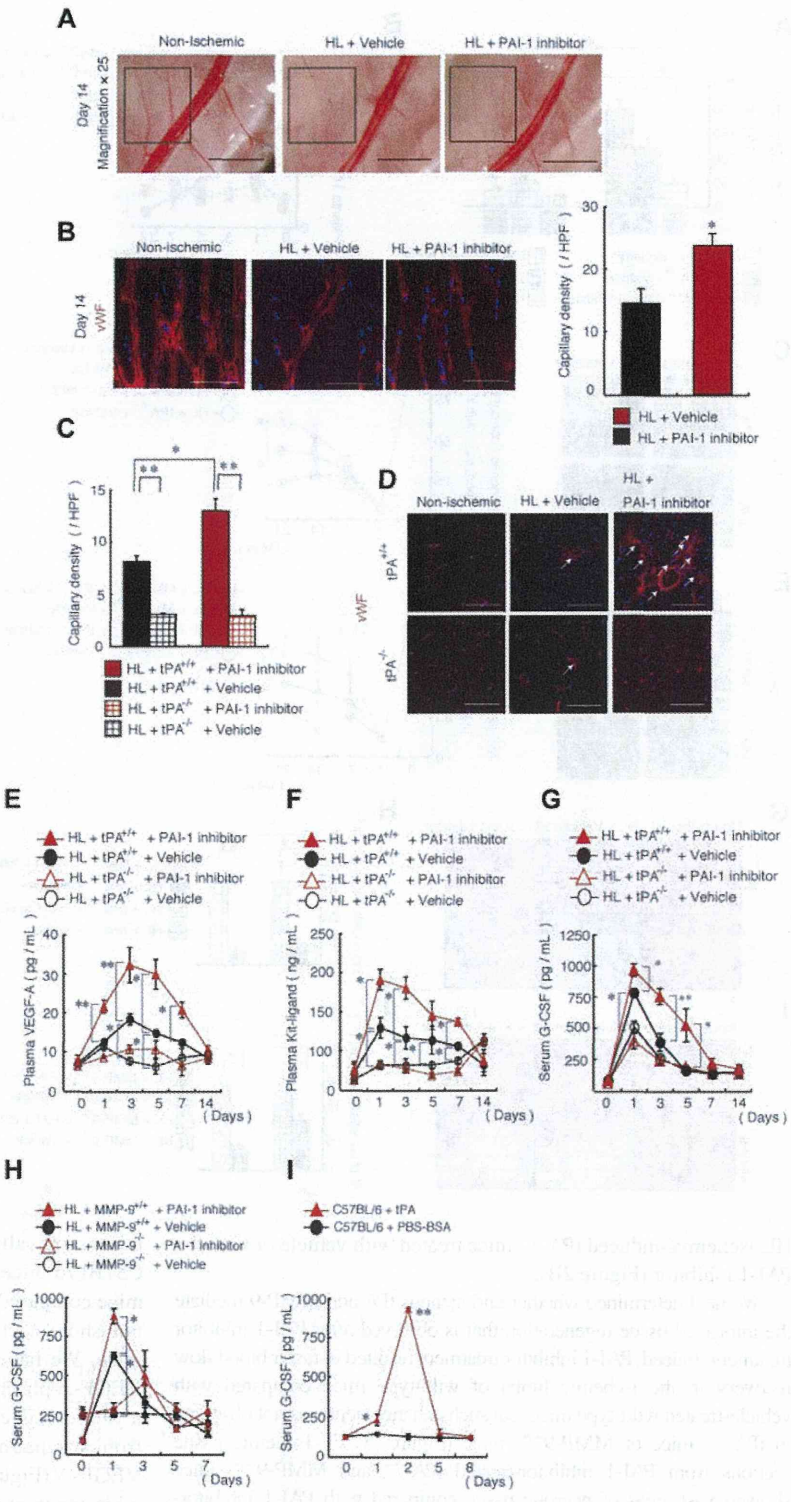

Figure 3. In vivo blockade of PAI-1 augments neoangiogenesis and growth factor release. (A) Macroscopic images of the lower limb region of nonischemic and PAI-1 inhibitor- or vehicle-treated wild-type mice were captured on day 14 after HL ischemia induction (magnification, 25×; scale bars, 2000 mm). The insert box depicts areas of neoangiogenesis. (B-G) HL ischemia was induced in C57BL/6, $tPA^{+/+}$, and $tPA^{-/-}$ mice, and the mice were then treated with or without PAI-1 inhibitor daily from days 0-6. (B-C) Capillary density was measured in sections of the hamstring (B) and adductor muscles (C) based on immunohistochemical staining of VWF per high power field (HPF). (B,D) Representative images of anti-VWF mAb immunohistochemical staining of ischemic muscle sections from HL-ischemia–induced C57BL/6, $tPA^{+/+}$, and $tPA^{-/-}$ mice either left untreated or treated with or without the PAI-1 inhibitor (n = 6/group) analyzed on day 14 after the procedure (scale bars, 200 mm). Arrows depict VWF^+ capillaries. (E-G) Plasma levels of VEGF-A (E) and KitL (F) and serum levels of G-CSF (G) in HL-ischemia–induced $tPA^{+/+}$ and $tPA^{-/-}$ mice treated with or without PAI-1 inhibitor were determined by ELISA (for VEGF-A, n = 9 for $tPA^{+/+}$ mice and n = 3 for $tPA^{-/-}$ mice; for KitL and G-CSF, n = 7 for $tPA^{+/+}$ mice and n = 3 for $tPA^{-/-}$ mice; for KitL, n = 3 for G-CSF). (H) G-CSF serum levels were analyzed by ELISA in HL-ischemia–induced $MMP\text{-}9^{+/+}$ and $MMP\text{-}9^{-/-}$ mice treated with or without PAI-1 inhibitor (H) and in C57BL/6 mice treated with a serpin-resistant tPA mutant (n = 4-5/group). Values represent the means ± SEM. $^*P < .05$; $^{**}P < .001$.

PAI-1 inhibition mobilizes neutrophils into the circulation and promotes neutrophil recruitment into ischemic tissues in vivo

The PAI-1 inhibitor-mediated increase in hematopoietic cytokines prompted us to examine whether the inflammatory response during ischemic recovery might be altered after PAI-1 inhibition. Isolation of leukocytes from ischemic muscle tissues, followed by MACS separation using the anti–Gr-1 Ab, revealed that approximately 40% of infiltrating leukocytes were neutrophils on day 5 of HL ischemia (Figure 4A). PAI-1 inhibitor treatment increased the number of $Gr\text{-}1^+$ neutrophils in ischemic sections of $tPA^{+/+}$ mice, but not in $tPA^{-/-}$ mice, compared with vehicle-treated mice (Figure 4B-C).

