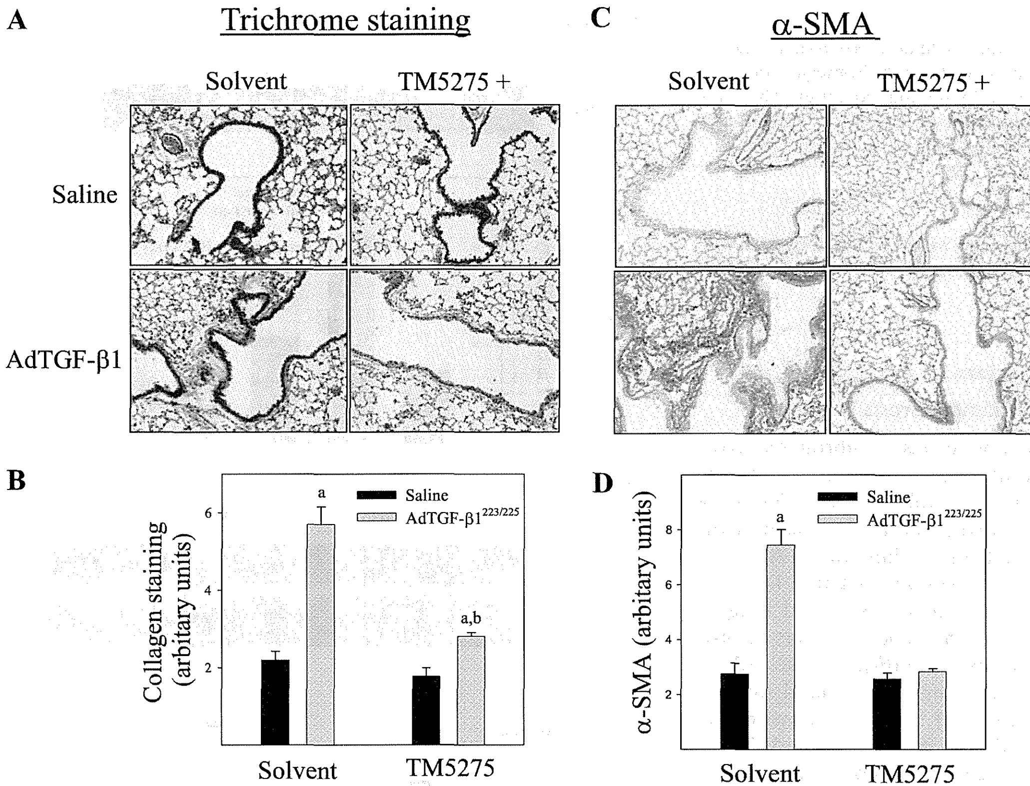

Figure 3. Effects of TM5275 on TGF-β1–induced accumulation of collagen and α–smooth muscle actin (α-SMA) in murine lung tissue. (*A*) Representative images of trichrome staining of collagen in murine lung. (*B*) Summary of semiquantified collagen staining data. (*C*) Representative immunohistochemical staining images of α-SMA in murine lung tissue. (*D*) Summary of semiquantification data of α-SMA immunostaining. [a]Significantly different from saline + solvent–treated control group. [b]Significantly different from group with AdTGF-$\beta 1^{223/225}$ alone ($P < 0.05$, $n = 4$–5).

differentiation of myofibroblasts, or induce the apoptosis of myofibroblasts. Most interestingly, Western blotting analysis showed that TM5275 almost completely blocked the AdTGF-$\beta 1^{223/225}$–induced expression of TGF-β1. These data clearly demonstrate the potent antifibrotic activity of TM5275 in our model of TGF-β1–induced lung fibrosis.

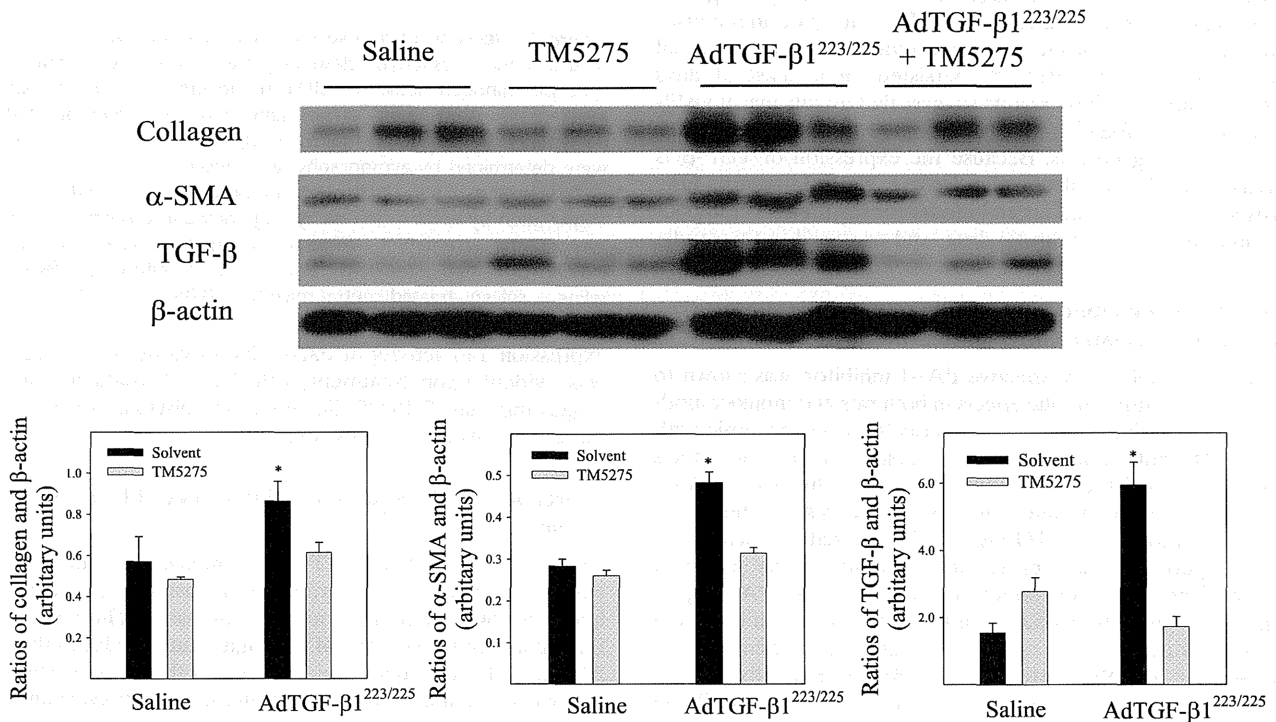

Figure 4. Western blot analyses of effects of TM5275 on the expression of collagen, α-SMA, and TGF-β proteins in lungs of TGF-β1–treated mice. *Top*, representative Western blotting images of collagen, α-SMA, and TGF-β. β-actin was used to show equal protein loading. *Bottom*, summaries of semiquantified data of collagen, α-SMA, and TGF-β band densities relative to β-actin, as determined by Image J software. *Significantly different from saline + solvent–treated control group ($P < 0.05$, $n = 4$–5).

