Discussion

In the present study, we found that all of the *SOXF* subfamily genes, *SOX7*, *SOX17*, and *SOX18*, are highly expressed in hESC-derived ECs enriched in HE and markedly down-regulated in pre-HPCs and HPCs to the levels comparable to that in CB CD34$^+$ cells. Overexpression of *Sox17* in ECs resulted in expansion of monotonous cells with a CD34$^+$CD43$^+$CD45$^{-/low}$ immunophenotype. These cells coexpressed hematopoietic marker antigens such as CD43 and a low level of CD45, as well as the HE marker VE-cadherin. These unique characteristics of *Sox17*-overexpressing ECs are reminiscent of HE cells. Overexpression of *Sox17* inhibited the hematopoietic differentiation of both pre-HPCs and HPCs and reprogrammed them into HE-like cells. In contrast, depletion of *SOX17* in pre-HPCs did not affect their hematopoietic differentiation. These findings suggest that SOX17 is one of the master regulators that define HE but must be down-regulated during the development of pre-HPCs to allow hematopoietic differentiation.

The effects of overexpression of *SOX17* in hESC-derived ECs and HPCs are very similar to that of overexpression of *Sox7* and *Sox18* in early hematopoietic precursors from mouse embryos and mouse ESCs.[14,15,28] However, it has been reported in mice that *Sox17* remains marginally expressed during blood specification and the overexpression of *Sox17* in early hematopoietic precursors induces massive apoptosis.[14] The contrasting effects of *Sox17* between humans and the mouse is somewhat surprising but could be partially attributed to the difference in expression during early hematopoiesis (see previous paragraph). The expression of all of the *SOXF* subfamily genes in ECs evokes the possibility that they have redundant function in the development of hematopoiesis from hESCs, as they do in postnatal angiogenesis in mice.[11] Nonetheless, the effects of knock-down of *Sox7* in mice and *SOX17* in the present study are different. *Sox7* knock-down in Brachyury$^+$Flk1$^-$ mesodermal precursors, which give rise on further differentiation to Flk1$^+$ cells containing hemangioblast precursors, profoundly inhibited the production of both hematopoietic progenitors and endothelial progenitors, leaving open the possibility that Sox7 inhibits the production of hematopoietic progenitors through inhibiting the formation of HE or hemangioblasts. In contrast, *SOX17* knock-down in ECs enriched in HE in the present study mainly compromised the development of mature hematopoietic cells and only mildly affected the proliferation of nonhematopoietic cells. Furthermore, depletion of *SOX17* in pre-HPCs did not significantly affect their hematopoietic differentiation. Therefore, the role of SOX17 at the developmental stage of blood specification could be more specific to the establishment of a hemogenic program in mesodermal or endothelial precursors compared with that of Sox7. Although we did not detect the effects of *SOX17* knock-down in pre-HPCs, recent studies have shown that Sox17 also plays an important role in the maintenance of fetal and neonatal HSCs, but not adult HSCs.[12] Sox17 has also been demonstrated to confer fetal HSC characteristics to adult hematopoietic progenitors.[13] SOX17 may again exert its critical function at a stage later than the pre-HPC stage, when pre-HPCs/HPCs differentiate into embryonic HSCs.

Very similar results have been demonstrated in the murine system with the transcription factor *HoxA3*. *HoxA3* is a gene uniquely expressed in the embryonic vasculature, but not in the yolk sac vasculature. HoxA3 restrains hematopoietic differentiation of the earliest endothelial progenitors and can induce reversion of the earliest hematopoietic progenitors into CD41-negative endothelial cells.[29] This reversible modulation of endothelial-hematopoietic state is accomplished by down-regulation of key hematopoietic transcription factors. Among these, Runx1 is able to erase the endothelial program set up by *HoxA3* and promote hematopoietic differentiation. *Sox17* was listed as one of the targets regulated by HoxA3. Given that SOX17 appeared to regulate directly the expression of RUNX1 in this study, it could be assumed that HoxA3 functions as an apical regulator of HE, eventually activating the transcription of *Runx1* via up-regulation of *Sox17* to initiate hematopoietic differentiation. It would be intriguing to address this question.

The direct targets for Sox17 have been characterized during endodermal differentiation of mouse ESCs using ChIP-on-chip analysis. The Sox17-binding consensus motif has also been identified using de novo motif analysis from the ChIP-on-chip data.[22] As expected, the genes bound by Sox17 in *Sox17*-overexpressing HE-like cells were quite different from those detected during endodermal differentiation and were related to the GO terms "vasculogenesis," "hemopoiesis," or "positive regulation of erythrocyte differentiation." Among these genes, *VE-cadherin/CDH5* encodes one of the well-known marker antigens of HE and is also expressed by embryonic HSCs.[24,25] Sox17 appears to bind directly to the promoters of *RUNX1*, *SCL/TAL1*, and *HHEX*, which encode key transcription factors essential to the development of HSCs from HE cells or hemangioblasts.[4,26,27] Other target genes included *BAZF/BCL6B*, *JUNB*, and *EGFL7*, which encode a POZ/BTB zinc finger protein, a basic HLH transcription factor, and a secreted angiogenic factor, respectively. These genes have been implicated in vasculogenesis and/or angiogenesis and BAZF/BCL6B and JUNB have also been implicated in hematopoiesis.[30-34] The profiles of these Sox17 targets during early hematopoietic development further support the critical role of SOX17 in the regulation of HE.