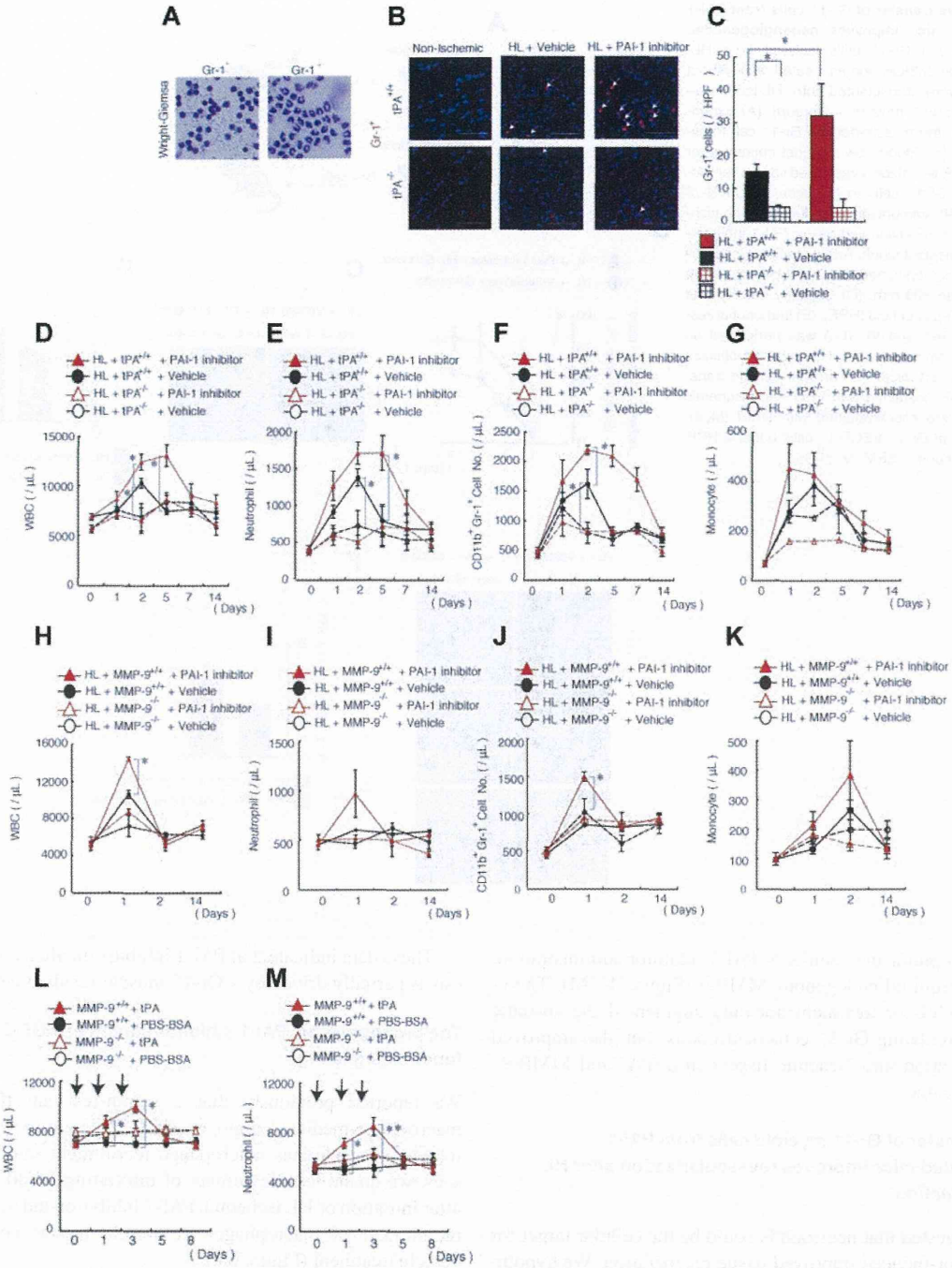

Figure 4. Pharmacologic PAI inhibition mobilizes neutrophils into the circulation and improves their tissue infiltration, a process dependent on endogenous tPA and MMP-9. (A) Wright-Giemsa staining of MACS-isolated infiltrating Gr-1$^+$ and Gr-1$^-$ cells derived from ischemic tissues of C57BL/6 mice on day 5 after HL induction. (B) Immunofluorescent staining of Gr-1 was performed on nonischemic muscle tissues or on HL-ischemic muscle tissues derived from vehicle- or PAI-1 inhibitor-treated HL-ischemic tPA$^{+/+}$ and tPA$^{-/-}$ mice 14 days after the HL procedure. PAI-1 inhibitor was administered daily on days 0-6 after the procedure. The arrows indicate Gr-1$^+$ cells (scale bars, 200 mm). Nuclei were counterstained with DAPI (blue). (C) Quantification of Gr-1$^+$ cells in ischemic muscle tissues (n = 3/group). (D-K) The total number of WBCs (D,H) and the number of neutrophils (E,I), CD11b$^+$Gr-1$^+$ cells (F,J), and monocytes (G,K) were determined in the peripheral blood of PAI-1 inhibitor-treated or vehicle-treated tPA$^{+/+}$ and tPA$^{-/-}$ mice (for B-G, n = 4) and in MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice (for H-K, n = 6) by counting (D,E,G,H,I,K) or by FACS analysis (F,J). (L-M) The total number of WBCs (L) and neutrophils (M) were counted in in MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice (n = 4). *$P < .05$; **$P < .001$ for recombinant tPA-treated versus vehicle-treated C57BL/6 mice. Values represent the means ± SEM. Data are expressed as the absolute number of each cell type per milliliter of blood. *$P < .05$.

We next analyzed blood samples to determine whether the augmented neutrophil influx in ischemic tissues was due to an overall increase in circulating blood cells. PAI-1 inhibitor-treated HL-ischemic tPA$^{+/+}$ mice, but not tPA$^{-/-}$ mice, showed an increase in the number of WBCs, including neutrophils, as determined by cell counting and FACS analysis using Abs against CD11b and Gr-1 (Figure 4D-F), but not monocytes, compared with vehicle-treated animals (Figure 4G). MMP-9 deficiency prevented the leukocyte and neutrophil increase, but not the monocyte increase, caused by PAI-1 inhibitor treatment (Figure 4H-K), indicating that PAI-1 inhibitor-mediated neutrophilia was dependent on endogenous MMP-9. Administration of recombinant tPA