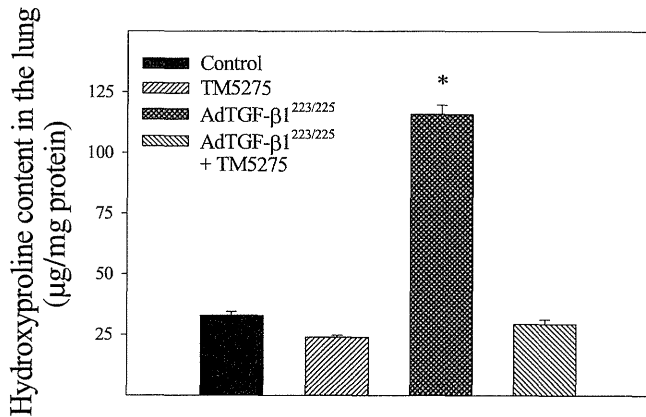

Figure 5. Effect of TM5275 on accumulation of hydroxyproline in lungs of TGF-β1–treated mice. The amount of hydroxyproline was determined by a color reaction assay, as described in MATERIALS AND METHODS. *Significantly different from saline + solvent–treated control group ($P < 0.05$, $n = 3$–6).

Effects of PAI-1 Inhibitor on TGF-β1–Induced Fibrotic Responses in Human Lung Fibroblasts

To explore further the therapeutic potential of TM5275 in the treatment of lung fibrotic diseases and the mechanism whereby TM5275 blocks AdTGF-β$1^{223/225}$–induced lung fibrosis, we assessed the effects of TM5275 on TGF-β1–induced fibrotic responses in human lung fibroblasts (CCL-210 cells). TGF-β1, not AdTGF-β$1^{223/225}$, was used in all *in vitro* studies, because the results of *in vivo* studies indicate that TGF-β1, and not adenovirus, caused fibrosis in murine lung tissue (Figure 1). The results show that treatment with TGF-β1 increased the activity of PAI-1 and inhibited the activity of uPA, whereas treating cells with TM5275 blocked the TGF-β1–induced activity of PAI-1 (13.7128 ± 0.3012, 19.9487 ± 1.1632, 13.5068 ± 0.3808, and 12.8755 ± 2.0121 arbitrary units for cells treated with solvent, TGF-β1, TM5275, and TGF-β1 plus TM5275, respectively) and stimulated the activity of uPA (1.0925 ± 0.0424, 0.3678 ± 0.0256, 1.2443 ± 0.1651, and 1.7916 ± 0.3619 arbitrary units for cells treated with solvent, TGF-β1, TM5275, and TGF-β1 plus TM5275, respectively). Neither TGF-β1 nor TM5275 alone exerted a significant effect on the activity of tPA. Most importantly, Western blot analyses showed that TM5275, when added to culture medium simultaneously with TGF-β1, almost completely blocked the TGF-β1–induced expression of α-SMA, fibronectin, and PAI-1 (Figure 6A).

To explore further whether TM5275 can suppress the progression of fibrosis after it is initiated, we pretreated CCL-210 cells with TGF-β1 for 1 or 6 hours, and then with 75 μM of TM5275 for 24 hours. The results show that the expression of PAI-1 and α-SMA was increased as early as 1 hour after treatment with TGF-β1 (Figure 6B). TM5275, however, significantly reduced the TGF-β1–induced expression of α-SMA and fibronectin, even after cells were pretreated with TGF-β1 for 6 hours (Figure 6B). These data further suggest that TM5275 can resolve preexisting fibrosis or block the progression of fibrosis.

Induction of Apoptosis by PAI-1 Inhibitor in Human Lung Fibroblasts Treated with or without TGF-β1

Although the traditional view, supported by our previous studies, suggests that PAI-1 promotes fibrosis by suppressing the degradation of extracellular matrix (ECM) (21, 27), emerging evidence suggests that PAI-1 may exert its profibrogenic effects by preventing apoptosis in (myo)fibroblasts (14, 28). Therefore, we examined whether TM5275 induced apoptosis in human lung fibroblasts. Our results indicate that TGF-β1 alone exerted no significant effect on the apoptosis of CCL-210 cells, whereas TM5275 significantly increased apoptotic cell numbers in both naive (TGF-β1–untreated) and activated (TGF-β1–treated) fibroblasts (Figure 7A). Interestingly, CCL-210 cells that were treated with TGF-β1 and therefore underwent myofibroblast differentiation seemed more sensitive to TM5275-induced apoptosis than were naive fibroblasts (Figure 7A). The apoptosis induced by TM5275 also activated caspase-3 (Figure 7B) and increased the expression of p53, a tumor repressor and initiator of apoptosis (Figures 7C and 7D). Together, these data suggest that TM5275 blocked TGF-β1–induced fibrosis, probably by inducing apoptosis in (myo)fibroblasts.

DISCUSSION

Fibrosis is a final stage of many diseases involved in almost all organ systems, including the lung, and no effective treatment is available for these devastating diseases. PAI-1 plays a key role in the development of fibrosis, and therefore is an ideal therapeutic target in the treatment of fibrotic diseases. Nonetheless, after the performance of intensive studies, no PAI-1 inhibitors have been developed for such purposes. Here we report for the first time, to the best of our knowledge, that TM5275, an orally effective novel small molecule PAI-1 inhibitor that demonstrated potent antithrombotic activity but low toxicity in several animal species/models (26), almost completely blocked TGF-β1–induced lung fibrosis in an animal model. *In vitro* studies further show that TM5275 induced the apoptosis of (myo)fibroblasts and significantly reduced fibrotic responses, even after cells were pretreated with TGF-β1 for 6 hours. These data suggest that TM5275 is a promising antifibrotic agent that can block the progression of fibrosis.