The results of the present study have unveiled a novel function of SOX17 in hematopoietic development. Because the overexpression of *Sox17* expands HE-like cells, it is possible that conditional expression of *SOX17* in hESC-derived endothelial progenitors facilitates hematopoietic development. Therefore, *SOX17* could be a novel target for manipulation to improve the yield of hematopoietic progenies from hESCs for regenerative cell therapies.

Acknowledgments

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Authorship

Contribution: Y. N.-T. performed the experiments, analyzed the results, produced the figures, and wrote the manuscript; M. Osawa,

M. Oshima, H.T., and S.M. assisted with the experiments including the hematopoietic analyses; M.E., T.A.E., T.T. and H.K. performed the microarray and ChIP-on-chip analyses; N.T., K.E., and H.N. generated the iPSCs; M. Osawa and A.I. conceived of and directed the project; and A.I. secured the funding and wrote the manuscript.

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

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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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$\beta1^{223/225}$. 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.

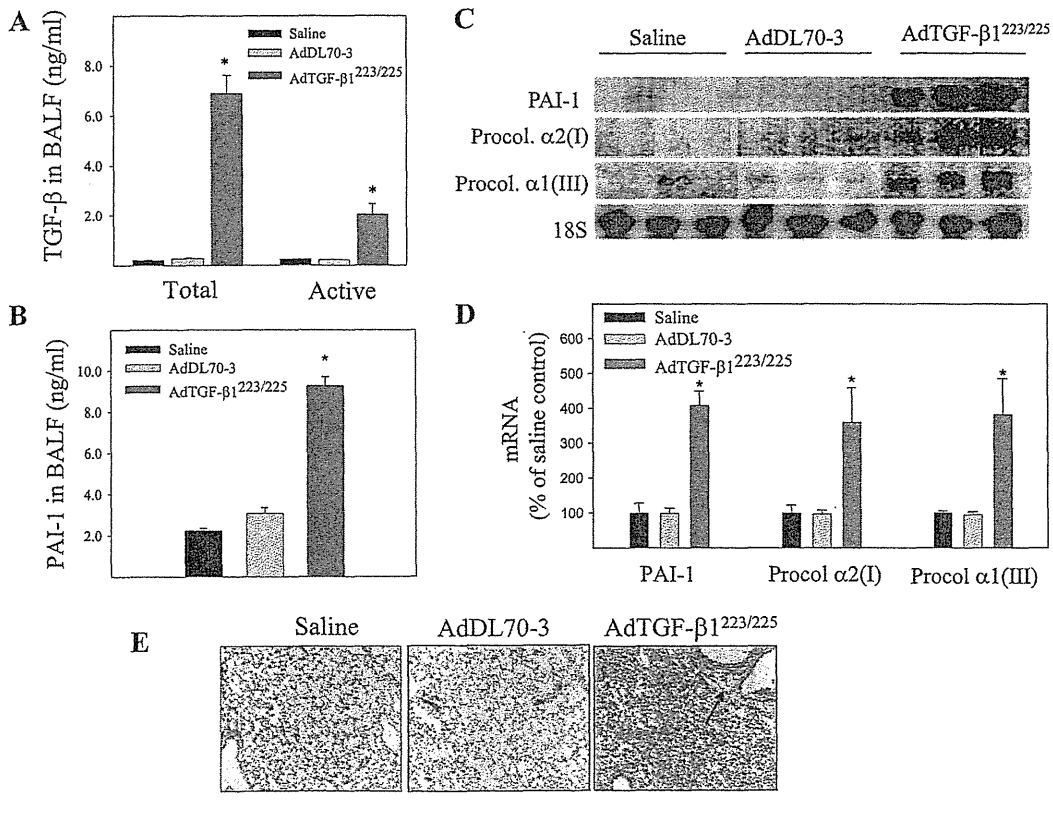


Figure 1. Effects of intranasal instillation of an adenovirus expressing constitutively active transforming growth factor–β1 (AdTGF-$\beta1^{223/225}$) on the expression of plasminogen activator inhibitor 1 (PAI-1) and the accumulation of collagen in murine lung tissue. AdTGF-$\beta1^{223/225}$, the 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