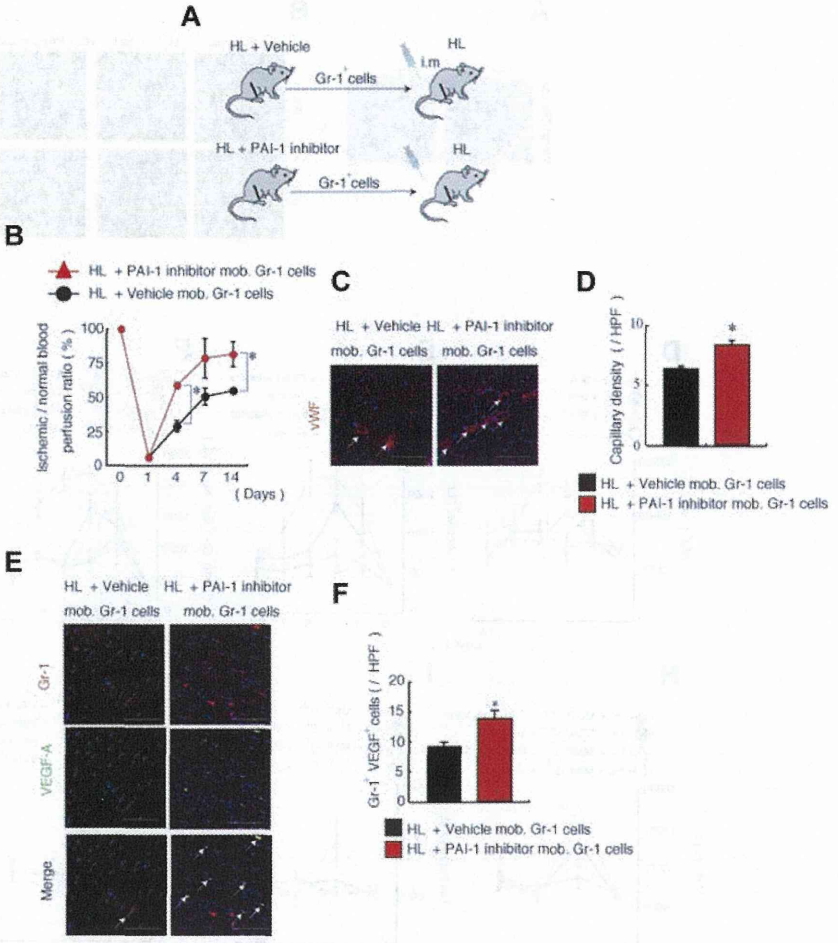

Figure 5. Adoptive transfer of Gr-1$^+$ cells from PAI-1 inhibitor-treated mice improves neoangiogenesis. (A-F) Muscle-derived Gr-1$^+$ cells isolated from HL-ischemia–induced-C57BL/6 donors treated with/without PAI-1 inhibitor were transplanted into HL-ischemia–induced recipients for 3 days (n = 6/group). (A) Experimental scheme of the muscle-derived Gr-1$^+$ cell transplantation assay. (B) Blood flow was determined after transplantation of PAI-1 inhibitor-mobilized versus vehicle-mobilized (mob.) Gr-1$^+$ cells in HL-ischemic C57BL/6 recipients. (C) VWF immunostaining of lower limb ischemic tissue of mice receiving vehicle- or PAI-1 inhibitor-mobilized cell transplantations. Arrows indicate capillaries. Nuclei were counterstained with DAPI (blue staining). Scale bars indicate 200 mm. (D) Capillary density was evaluated per high-power field (HPF). (E) Immunofluorescent staining of Gr-1 and VEGF-A was performed on sections derived from vehicle or PAI-1 inhibitor-mobilized Gr-1 cell-transplanted mice. The arrows indicate transplanted Gr-1$^+$ cells costained with VEGF-A in ischemic tissues. Nuclei were counterstained with DAPI (blue). (F) Quantification of Gr-1$^+$ VEGF-1$^+$ cells under a HPF. Data represent means ± SEM. *$P < .05$.

induced neutrophilia and, similar to PAI-1 inhibitor administration, this process required endogenous MMP-9 (Figure 4L-M). Therefore, PAI-1 inhibitor treatment not only augmented the absolute number of circulating Gr-1$^+$ cells/neutrophils, but also improved their incorporation into ischemic tissues in a tPA- and MMP-9–dependent manner.

Adoptive transfer of Gr-1$^+$ myeloid cells from PAI-1 inhibitor-treated mice improves revascularization after HL ischemia induction

Our data suggested that neutrophils could be the cellular target for PAI-1 inhibitor-induced improved tissue regeneration. We hypothesized that PAI-1 induction during HL ischemia may alter the ability of neutrophils to stimulate angiogenesis. To test this hypothesis, muscle-derived Gr-1$^+$ cells were obtained from HL-ischemia–induced donor mice that had been treated with or without the PAI-1 inhibitor. These Gr-1$^+$ cells were transplanted into HL recipient mice (Figure 5A) by intramuscular injection. In contrast to cells from vehicle-treated mice, Gr-1$^+$ cells isolated from muscle tissues of PAI-1 inhibitor-treated mice accelerated ischemic reperfusion (Figure 5B) and increased capillary density in ischemic tissues of HL-ischemic recipients (Figure 5C-D). We showed that neutrophils release the proangiogenic factor VEGF-A.[21] Consistent with that result, the absolute number of Gr-1$^+$VEGF-1$^+$ cells was higher in ischemic recipient tissues transplanted with Gr-1$^+$ cells from PAI-1–treated mice (Figure 5E-F).

These data indicate that PAI-1 inhibitor-mediated neoangiogenesis is partially driven by a Gr-1$^+$ muscle-residing cell population.

The proangiogenic PAI-1 inhibitor enhances FGF-2 and VEGF-A function/signaling

We reported previously that a serpin-resistant tPA promoted macrophage-mediated angiogenesis.[22] To determine whether PAI-1 inhibition accelerates macrophage recruitment into ischemic tissues, we quantified the number of infiltrating F4/80$^+$ cells 3 days after initiation of HL ischemia. PAI-1 inhibition did not increase the recruitment of macrophages in muscle tissues compared with vehicle treatment (Figure 6A).

To identify the molecular mechanisms underlying the enhanced angiogenesis observed after PAI-1 inhibition, we examined the expression of angiogenesis-related factors in ischemic muscle tissues derived from PAI-1 inhibitor- and vehicle-treated animals. FGF-2 signaling has been associated with neutrophil-mediated angiogenesis[23] and PAI-1 activity.[22] Immunohistochemical analysis of ischemic muscle tissues demonstrated that the number of F4/80$^+$ cells coexpressing FGF-2 or VEGF-A was not significantly different from vehicle- and PAI-1 inhibitor-treated tissues (Figure 6B). In contrast, the number of ischemic tissue-resident Gr-1$^+$ cells coexpressing both FGF-2 and VEGF-A was higher in sections derived from PAI-1 inhibitor-treated mice (Figure 6C).