The mechanism whereby TM5275 blocked TGF-β1–induced lung fibrosis is unclear at present. TM5275 was administrated 4 days after mice were challenged with AdTGF-β$1^{223/225}$. Although we did not monitor fibrotic changes until 7 days after the instillation of AdTGF-β$1^{223/225}$, Sime and colleagues (29), who generated the AdTGF-β$1^{223/225}$ adenovirus, reported that an extensive deposition of ECM proteins occurred, including collagen, elastin, and fibronectin, starting on Day 3 after the instillation of AdTGF-β$1^{223/225}$. The induction of α-SMA, a marker of fibroblast activation, was also evident from Day 3 onward (29), suggesting that fibrosis began to develop as early as 3 days after the instillation of AdTGF-β$1^{223/225}$ in this model. Importantly, a comparable amount of total (latent + active) TGF-β1 was detected in BALF after the instillation of AdTGF-β$1^{223/225}$ in our study, as it was in the study of Sime and colleagues (29), suggesting that fibrotic tissue had likely been deposited in lung tissue by Day 4 after the instillation of AdTGF-β$1^{223/225}$, when treatment with TM5275 began. Our new *in vitro* data further show that TGF-β1 induced fibrotic responses, including the expression of PAI-1 and α-SMA, as early as 1 hour after treatment. TM5275, on the other hand, significantly reduced such fibrotic responses, even after cells were pretreated with TGF-β1 for 6 hours (Figures 6B and 6C). Based on these data, we conclude that TM5275 blocks TGF-β1–induced lung fibrosis, at least in part, by resolving established fibrosis or blocking the progression of fibrosis.

TGF-β plays a critical role in the development of fibrosis. A positive feedback loop between PAI-1 and TGF-β1 was described elsewhere (30, 31). Matsuo and colleagues reported that upon unilateral ureteral obstruction, PAI-1 transgenic mice showed increased numbers of interstitial myofibroblasts and higher concentrations of TGF-β1 mRNA (30). Seo and colleagues further showed that knockout of the PAI-1 gene

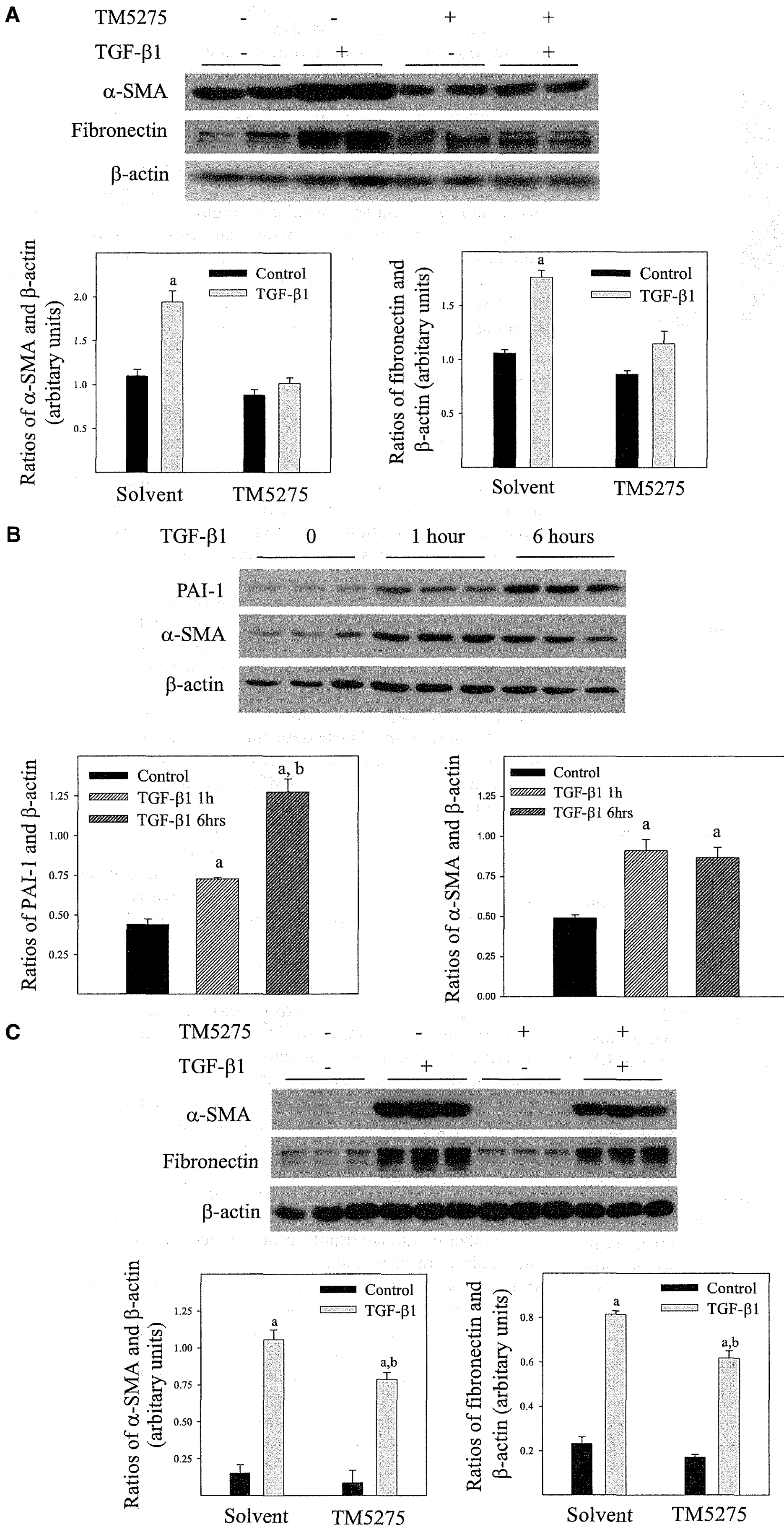

Figure 6. Effects of TM5275 on TGF-β1–induced expression of α-SMA, fibronectin, and PAI-1 in human lung fibroblasts. (*A*) Human lung fibroblasts (CCL-210 cells) were treated with 1 ng/ml of TGF-β1 in the presence or absence of 75 μM of TM5275 for 24 hours. (*B*) CCL-210 cells were treated with 1 ng/nl of TGF-β1 for 1 or 6 hours. (*C*) CCL-210 cells were pretreated with 1 ng/ml of TGF-β1 for 6 hours, and then with 75 μM of TM5275 for 24 hours. *Top*, representative Western blotting images. *Bottom*, summaries of the semiquantified data of band densities relative to β-actin band densities. [a]Significantly different from solvent alone group. [b]Significantly different from TGF-β1 alone–treated group ($P < 0.05$, $n = 6$).

