increase early increments in PF PA activity, but also potentiate the neutralization of newly supplied PAI-1 after IPFT (Figure 7). Although mAbs were present in PFs up to 24 hours after IPFT (Figures 2E and 5A, *inset*), intrapleural PA and fibrinolytic activities were inhibited at 24 hours (Figure 6B, Figure E7A) by endogenous PAI-1 (Figure 7, *pink dotted line*). Therefore, although mAb-assisted neutralization of PAI-1 combined with scuPA enhances the efficacy of IPFT and permits an eightfold reduction in the dose of the fibrinolysin (Figures 5 and 7), PA activity becomes inhibited at 2–24 hours, most likely due to the critical contribution of PAI-1–independent inactivation (18) into intrapleural scuPA processing. Because mAbs did not induce notable bleeding complications (either local or systemic) with any of the scuPA doses tested (0.25 and 0.0625 mg/kg), local neutralization of PAI-1 in scuPA-based IPFT was well tolerated after intrapleural administration in rabbits.

Fibrinolytic activity (and successful IPFT) in TCN-induced pleural injury strictly depends on the balance of endogenous plasmin and its inhibitors/regulators. Changes in the fibrinolytic activity over time at different doses of scuPA, with or without mAbs or IgG, were similar (Figures 6A and E7B). PAI-1 neutralization and the accelerated formation of αM/uPA could protect a fraction of the endogenous plasmin from slow inactivation by PAI-1 (33) and αM (34). Thus, inactivation of PAI-1 could induce observed elevation of fibrinolytic activity with mAbs at 10 and 20 minutes (Figures 6A and E7B), which contributes to the salutary effects of the PAI-1–targeting intervention. The lack of correlation between AUC and GLIS (Figure 6C) strongly suggests the likelihood that late (at 2–24 h), relatively low-grade, but durable fibrinolysis contributes to successful IPFT. We infer (Figure 7) that, as long as the overall intrapleural PA activity is positive, the activation of endogenous plasminogen generates low-grade (Figures 6A and E7B) fibrinolytic activity, which contributes to successful clearance of intrapleural adhesions (Figures 2 and 5). The half-life of PA activity can be increased as a result of either: increasing the dose of scuPA (Figure 7, *green line*) (18, 24); or neutralizing the endogenous PAI-1 (Figure 7, *pink dotted line*) or both. Thus, in the presence of mAbs that redirect the PAI-1 reaction to the substrate branch (Figure 1), positive intrapleural PA activity is durably maintained due to a decrease in the level of endogenous PAI-1 activity (Figure 7). As a result, in the presence of mAbs, even an ineffective dose of scuPA (0.0625 mg/kg) maintains fibrinolytic activity long enough to effectively degrade fibrinous adhesions in TCN-induced injury (Figure 5A). The proposed mechanism (Figure 7) explains intrapleural fibrinolysis

during IPFT of TCN-induced pleural injury in rabbits. However, it does not predict the exact time of fibrinolysis needed for successful IPFT, as well as whether or not this time correlates with the fibrinolytic activity at 0–80 minutes (Figures 6A, 6C, and E7B) or with "fibrinolytic potential" (baseline fibrinolytic activity after activation of endogenous plasminogen; Figures 6B and E6A; 0 h uPA).

Although the mechanism of intrapleural tPA processing could deviate from that of scuPA (18), the similarity in the effects of MA-33H1F7 and MA-8H9D4 on the "suicide" inhibition of tPA and uPA by PAI-1 (20–22) support testing a similar approach to enhance tPA-based IPFT. The availability of a number of anti–PAI-1 mAbs, antigen-binding fragments, and single-chain variable fragments, with stoichiometries of inhibition ranging from 1.5–2.0 to greater than 100 allows interventional flexibility, which could expedite future clinical trials. Moreover, mAb-mediated modulation of the PAI-1 reaction (Figure 1) is effective against stable ternary "molecular-sandwich"–type complexes (35), which is another potential advantage of this approach.

Here, for the first time, we show that neutralization of endogenous PAI-1 is well tolerated in TCN-induced pleural injury in rabbits, and results in an eightfold decrease in the effective dose of scuPA-based IPFT due to an increase in the half-life of intrapleural PA activity. These results concur with both the mechanism of intrapleural processing of scuPA (18), and the emerging role of endogenous active PAI-1 as a biomarker and a molecular target for IPFT in pleural injury (17, 18). Therefore, PAI-1 targeting represents a promising approach to the development of reliable, safe, and efficacious IPFT amenable to testing in clinical trials. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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