FGF-2 can signal through syndecan-4 independently of FGF receptors.[22] Therefore, in the present study, we investigated whether

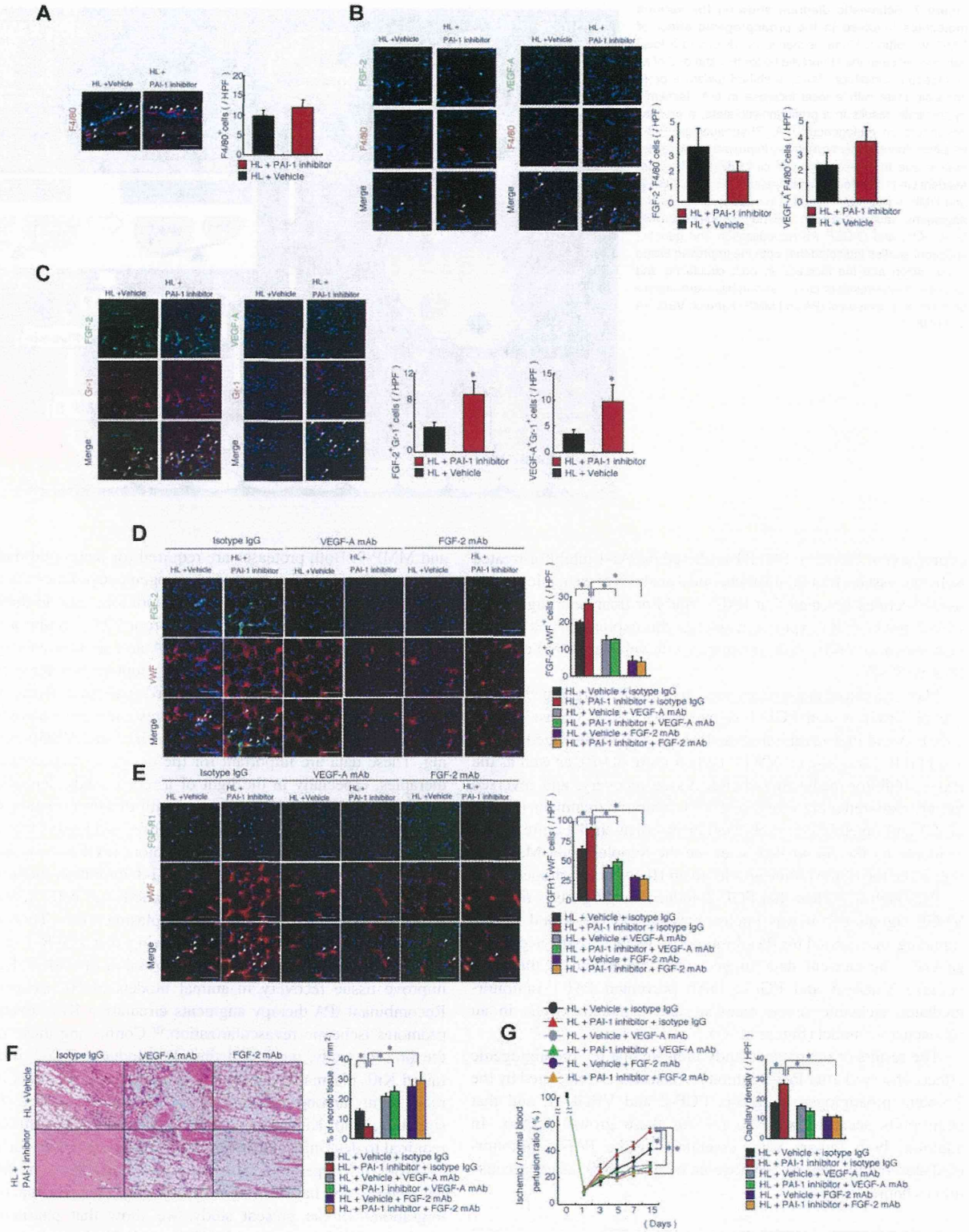

Figure 6. PAI-inhibition induces angiogenesis during HL-ischemic recovery via FGF-2- and VEGF-A–mediated pathways. (A-C) HL-ischemia–induced C57BL/6 mice were treated with the PAI-1 inhibitor or vehicle. Ischemic sections of PAI-1 inhibitor or vehicle-treated mice 3 days after the HL procedure were costained for F4/80 (A), F4/80 and VEGF-A or F4/80 and FGF-2 (B), or Gr-1 and VEGF-A or Gr-1 and FGF-2 (C). Nuclei were counterstained with DAPI (blue). Left panels are representative immunofluorescent images. Arrows indicate VEGF-A$^+$, FGF-2$^+$, F4/80$^+$, or Gr-1$^+$ cells. Right panel shows the quantification of the indicated cell populations per high-power field (HPF; n = 5/group for each experiment). (D-G) HL-ischemia–induced C57BL/6 mice were treated with the PAI-1 inhibitor and coinjected with neutralizing doses of anti–FGF-2, anti–VEGF-A, or anti-IgG control Abs (n = 4/group). (D-E) Ischemic muscle tissues from Ab-treated animals 14 days after the HL procedure were immunofluorescently costained for FGF-2/VWF and FGF-R1/VWF. Nuclei were counterstained with DAPI (blue staining). Arrows indicate FGF-2$^+$/VWF$^+$ and FGF-R1$^+$/VWF$^+$ cells (scale bars, 200 mm). Right panel shows the indicated cell populations quantified per HPF. (F) Left panel, ischemic muscle tissue sections were stained with H&E (scale bars, 200 mm). Right panel shows the quantification of necrotic areas in ischemic H&E-stained tissue sections. (G) Blood flow was determined at the indicated time points. (H) Ischemic muscle tissue sections stained with Abs against VWF antigen on day 14 were used to determine capillary density. Data represent means ± SEM. *$P < .05$.

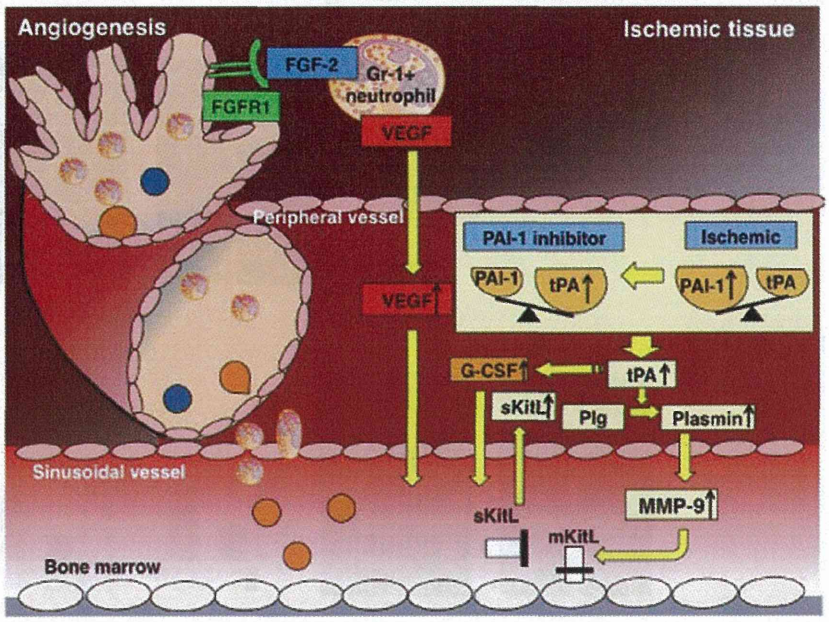

Figure 7. Schematic diagram showing the various molecules involved in the proangiogenic effect of PAI-1 inhibition. Under ischemic conditions, the local balance between the fibrinolytic factor tPA and one of its endogenous inhibitors, PAI-1, is shifted toward a profibrinolytic state with a local increase in tPA. Ischemia systemically results in a profibrinolytic state, a process dependent on endogenous tPA. Pharmacologic PAI-1 inhibition during ischemic recovery improved tissue regeneration due to an expansion of circulating and tissue-resident Gr-1$^+$ neutrophils coexpressing VEGF-A, FGF-2, and TIMP-1–free MMP-9, and to increased release of the angiogenic factor VEGF-A, the hematopoietic growth factor KitL, and G-CSF. Ab neutralization and genetic-knockout studies indicated that both the improved tissue regeneration and the increase in both circulating and ischemic tissue-resident Gr-1$^+$ neutrophils were dependent on the activation of tPA and MMP-9 and on VEGF-A and FGF-2.

expression of FGF-2 or FGFR1 is altered in PAI-1 inhibitor-treated ischemic tissues. Immunohistochemical analysis of ischemic muscle tissue sections revealed that PAI-1 inhibitor treatment augmented FGF-2 and FGF-R1 expression and that this expression colocalized more often to VWF$^+$ cells compared with vehicle-treated controls (Figure 6D-E).