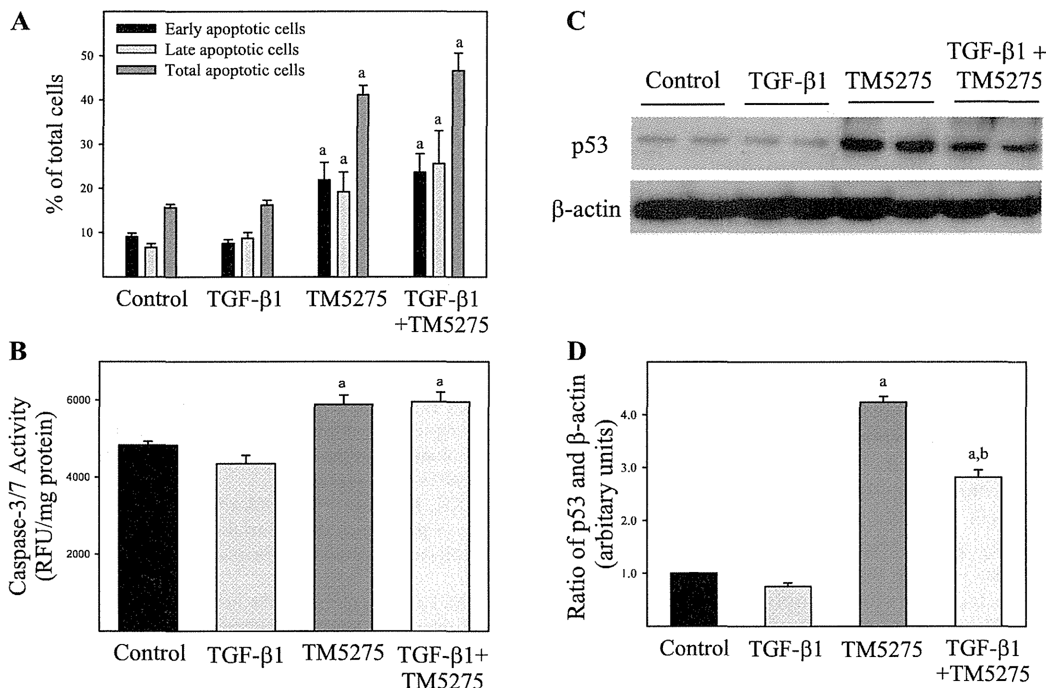

Figure 7. Induction of apoptosis by TM5275 in human lung fibroblasts treated with or without TGF-β1. CCL-210 cells were treated with 1 ng/ml of TGF-β1 in the presence or absence of 75 μM of TM5275 for 24 hours. (*A*) Apoptotic cell death was analyzed by flow cytometry techniques, using an Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit from Invitrogen. Early and late stages of apoptotic cells were calculated separately or in combination. The results are expressed as percentages of total cell number. (*B*) The activity of caspase-3/7 was determined with an Apo-ONE Homogenous Caspase-3/7 Assay Kit from Promega. (*C*) Concentrations of p53 protein were determined by Western blot analysis, and the results were semiquantified with Image J software (*D*). [a]Significantly different from corresponding untreated control group. [b]Significantly different from corresponding group treated with TM5275 alone ($P < 0.05$, $n = 7$–8). RFU, relative fluorescence units.

suppressed the expression of high glucose-induced TGF-β1 mRNA, whereas recombinant PAI-1 restored the inducibility of TGF-β1 by high glucose in PAI-1 knockout mesangial cells (31). Furthermore, they showed that recombinant PAI-1 protein stimulated TGF-β promoter activity, and that the induction of fibronectin and collagen I by recombinant PAI-1 was abrogated by the TGF-β1 receptor inhibitor or anti–TGF-β antibody (31). Together, the data strongly suggest that PAI-1 positively regulates TGF-β1 gene expression. In the present study, the intranasal instillation of AdTGF-$\beta1^{223/225}$, but not control virus (AdDL70-3), induced endogenous TGF-β1 as well as the expression of PAI-1 in murine lungs, suggesting that the induction was caused by TGF-β1 and not adenovirus. Although the precise mechanism whereby TGF-β1 (AdTGF-$\beta1^{223/225}$) induced its own gene expression is unclear, our data suggest that PAI-1 may be involved, because TM5275 completely blocked such an induction. These results further support the notion of a positive feedback loop between PAI-1 and TGF-β1. The data also suggest that TM5275 blocks TGF-β1–induced lung fibrosis in mice, at least in part, by breaking up this positive feedback loop.

Myofibroblasts are the major producers of ECM, and therefore contribute importantly to the development of fibrosis. Upon the resolution of normal wound healing, myofibroblasts undergo apoptosis. The dysregulation of apoptosis, therefore, leads to impaired wound healing (fibrosis) because of the prolonged activity of myofibroblasts. The mechanisms regulating the apoptosis of myofibroblasts are largely unknown at present. Interestingly, plasmin was reported to induce apoptosis, whereas PAI-1 protects (myo)fibroblasts from the apoptosis induced by different stimuli (14, 28, 32–35). The density of myofibroblasts is greater in PAI-1–overexpressing mice (30) and lower in PAI-1 knockout mice (36) upon fibrotic stimulation, which further suggests that PAI-1 may promote fibrosis by protecting myofibroblast from apoptosis. In this study, TGF-β1 increased the expression of α-SMA, a marker of myofibroblasts, in murine lungs and in human lung fibroblasts, whereas TM5275 suppressed the TGF-β1–induced expression of α-SMA both *in vivo* and *in vitro*. These data suggest that another potential mechanism, whereby TM5275 blocks TGF-β1–induced lung fibrosis, induces the apoptosis of myofibroblasts. This notion is further supported by our flow cytometry data, which show that TM5275 induced apoptosis in both naive fibroblasts (TGF-β1–untreated) and myofibroblasts (TGF-β1–treated fibroblasts). Moreover, the flow cytometry data indicate that myofibroblasts were more sensitive to TM5275-induced apoptosis than were naive fibroblasts. Because myofibroblasts are the major producers of ECM and produce much more ECM than naive fibroblasts, and because myofibroblasts can be derived from different types of cells in addition to resident fibroblasts, a drug targeting myofibroblasts should demonstrate better therapeutic potential than a drug targeting naive fibroblasts. These data suggest that TM5275 is a promising antifibrotic agent. Nonetheless, whether TM5275 has the same selectivity *in vivo* is unknown, as is the mechanism underlying such selectivity, and further investigation is warranted. The results from these studies will aid in the development of more effective antifibrotic drugs.

Different hypotheses were proposed to elucidate the mechanisms whereby PAI-1 protects cells from apoptosis. One potential mechanism involves the inhibition of caspase-3 activity by directly binding to caspse-3 protein (32, 37, 38). In this study, TM5275, with or without TGF-β1, slightly but significantly increased the activity of caspase-3/7. Although the underlying mechanism is unknown, the results suggest that TM5275 induced the apoptosis of (myo)fibroblasts in part by the activation of caspase-3/7 pathways. Horowitz and colleagues showed that in addition to binding to and inhibiting the activity of caspase-3,

plasminogen and plasmin induced the apoptosis of fibroblasts, which was associated with pericellular fibronectin proteolysis (28). They also showed that PAI-1 protected fibroblasts from the apoptosis induced by plasminogen but not by plasmin, suggesting that PAI-1 protects fibroblasts from apoptosis by inhibiting the activation of plasminogen (28). Although we did not measure the activity of plasmin, our data show that TM5275 treatment restored or increased the activity of uPA and tPA in both TGF-β1–challenged mice and cultured human lung fibroblasts, suggesting that the induction of (myo)fibroblast apoptosis by TM5275 may result from increased plasmin activity.