FGF-2–induced angiogenesis requires VEGF signaling.[24] Blockade of VEGF-A and FGF-2 signaling with Abs against murine VEGF-A and FGF-2 inhibited the PAI-1 inhibitor-mediated FGF-2 and FGFR-1 increase on VWF$^+$ cells (Figure 6D-E), as well as the PAI-1 inhibitor-mediated ischemic tissue recovery, and reversed the necrosis-reducing effect of PAI-1 inhibitor treatment (Figure 6F-G) and myeloid cell mobilization (supplemental Figure 1A-C, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) in an HL-ischemic model.

Although it is clear that FGF-2–induced angiogenesis requires VEGF signaling,[24] it was unclear whether VEGF-A and FGF-2 signaling are required for PAI-1 inhibitor-mediated tissue neoangiogenesis. Our present data suggest that this might be the case, because VEGF-A and FGF-2 mAb prevented PAI-1 inhibitor-mediated ischemic tissue recovery and neoangiogenesis in an HL-ischemic model (Figure 6F-G).

The results of the present study indicate that the proangiogenic effects observed after PAI-1 inhibitor treatment are mediated by the 2 potent proangiogeneic factors FGF-2 and VEGF-A, and that neutrophils seem to be a source for these growth factors. In addition, both factors were essential for the PAI-1 inhibitor-mediated recruitment of "angiogenic hematopoietic effector cells" into ischemic tissues.

Discussion

The present study identifies activation of the FGF-2 and VEGF-A pathways as the cause of the proangiogeneic effect of drug-induced PAI-1 deficiency in a murine model of HL ischemia. Our data support a mechanism whereby drug-induced PAI-1 inhibition enhances angiogenesis through up-regulation of endogenous tPA and MMP-9. Both proteases are required for neutrophil mobilization and for the release of proangiogenic cytokines, including FGF-2 and VEGF-A (Figure 7). In addition, muscle-infiltrating CD11b$^+$Gr-1$^+$ neutrophils harvested from PAI-1 inhibitor-treated mice coexpressed FGF-2 and VEGF-A and showed an improved capacity to stimulate angiogenesis on adoptive transfer compared with equal numbers of carrier-treated ischemic tissue-derived neutrophils. Ab-blocking experiments revealed that PAI-inhibitor–induced tissue regeneration required FGF-2 and VEGF-A signaling. These data are important for the design of future cell-based therapies, especially in the light of a recent study demonstrating that BM cells harvested from mice with distant ischemia show a reduced capacity to stimulate angiogenesis on adoptive transfer.[25]

FGF family members and its receptors (FGFRs) can promote angiogenesis. In the present study, PAI-1 inhibition-induced augmentation of FGFR1 expression on endothelial cells in ischemic tissues coincided with increased KitL plasma levels. This result is consistent with a previous study showing that FGFR-1–deficient embryoid bodies show decreased expression of KitL.[26] KitL can improve tissue recovery in animal models of HL ischemia.[27-29] Recombinant tPA therapy augments circulating KitL levels[30] and promotes ischemic revascularization.[19] Confirming these data, in the present study, we found that drug-induced PAI-1 inhibition raised KitL plasma levels via tPA augmentation. Our data imply a relationship among the fibrinolytic factors PAI-1/tPA, FGFR1 signaling, and KitL production. However, further studies are required to determine how these pathways interact with each other.

PAI-1 in cooperation with integrins, coagulation, fibrinolysis, and endocytosis has been shown to be important for macrophage migration.[7] In the present study, we show that pharmacologic blockade of PAI-1 increased MMP-9– and tPA-dependent augmentation of tissue-residing neutrophils, but not F4/80$^+$ macrophages, under ischemic conditions. Therefore, PAI-1 seems to act as a negative regulator of neutrophil recruitment during HL-ischemic recovery. Supporting our observations, Renckens et al demonstrated that PAI-1 gene-deficient mice showed an enhanced early influx of neutrophils to the site of inflammation in a murine model of turpentine-induced tissue injury.[31] In this model, no difference

was found between PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice in factors known to attract neutrophils, including keratinocyte-derived chemokine and macrophage inflammatory protein-2.

Among the factors that can enhance the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils[32] is the hematopoietic growth factor G-CSF. In the present study, we demonstrate for the first time that recombinant tPA and endogenous tPA that is enhanced by PAI-1 inhibition promote the release of G-CSF. G-CSF has been shown to improve tissue recovery in animal models of HL[21] and myocardial[33] and focal cerebral ischemia injuries in both mice and humans[34] by modulating various cell types, including endothelial cells and neutrophils.[21,32] Various studies have demonstrated the importance of MMP-9 for neutrophil-driven neoangiogenesis in an HL-ischemic model.[2,19,21,35] A recent study demonstrated that tissue-infiltrating neutrophil pro-MMP-9 induces angiogenesis catalytically via an FGF-2/FGFR2 pathway.[23] Consistent with that study, we have shown previously that pharmacologic PAI-1 inhibition results in the accumulation of FGF-2– and VEGF-A–expressing Gr-1^{+} neutrophils within ischemic muscle tissues through an effect on endogenous tPA and MMP-9, and in an increase of plasma VEGF-A via up-regulation of endogenous tPA.[19] Neutrophils can secrete tissue inhibitors of metalloproteinase (TIMP)–free MMP-9 that can act in concert with, for example, macrophages to liberate proangiogenic growth factors such as VEGF and FGF-2 that are sequestered to the extracellular matrix.

PAI-1 can inhibit cell adhesion and migration by inhibiting the activity of uPA receptor (uPAR)–bound uPA and by preventing integrin association to vitronectin. Studies with uPAR$^{-/-}$ mice have emphasized the critical role of this receptor in leukocyte trafficking.[31] Indeed, uPAR$^{-/-}$ mice displayed a profoundly reduced neutrophil recruitment to the peritoneal cavity after IP administration of thioglycollate.[36] Our present results are consistent with the findings that neutrophil extravasation into the interstitium after lung ischemia-reperfusion injury after lung transplantation was blocked in tPA-deficient mice.[37] At the molecular level, this blockage was associated with reduced expression of platelet endothelial cell adhesion molecule-1 mediated through the tPA/low-density lipoprotein receptor–related protein/NF-κB signaling pathway.