p53 is a master controller of apoptosis. Here we showed for the first time, to the best of our knowledge, that the expression of p53 was increased in human lung fibroblasts by TM5275, with or without TGF-β1 treatment. TGF-β1 alone exerted no significant effect on p53 expression, but slightly reduced the TM5275-stimulated expression of p53. The data suggest that the activation of p53 is involved in TM5275-induced apoptosis in human lung fibroblasts, although the mechanism underlying the activation of p53 pathway by TM5275 remains unclear. Our results are consistent with data reported by other investigators (39, 40), indicating that the activation of p53 was associated with the apoptosis of fibroblasts induced by gallic acid or silica. p53 was also shown to prevent apoptosis or lung injury. Davis and colleagues showed that bleomycin induced more apoptosis in macrophages and lung injury in p53 null mice than in p53 heterozygous or wild-type mice (41). Ghosh and colleagues also reported that knockdown of the p53 gene in epithelial cells (surfactant protein C–expressing cells), using dominant-negative techniques, enhanced the sensitivity of mice to bleomycin-induced lung fibrosis (42). These results suggest that p53 protects macrophages and lung epithelial cells from apoptosis and thereby lung injury and fibrosis. Such contradictory effects of p53 in apoptosis and lung injury/fibrosis can be explained by the diverse functions of p53 and the role of different types of cells in the development of fibrosis. Because (myo)fibroblasts are the major producers of ECM, the induction of (myo)fibroblast apoptosis is expected to attenuate fibrosis progression. Nonetheless, whether TM5275 induces p53 and apoptosis in lung (myo) fibroblasts *in vivo*, and whether the induction of p53 and the apoptosis of (myo)fibroblasts underlie the protective effects of TM5275, remain to be explored further.

Finally, we point out that PAI-1 performs multiple functions and is involved in the pathogenesis of various diseases, including thrombosis, arthrosclerosis, cancer, and fibrosis. Therefore, the development of a specific PAI-1 inhibitor has been a major focus of many studies. Several strategies, involving PAI-1 inhibitory antibody (43, 44), mutant PAI-1 proteins (27, 45, 46), and PAI-1 small interfering RNA (13), were proposed to inhibit the activity of PAI-1 in the treatment of these diseases. However, the application of these large molecules in clinical settings will be limited because of potential delivery problems. Small molecule PAI-1 inhibitors offer great therapeutic potential, because they can be delivered easily to the body, and most are orally effective (47–50). Several of these small molecule PAI-1 inhibitors proved effective in the treatment of thrombosis (the major area of interest in PAI-1 inhibitor research) (47–50), but whether they demonstratee therapeutic potential for lung fibrotic diseases remains unclear. In this study, we show for the first time, to the best of our knowledge, that TM5275 at a dose of 40 mg/kg/day for 10 days, a dose much lower than that (2,000 mg/kg/day for 2 weeks) used in the previous toxicity study and shown to cause no obvious toxicity (26), almost completely blocked TGF-β1–induced lung fibrosis. These data suggest that TM5275 is a relatively safe and potent antifibrotic agent, with promising therapeutic potential in lung fibrotic diseases.

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A Small Molecule Inhibitor to Plasminogen Activator Inhibitor 1 Inhibits Macrophage Migration

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Objective—Macrophage (Mϕ) migration rests on the adhesion/detachment between Mϕ surface components and extracellular matrixes, and the contribution of numerous inflammatory disorders. Plasminogen activator inhibitor (PAI)-1, a serine protease inhibitor, influences Mϕ motility through an action distinct from its classical modulation of the plasmin-based fibrinolytic process. We rely here on a small molecule PAI-1 inhibitor (TM5275) to investigate the role of PAI-1 in Mϕ migration in the pathogenesis of renal injury.

Approach and Results—Mϕ migration was inhibited both in vitro and in vivo by TM5275. It was also reduced in T-cell–deficient nude mice, but not in PAI-1–deficient mice. Mϕ migration hinged on the interaction of PAI-1 with low-density lipoprotein receptor–related protein, an interaction prevented by TM5275, but not with vitronectin, urokinase-type plasminogen activator, or tissue-type plasminogen activator. Fed to rats with anti–Thy-1–induced nephritis, TM5275 significantly decreased Mϕ accumulation and ameliorated the progression of renal injury.

Conclusions—These findings suggest that a small molecule PAI-1 inhibitor represents a novel class of anti-inflammatory agents targeting Mϕ migration by the inhibition of the interaction of PAI-1 with low-density lipoprotein receptor–related protein. ***(Arterioscler Thromb Vasc Biol.* 2013;33:935-942.)**

Key Words: inflammation ■ low-density lipoprotein receptor–related protein ■ macrophage migration ■ plasminogen activator inhibitor 1 ■ Thy-1 nephritis

The fibrinolytic system not only removes fibrin deposits from the vasculature, but is also implicated in extracellular matrix remodeling.[1–3] The primary fibrinolytic enzyme, plasmin, is a potent, broadly acting serine protease, formed when circulating plasminogen is cleaved and activated by 1 of 2 plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). The serine protease inhibitor plasminogen activator inhibitor (PAI-1) is the main plasma inhibitor of uPA and tPA. It inhibits plasmin-mediated fibrinolysis.[4] Interestingly, recent studies in mice deficient in or overexpressing the PAI-1 gene implicate that, in addition to its original action on the fibrinolytic process, PAI-1 is involved in several broad biological abnormalities, such as fibrosis, cell regeneration, and metabolic or neurological disorders.[5,6]

More recently, PAI-1 has been shown to affect significantly on cell adhesion, detachment, and migration by an interaction with various molecules, such as tPA, uPA, vitronectin (VN), and low-density lipoprotein receptor–related protein (LRP).[7–9] Indeed, genetic inactivation of PAI-1 or LRP1 can reduce macrophage (Mϕ) migration[10] and ameliorate inflammatory or allergic diseases in rodents.

We have developed an original approach to identify and synthesize orally active inhibitors of PAI-1. Compounds selected virtually by structure-based drug design underwent a docking simulation to select candidates that fit within the cleft accessible to insertion of the reactive center loop comprising s3A in PAI-1 3-dimensional structure. These compounds have been shown to inhibit coagulation in 2 different rodent models and 1 monkey model of thrombosis, and prevent the fibrotic process in 2 different rodent models of lung fibrosis.[11]

In this study, we use such a newly developed small molecule PAI-1 inhibitor, TM5275, as a chemical probe and investigate the effect of the inhibitor on Mϕ function, such as migration and cytokine secretion. We demonstrate that PAI-1 functions

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as a chemotactic factor and attracts Mϕ in vivo and in vitro, an action effectively prevented by the PAI-1 inhibitor TM5275. On closer inspection, Mϕ migration hinges on the interaction of PAI-1 with LRP, but not with VN, uPA, or tPA. TM5275 inhibits Mϕ migration in vitro by preventing the interaction between PAI-1 and LRP, and TM5275 also prevents Mϕ migration in vivo. Given to a rat Thy-1 nephritis model, TM5275 significantly decreases the number of infiltrated Mϕ and ameliorates the progression of renal injury. This small molecule PAI-1 inhibitor is thus a novel class of anti-inflammatory agent targeting Mϕ migration.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

TM5275 Inhibits Mϕ Migration Into the Peritoneum

The effect of TM5275 on in vivo Mϕ migration was investigated. Mϕ migration into the mouse peritoneum was induced by the intraperitoneal injection of a thioglycollate solution for 4 days. Mice with or without TM5275 were used for an in vivo assay. After 4 days, the PAI-1 level increased significantly in the peritoneal fluid (Figure 1A) of control mice but fell significantly ($P<0.01$) in those given TM5275. Results were compared with dexamethasone (corticosteroid), a well-known inhibitor of Mϕ migration,[12] and with clopidogrel, an analog of ticlopidine that inhibits adenosine diphosphate–mediated platelet aggregation. TM5275 as well as dexamethasone, but not clopidogrel, inhibited Mϕ migration in a dose-dependent manner in vivo (Figure 1B). To confirm that TM5275 inhibited Mϕ migration through its interaction with a PAI-1 moiety, we performed the same experiment in PAI-1–deficient mice and their wild-type littermate controls. As expected, quantitative Mϕ accumulation in PAI-1–deficient mice was significantly reduced as compared with wild-type mice, whereas TM5275 failed to inhibit Mϕ infiltration (Figure 1C), indicating that the PAI-1 modulates Mϕ migration.

Is TM5275-mediated inhibition of Mϕ infiltration a direct effect on Mϕ or an indirect effect through other immune cells, such as inflammatory T-cells known to activate Mϕ?[13] TM5275 was therefore tested in T-cell–deficient nude mice. It inhibited Mϕ infiltration into the peritoneum, despite the absence of T-cells (Figure 1D), indicating that at least T-cells are not required for the PAI-1–induced Mϕ migration.

Taken together, these data suggest that PAI-1 facilitates Mϕ migration in vivo in the peritoneum of mice given thioglycollate. This effect is effectively prevented by a PAI-1 inhibitor TM5275.

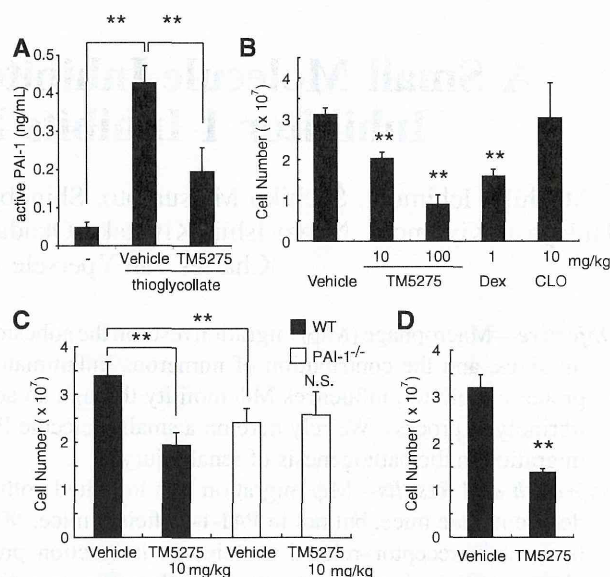

Figure 1. TM5275 inhibits the plasminogen activator inhibitor (PAI)-1–induced macrophage (Mϕ) migration in vivo. **A**, PAI-1 concentration was elevated by thioglycollate. TM5275 significantly decreased intraperitoneal levels of active plasminogen activator inhibitor 1 (PAI-1). **B**, The number of Mϕ migrating into the peritoneal cavity in vivo on stimulation by thioglycollate was assessed. TM5275 inhibited Mϕ migration in a dose-dependent manner. Dexamethasone (Dex) and Clopidogrel (CLO) were used as positive and negative controls, respectively. **C**, The number of Mϕ migrating into the peritoneal cavity in vivo on stimulation by thioglycollate was assessed in wild-type (WT) or PAI-1–deficient mice. TM5275 inhibited Mϕ migration in the WT mice but not in the PAI-1–deficient mice. **D**, The number of Mϕ migrating into the peritoneal cavity in vivo on stimulation by thioglycollate was assessed in T-cell–deficient nude mice. TM5275 inhibited Mϕ migration in T-cell–deficient nude mice. Data are shown as the mean and corresponding SEM. **$P<0.01$ by 1-way ANOVA and Dunnett test, n=5 to 11. N.S. indicates not significant.

PAI-1 Induces Mϕ Migration Via LRP

The benefit of TM5275 on the Mϕ migration was tested in vitro in a modified Boyden chamber with a human acute monocytic leukemia cell line, THP-1, which differentiates into Mϕ-like cells on incubation for 2 days with 50 nmol/L phorbol 12-myristate 13-acetate, (PMA). TM5275 inhibited the PAI-1–induced THP-1 migration in a dose-dependent manner (Figure 2A). By contrast, it proved ineffective for the THP-1 migration induced by the fetal bovine serum, which contained matrix components, such as VN and fibronectin, all factors known to affect Mϕ migration as chemoattractants (Figure 2B). These results suggest that TM5275 inhibits only PAI-1–dependent Mϕ migration.

Several candidate proteins for Mϕ migration, for example, tPA, uPA, VN, and LRP, were investigated to elucidate the molecular mechanism(s) underlying the THP-1–derived Mϕ migration. We used endotoxin-free PAI-1 mutants, that is, PAI-1 R76E (a mutant with no interaction with LRP), PAI-1 Q123K (a mutant with no binding ability to VN), and PAI-1 T333R (a mutant with no inhibitory activity against uPA/tPA). Among these PAI-1 proteins, only the PAI-1 R76E mutant affected Mϕ migration (Figure 2C), indicating that the PAI-1–induced cell migration hinges on its interaction with LRP1, whereas VN, uPA, or tPA is not necessarily required. In our assays, the expression of LRP1 mRNA and protein was very low in monocytic THP-1 cells without treatment with PMA but rose significantly after PMA treatment (Figure 2D and 2E). PAI-1 is thus unable to induce the transmigration of monocytic THP-1 cells without previous treatment with PMA (data not shown). The critical role of LRP1 in PAI-1–induced Mϕ migration was confirmed with an LRP1 antagonist,