Reichel et al showed that extravasated plasmin(ogen) mediates neutrophil recruitment in vivo via activation of perivascular mast cells and secondary generation of lipid mediators.[38]

The combined data suggest that strategies aimed at inactivation of PAI-1 (eg, the use of the small-molecule TM5275) could be an immediately clinically applicable therapeutic option for improving angiogenesis in ischemic patients. The results of the present study shed new light on the mechanism by which PAI-1 and tPA enhance neovascularization by modulation of the local and systemic growth factor environment and by alteration of neutrophil migration.

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Authorship

Contribution: Y.T., C.N., and K.S.-K. designed and performed the experiments, analyzed the data, and wrote the manuscript; M.O.-K. and M.I. designed and performed the experiments; A.S., I.G., H.K., and Y.S. developed the analytical tools; T.D., T.M., and Y.T. provided reagents; K.O., K.S., and H.N. provided technical support and conceptual advice; and B.H. and K.H. designed the experiments, analyzed the data, and wrote the manuscript.

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Correspondence: Koichi Hattori, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; e-mail: khattori@ims.u-tokyo.ac.jp.

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Therapeutic Value of Small Molecule Inhibitor to Plasminogen Activator Inhibitor–1 for Lung Fibrosis

Wen-Tan Huang[1], Praveen K. Vayalil[1,2], Toshio Miyata[3], James Hagood[4], and Rui-Ming Liu[1]

[1]Department of Environmental Health Sciences, School of Public Health, and [2]Center for Free Radical Biology, Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama; [3]United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, Tohoku, Japan; and [4]Division of Respiratory Medicine, University of California at San Diego and Rady Children's Hospital, San Diego, California

Fibrosis is a final stage of many lung diseases, with no effective treatment. Plasminogen activator inhibitor–1 (PAI-1), a primary inhibitor of tissue-type and urokinase-type plasminogen activators (tPA and uPA, respectively), plays a critical role in the development of fibrosis. In this study, we explored the therapeutic potential of an orally effective small molecule PAI-1 inhibitor, TM5275, in a model of lung fibrosis induced by transforming growth factor–β1 (TGF-β1), the most potent and ubiquitous profibrogenic cytokine, and in human lung fibroblasts (CCL-210 cells). The results show that an intranasal instillation of AdTGF-β1$^{223/225}$, an adenovirus expressing constitutively active TGF-β1, increased the expression of PAI-1 and induced fibrosis in murine lung tissue. On the other hand, treating mice with 40 mg/kg of TM5275 for 10 days, starting 4 days after the instillation of AdTGF-β1$^{223/225}$, restored the activities of uPA and tPA and almost completely blocked TGF-β1–induced lung fibrosis, as shown by collagen staining, Western blotting, and the measurement of hydroxyproline. No loss of body weight was evident under these treatment conditions with TM5275. Furthermore, we show that TM5275 induced apoptosis in both myofibroblasts (TGF-β1–treated) and naive (TGF-β1–untreated) human lung fibroblasts, and this apoptosis was associated with the activation of caspase-3/7, the induction of p53, and the inhibition of α–smooth muscle actin, fibronectin, and PAI-1 expression. Such an inhibition of fibrotic responses by TM5275 occurred even in cells pretreated with TGF-β1 for 6 hours. Together, the results suggest that TM5275 is a relatively safe and potent anti-fibrotic agent, with therapeutic potential in fibrotic lung disease.

Keywords: PAI-1 inhibitor; lung fibrosis therapy; (myo)fibroblast apoptosis; TGF-β1; animal model

Pulmonary fibrosis is a characteristic feature and final stage of many lung diseases, including idiopathic pulmonary fibrosis, cystic fibrosis, acute respiratory distress syndrome, severe acute respiratory syndrome, sarcoidosis, silicosis, and asbestosis. The most enigmatic and fatal form of pulmonary fibrosis is idiopathic pulmonary fibrosis (IPF). Despite decades of intensive study, no effective treatment for these devastating lung diseases has been developed because of a poor understanding of their complex pathological process. In the past, lung fibrosis was speculated to result from an unremitting inflammatory response to an exogenous insult, leading to the activation and proliferation of fibroblasts, and eventually culminating in progressive fibrosis. Therefore, anti-inflammatory agents, alone or in combination with cytotoxic drugs, have been used in the clinic as a standard therapeutic regimen for the treatment of lung fibrotic diseases. However, little evidence indicates that these agents alter the natural history of the disease or improve the survival of patients (1–4). Although other therapeutic strategies were used in clinical trials, including interferon-γ and anti–transforming growth factor–β or anti–connective tissue growth factor antibody, the efficacies of these treatments remain unclear (5–8).

CLINICAL RELEVANCE

The findings of this study may lead to the development of new therapeutic drugs for lung fibrotic diseases. The results also shed new light on the mechanism whereby plasminogen activator inhibitor–1 promotes fibrosis.

Plasminogen activator inhibitor 1 (PAI-1) is a primary inhibitor of urokinase-type and tissue-type plasminogen activators (uPA and tPA, respectively), and plays a critical role in wound healing and tissue remodeling. The expression of PAI-1 is increased in many fibrotic diseases, including IPF (9–14), and in experimental models of fibrosis (15, 16). Knockout of the PAI-1 gene or the administration of PAI-1 small interfering RNA attenuates, whereas the overexpression of PAI-1 protein enhances, fibrotic responses induced by different stimuli (13, 17–19). All these lines of evidence suggest that PAI-1 plays an essential role in the development of lung fibrosis. Nonetheless, the mechanism whereby PAI-1 promotes fibrosis is still poorly understood, and most importantly, no PAI-1 inhibitor has been developed for the treatment of lung fibrotic diseases.

In this study, we explored the therapeutic potential of an orally effective small molecule PAI-1 inhibitor, TM5275, for lung fibrosis, using human lung fibroblasts and a well-established lung fibrosis model induced by transforming growth factor–β1 (TGF-β1), the most potent and ubiquitous profibrogenic cytokine. The results show that an oral administration of TM5275, 4 days after mice were challenged with TGF-β1, almost completely blocked TGF-β1–induced lung fibrosis, with no significant effect on body weight. We further show that TM5275 induced (myo)fibroblast apoptosis and suppressed TGF-β1–induced fibrotic responses in human lung fibroblasts. Together, the results suggest that TM5275, a small molecule PAI-1 inhibitor, is a promising therapeutic agent for lung fibrotic diseases.