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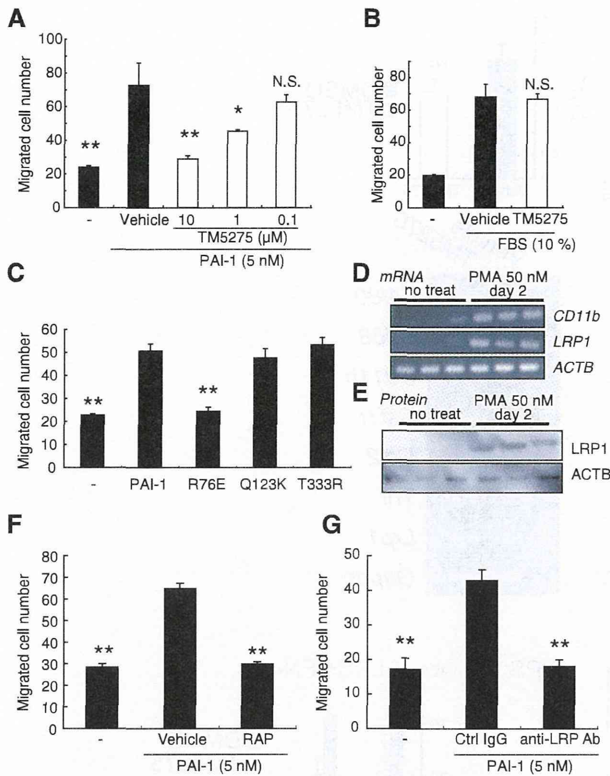

Figure 2. TM5275 inhibits plasminogen activator inhibitor (PAI)-1–induced macrophage (Mϕ) migration in vitro. PAI-1–induced cell migration depends on the interaction of PAI-1 with low-density lipoprotein receptor–related protein (LRP), but not with vitronectin (VN), urokinase-type plasminogen activator (uPA), or tissue-type plasminogen activator (tPA). **A**, Chemotaxis assays were performed with a modified Boyden chamber. Cells were added into the upper well of a Boyden chamber. Plasminogen activator inhibitor 1 (PAI-1) was fortified in the serum-free medium of the lower well. Human monocytic THP-1 cells were treated with Phorbol 12-myristate 13-acetate (PMA) for 2 days to differentiate into Mϕ and reseeded. Cells were pretreated with inhibitors for 30 minutes at 37°C. Effects of TM5275 on PAI-1–induced Mϕ migration were determined by the chemotaxis assay. TM5275 inhibited the PAI-1–induced Mϕ migration in a dose-dependent manner. **B**, Effects of TM5275 on the fetal bovine serum (FBS)-induced Mϕ migration were determined by the chemotaxis assay. 10 μmol/L of TM5275 did not inhibit the FBS-induced Mϕ migration. **C**, Chemotactic effects of the following PAI-1 variants were compared at the same concentration (5 nmol/L): PAI-1 R76E (a mutant with no interaction with LRP), PAI-1 Q123K (a mutant with no binding ability to vitronectin), and PAI-1 T333R (a mutant with no inhibitory activity against uPA/tPA). mRNA (**D**) and protein (**E**) expressions in the PMA-induced differentiated THP-1 cells. THP-1 cells were treated with 50 nmol/L PMA for 2 days. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA expressions for CD11b antigen (*CD11b*) and *LRP1* were performed. Actin beta (ACTB) was used as an invariant control in the experiment. Chemotaxis assays were performed with a modified Boyden chamber. Cells were added into the upper well of a Boyden chamber, and PAI-1 was fortified in the serum-free medium of the lower well. Human monocytic THP-1 cells were treated with PMA for 2 days to differentiate into Mϕ and reseeded. Cells were pretreated with receptor-associated protein (RAP; **F**) or anti-LRP1 antibody (anti-LRP Ab; **G**) for 30 minutes at 37°C. Effects of RAP and anti-LRP1 antibody on the PAI-1–induced Mϕ migration were determined by the chemotaxis assay. RAP and anti-LRP1 antibody significantly inhibited the PAI-1–induced Mϕ migration. Data are shown as the means and corresponding SEM. $^{**}P<0.01$ by 1-way ANOVA and Dunnett test, n=3 to 12. N.S. indicates not significant.

receptor-associated protein,[9] and an LRP1 neutralizing antibody. As shown in Figure 2F and 2G, receptor-associated protein and anti-LRP1 antibody indeed inhibited PAI-1–induced Mϕ migration.

The inhibitory action of TM5275 on the direct molecular interaction between PAI-1 and LRP1 was further examined. Previous studies showed that PAI-1 binds both to cluster II (Cl II) and to cluster IV (Cl IV) LRP.[14,15] LRP1 protein Cl II and Cl IV were thus immobilized on a 96-well ELISA plate, and Alexa488-labeled PAI-1 was added either in the presence or absence of 10 μmol/L TM5275 for 3 hours. The amount of PAI-1 bound to LRP1 was eventually assessed by the fluorescence intensity of Alexa488. The apparent binding of PAI-1 to LRP1 was significantly reduced by TM5275 (Figure 3). Receptor-associated protein (1 μmol/L) also inhibited the binding between PAI-1 and LRP1 (Figure in the online-only Data Supplement). A dose–response analysis demonstrated that TM5275 inhibited the binding between PAI-1 and LRP1 at a much lower concentration (IC50=3.13 μmol/L and 3.02 μmol/L for Cl II and Cl IV, respectively) than that required to inhibit the PAI-1 activity (IC50=6.95 μmol/L).

The effect of TM5275 on other Mϕ functions, such as differentiation and lipopolysaccharide (LPS)-induced cytokine secretion, was investigated. Monocytic THP-1 cells were incubated with PMA either in the presence or absence of 10 μmol/L TM5275, and the expression of Mϕ-specific mRNAs *F4/80*, *Cd68*, *Cd11b*, *Csf1r*, *Ccl2*, *Tnf*, and *Lrp* was analyzed (Figure 4A). TM5275 had virtually no influence on the expression of these Mϕ marker genes. The effects of TM5275 on interleukin-6 (IL-6) secretion and NO synthesis were further tested in the THP-1–derived Mϕ because PAI-1 expression can be modulated by various cytokines.[16,17] THP-1–derived Mϕ were stimulated either with LPS only or with LPS plus interferon-γ. Analysis of the cell culture supernatant disclosed that TM5275 had no effect on IL-6 secretion and NO synthesis (Figure 4B and 4C).