MATERIALS AND METHODS

Animal Treatment

Male C57BL/6 mice (6–8 weeks old) were challenged with 10^9 plaque-forming units of AdTGF-β1$^{223/225}$, an adenovirus expressing constitutively active TGF-β1, AdDL70-3, a virus vector, or saline by intranasal instillation. Four days later, the mice were treated with 40 mg/kg of TM5275 (dissolved in 2% DMSO) or solvent by gavage daily for 10 days. Mice were killed 7 or 14 days after the instillation of AdTGF-

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Correspondence and requests for reprints should be addressed to Rui-Ming Liu, Ph.D., Department of Environmental Health Sciences, School of Public Health, University of Alabama at Birmingham, Birmingham, AL 35294-0022. E-mail: rliu@uab.edu

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$\beta 1^{223/225}$. Bronchoalveolar lavage was performed, the left lungs were fixed with 4% paraformaldehyde, and the rest of the lung tissue was frozen immediately in liquid nitrogen. All animals were maintained on a 12-hour light/dark cycle at 22°C in the specific pathogen free facility, and all procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham.

Cell Culture and Treatment

CCL-210 cells, which are normal human lung fibroblasts from the American Type Culture Collection (Manassas, VA), were cultured in Eagle's minimum essential medium formulated by the American Type Culture Collection, as we described previously (20). At 70–80% confluence, cells were treated with 1 ng/ml of TGF-β1 (R&D, Minneapolis, MN) for various periods of time, and then with 75 μM TM5275 for 24 hours.

ELISA

The amounts of total and active TGF-β proteins in the bronchoalveolar lavage fluid (BALF) were measured using a ELISA kit (catalogue number 84-7344-88) from eBioscience (San Diego, CA), according to the protocol provided by the manufacturer. The amounts of PAI-1 antigen in the BALF were determined using a ELISA kit from Molecular Innovations (Novi, MI), as we described previously (21).

Northern Blot Hybridization

Total RNA was isolated from lung tissue, using TRIzol reagent. The mRNA of procollagen α2 (I), procollagen α1 (III), and PAI-1 was assessed by Northern blot hybridization, as we described previously (22).

Lung Histology and Collagen Staining

The deposition of collagen in the lung was revealed by Masson trichrome staining, and quantified by morphometric techniques, as we described previously (23).

Immunohistochemical Staining

α–smooth muscle actin (α-SMA) in murine lung tissue was stained with monoclonal anti-mouse α-SMA antibody (catalogue number CM001B; Biocare Medical, Concord, CA) and semiquantified, as we previously described (23).

Measurement of Hydroxyproline

The hydroxyproline content in lung tissue was determined as described elsewhere (24), and calculated according to the standard curves derived from 4-hydroxy-L-proline.

Reverse Zymography and Zymography

The activities of PAI-1 and tPA/uPA were determined by reverse zymography and zymography, respectively, as we described previously (21). The intensities of bands were semiquantified using Image J software (from the National Institutes of Health website).

Western Blot Analyses

Western blot analyses of protein abundance were conducted as we described previously (25), using the antibodies collagen 1α1 (catalogue number sc8784; Santa Cruz Biotechnology, Santa Cruz, CA), PAI-1 (ASMPAI-GF; Molecular Innovations), α-SMA (catalogue number CM001B; Biocare Medical), fibronectin (catalogue number 610077; BD Biosciences, Franklin Lakes, NJ), and β-actin (protein loading control). Protein bands were semiquantified using Image J software.

Apoptosis Analysis

Apoptosis was analyzed by flow cytometry techniques, using an Alexa Fluor 488 Annexin V Kit (Invitrogen, Carlsbad, CA), following the protocol provided by the manufacturer.

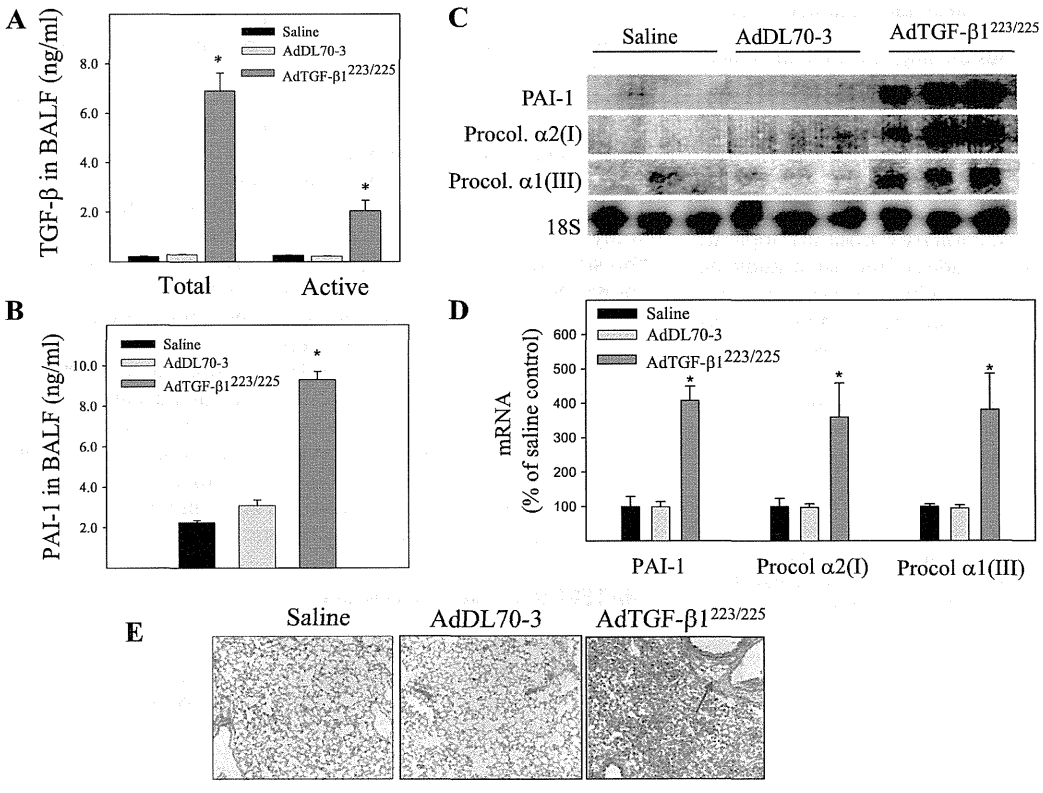

Figure 1. Effects of intranasal instillation of an adenovirus expressing constitutively active transforming growth factor–β1 (AdTGF-$\beta 1^{223/225}$) on the expression of plasminogen activator inhibitor 1 (PAI-1) and the accumulation of collagen in murine lung tissue. AdTGF-$\beta 1^{223/225}$, the adenovirus vector (AdDL70-3), or saline was administered to murine lungs by intranasal instillation. Seven days after instillation, the mice were killed. The amounts of total and active transforming growth factor–β1 (TGF-β1) (*A*) as well as total PAI-1 protein (*B*) in the bronchoalveolar lavage fluid (BALF) were determined by ELISA. (*C*) Representative Northern blotting image of procollagen (Procol) and PAI-1 mRNAs in murine lung tissue. 18S was used as an RNA loading control. (*D*) Quantitative data of the radioactivity of Northern blots from Instant Image (Packard Instrument Co., Meriden, CT). (*E*) Trichrome staining of collagens in murine lung tissue (*arrow* indicates collagen). *Significantly different from saline-treated mice ($P < 0.05$, $n = 5$–8).

Activity of Caspase-3/7

The activity of caspase-3/7 was determined with an Apo-ONE Homogenous Caspase-3/7 Assay (catalogue number G7790; Promega, Madison, WI), according to the protocol provided by the manufacturer. The fluorescence was measured at excitation/emission wavelengths of 485/528 nm, and the results were normalized according to protein concentrations.

Statistical Analysis

Data are presented as means ± SEM, and were evaluated by one-way ANOVA. Statistical significance was determined *post hoc* by Tukey's test.

RESULTS

Intranasal Instillation of AdTGF-β1$^{223/225}$ Increased the Expression of PAI-1 and Induced Lung Fibrosis in Mice

TGF-β1 is the most potent and ubiquitous profibrogenic cytokine, and is increased in almost all fibrotic diseases. To explore the therapeutic potential of the PAI-1 inhibitor TM5275 in lung fibrotic diseases, we first examined whether an intranasal instillation (a noninvasive lung drug-delivery technique) of AdTGF-β1$^{223/225}$, an adenovirus expressing constitutively active TGF-β1, would effectively induce the expression of PAI-1 and lung fibrosis in mice. The results show that the concentrations of protein in both active and latent forms of TGF-β1 in the BALF were dramatically increased 7 days after an intranasal instillation of AdTGF-β1$^{223/225}$ (Figure 1A). Associated with the increase in expression of TGF-β1, the instillation of AdTGF-β1$^{223/225}$ significantly increased concentrations of PAI-1 protein and mRNA in BALF and lung tissue (Figures 1B–1D), as well as the expression of procollagen mRNA and deposition of collagen in the lung (Figures 1C–1E). Virus vector alone (AdDL70-3), on the other hand, exerted no significant effect on the expression of TGF-β or PAI-1, and neither did it increase the deposition of collagen in the lung, suggesting that fibrotic responses are induced by TGF-β1 and not adenovirus. Importantly, such fibrotic responses induced by an intranasal instillation of AdTGF-β1$^{223/225}$ persisted for at least 21 days (data not shown). These results suggest that an intranasal instillation of AdTGF-β1$^{223/225}$ is an effective and reliable means of inducing lung fibrosis. Because the expression of TGF-β is increased in almost all fibrotic diseases, TGF-β–induced lung fibrosis serves as a good animal model for testing the therapeutic potential of an antifibrotic drug, and was used in this study.

Effects of PAI-1 Inhibitor on Activities of PAI-1 and tPA/uPA in Lungs of Mice Treated with or without TGF-β1

TM5275, a novel, orally effective PAI-1 inhibitor, was shown to exert potent antithrombotic effects in both rats and monkey models, without causing overt toxicity or effects on bleeding time (26). To test its antifibrotic potential, we first examined the effects of an oral administration of TM5275 on the activities of PAI-1, tPA, and uPA in murine lung tissue. The results show that an administration of AdTGF-β1$^{223/225}$ increased the activity of PAI-1 (Figure 2A) and suppressed the activity of uPA, although it exerted no significant effect on the activity of tPA (Figure 2B). Treatment with TM5275, on the other hand, inhibited the TGF-β1–induced activity of PAI-1 (Figure 2A), and completely reversed the inhibitory effect of TGF-β1 on uPA activity (Figure 2B). We also observed that the activities of tPA and uPA in mice treated with TGF-β1 plus TM5275 were significantly higher than those treated with TM5275 alone. These results suggest that the expression of tPA and uPA proteins may be increased in TGF-β1–treated mice, even though their activities were inhibited because of a simultaneous increase in the expression and activity of PAI-1. No obvious body weight loss was evident upon treatment with TM5275 (data not shown), suggesting that TM5275 did not cause obvious toxicity under the conditions used in this study.

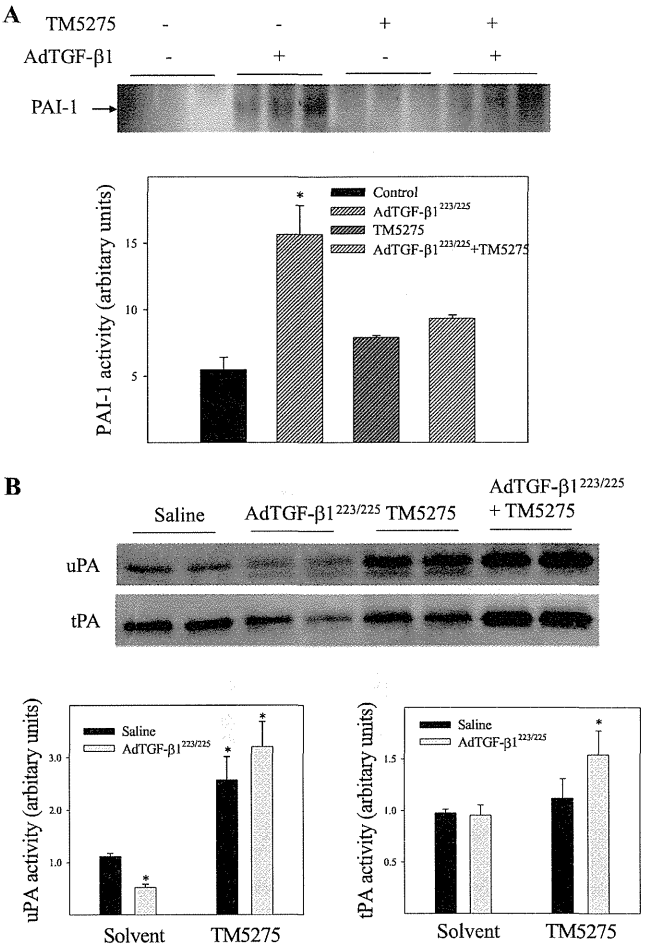

Figure 2. Effects of small molecule PAI-1 inhibitor TM5275 on the activities of PAI-1, tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA) in the lungs of mice intranasally instilled with AdTGF-β1$^{223/225}$ or saline. (*A*) The activity of PAI-1 was determined by reverse zymography. (*B*) The activities of tPA and uPA were determined by zymography, and photo-negative images of the gels are presented here. Dark bands attributable to an inhibition of casein degradation by PAI-1 in reverse zymography and protein lytic bands in zymography were semiquantified with Image J software. Semiquantified data are presented at the *bottoms* of *A* and *B*. *Significantly different from saline + solvent–treated control mice ($P < 0.05$, $n = 4$–6).

Effects of PAI-1 Inhibitor on TGF-β1–Induced Lung Fibrosis in Mice

The effects of TM5275 on TGF-β1–induced lung fibrosis were explored further by measuring the deposition of collagen and hydroxyproline content in murine lung tissue. The results show that an administration of TM5275 significantly reduced the TGF-β1–induced accumulation of collagen in the lung, as shown by trichrome staining, Western blotting, and hydroxyproline measurement (Figures 3–5). Immunohistochemical staining and Western blotting further showed that TM5275 suppressed TGF-β1–induced α–smooth muscle actin (α-SMA), a marker of myofibroblast differentiation in murine lungs (Figures 3 and 4), suggesting that TM5275 may prevent the TGF-β1–induced