Taken together, these data demonstrate that PAI-1 directly facilitates Mϕ migration. TM5275 effectively inhibits Mϕ migration by preventing the interaction between PAI-1 and

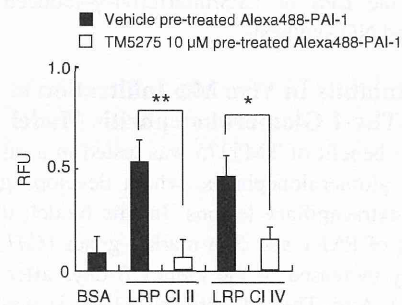

Figure 3. Effects of TM5275 on the interaction between plasminogen activator inhibitor (PAI)-1 and low-density lipoprotein receptor–related protein 1 (LRP1). Recombinant LRP1 cluster II (Cl II) and Cl IV protein were immobilized on a ELISA plate, and then blocked with 3% BSA for 2 hours at 37°C. The plate was incubated with Alexa fluor488-labeled recombinant PAI-1 protein, which was pretreated for 3 hours at 37°C with or without TM5275 (10 μmol/L). The direct binding between PAI-1 and LRP1 was measured as the fluorescent intensity. Data are shown as the means and corresponding SEM. $^{**}P<0.01$ or $^{*}P<0.05$ by 1-way ANOVA and Dunnett test, n=6.

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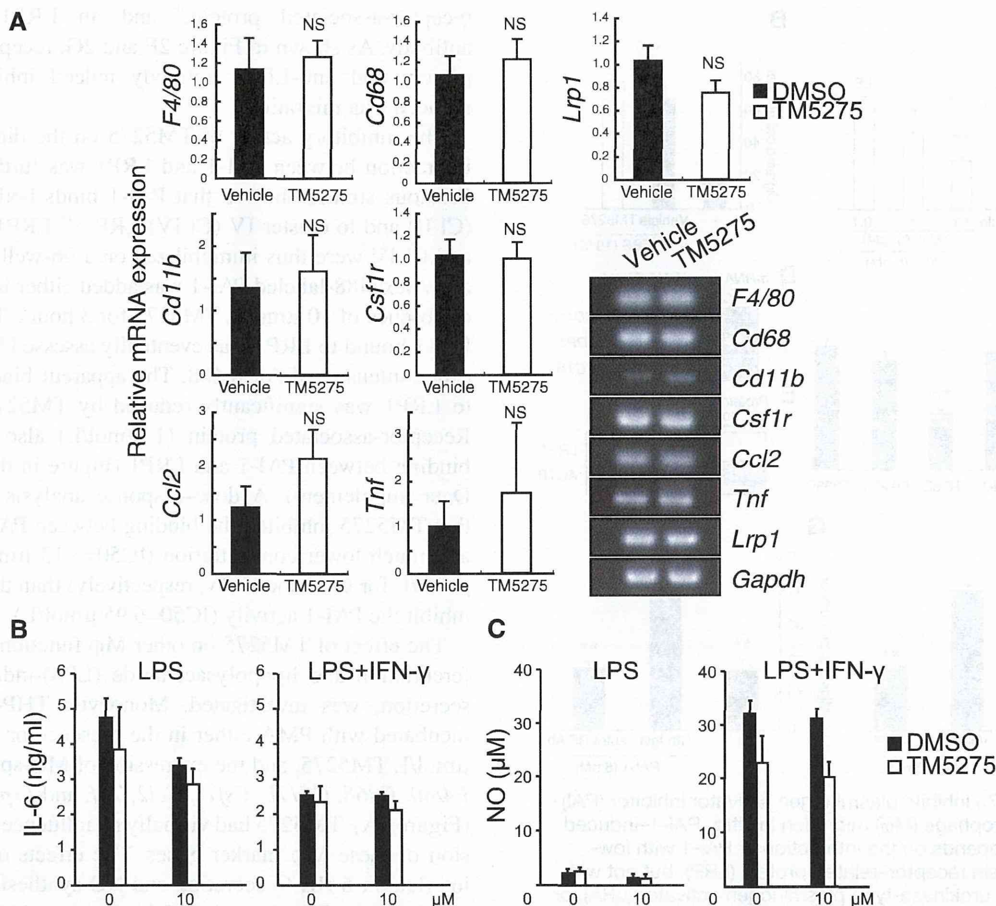

Figure 4. Effects of TM5275 on gene expressions and cytokine secretion in the phorbol 12-myristate 13-acetate, phorbol myristate acetate (PMA)-induced differentiated THP-1 cells. THP-1 cells were treated with 50 nmol/L PMA for 2 days. **A**, Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and quantitative real-time PCR analysis of *F4/80, Cd68, Cd11b, Csf1r, Ccl2, Tnf, Lrp* mRNA expressions were performed. Actin beta (*Actb*) was used as an invariant control in the experiment. **B**, PMA-treated THP-1 cells were stimulated by lipopolysaccharide (LPS) or LPS plus interferon γ (IFN-γ). Interleukin-6 (IL-6) and nitric oxide (NO) in the conditioned medium were measured. TM5275 had little effect on expressions of these marker genes, IL-6 secretion and NO synthesis. Data are shown as the mean and corresponding SEM.

one of its binding molecules, LRP. By contrast, TM5275 does not affect the LPS or LPS/interferon-γ–induced cytokine secretion and NO synthesis.

TM5275 Inhibits In Vivo Mϕ Infiltration in a Rat Anti–Thy-1 Glomerulonephritis Model

The clinical benefit of TM5275 was tested in a rat model of anti–Thy-1 glomerulonephritis, which develops glomerular endo- and extracapillary lesions. In this model, the mRNA expressions of PAI-1 and Mϕ marker genes (*Cd11b, Cd68*) significantly increased in the kidney 6 days after induction (Figure 5A). Anti–Thy-1.1 antibody (1-22-3) was administered intravenously to Sprague-Dawley rats (age, 6 weeks) subsequently treated with either vehicle, clopidogrel (30 mg/kg per day), or a PAI-1 inhibitor (TM5275 30 mg/kg per day), all delivered orally by gavage.

TM5275 treatment initiated 6 days after the antibody treatment significantly reduced proteinuria (Table I in the online-only Data Supplement). TM5275, but not clopidogrel, significantly reduced the Mϕ infiltration 7 days after disease induction, as determined by Mϕ marker genes (*Cd11b, Cd68*) mRNA expression (Figure 5A) and immunohistochemical analysis (Figure 5B and 5C). TM5275 provision significantly reduced ED-1 (C68) positive cells attracted in the glomerulus by the anti–Thy-1 antibody treatment.

Consistent with previous reports,[18–21] injection of anti–Thy-1 caused acute mesangiolysis, capillary ballooning, and microaneurysm formation at day 7 (Figure 6). Treatment with TM5275 significantly suppressed extracellular matrix formation on day 7 as determined by Masson Trichrome staining (Figure 6A). Glomerular injury (Figure 6B) and microaneurysm formation (Figure 6C) were also significantly suppressed. Phosphotungstic acid hematoxylin staining further revealed a reduced deposition of fibrin in the glomerulosclerotic lesions (Figure 6D and 6E). In normal rat kidney, staining for desmin in the podocytes was negligible, whereas staining for Wt1 was readily apparent (Figure 6F–6I). In anti–Thy-1 antibody–induced nephritis, increased staining of desmin and reduced

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