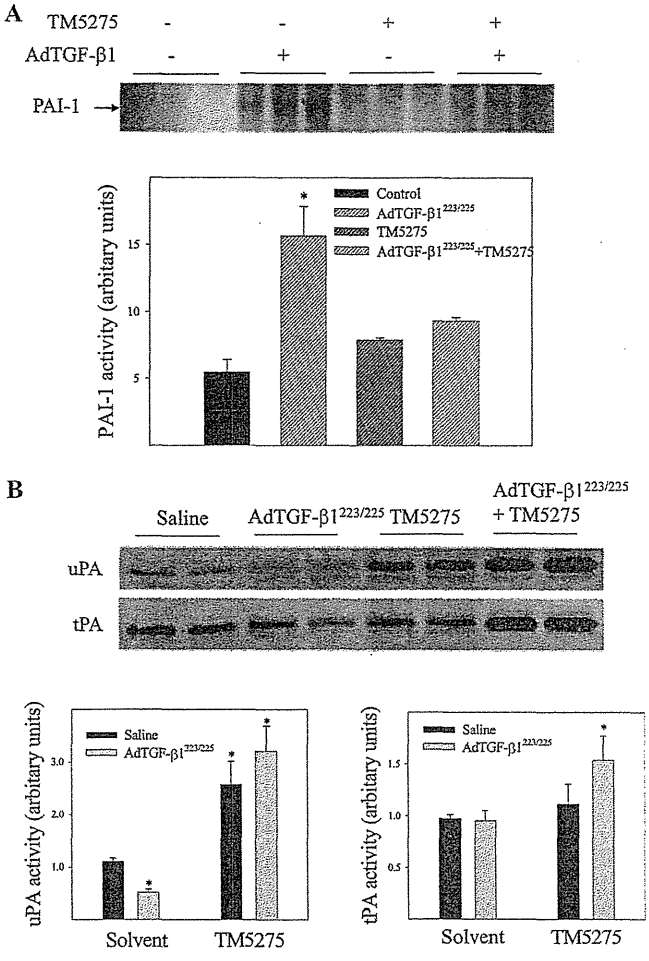


Figure 2. Effects of small molecule PAI-1 inhibitor TM5275 on the activities of PAI-1, tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA) in the lungs of mice intranasally instilled with AdTGF-β1$^{223/225}$ or saline. (*A*) The activity of PAI-1 was determined by 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).

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.

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

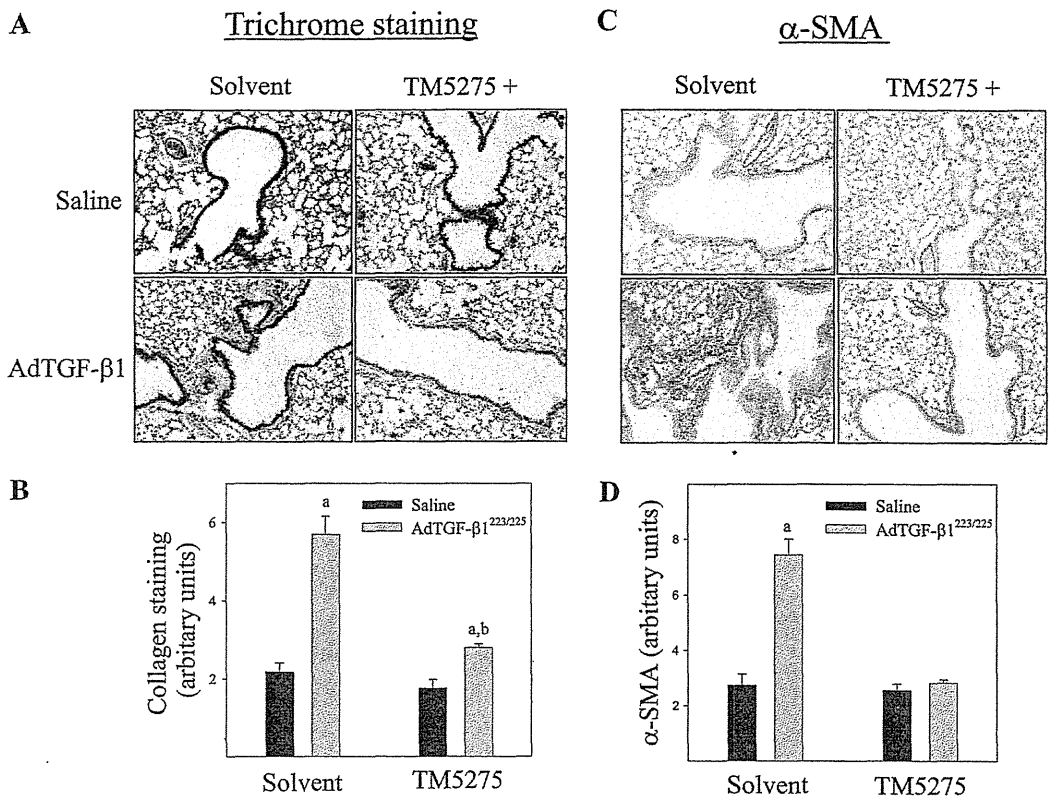


Figure 3. Effects of TM5275 on TGF-β1–induced accumulation of collagen and α–smooth muscle actin (α-SMA) in murine lung tissue. (*A*) Representative images of trichrome staining of collagen in murine lung. (*B*) Summary of semiquantified collagen staining data. (*C*) Representative immunohistochemical 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-$\beta1^{223/225}$ 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-$\beta1^{223/225}$–induced expression of TGF-β1. These data clearly demonstrate the potent antifibrotic activity of TM5275 in our model of TGF-β1–induced lung fibrosis.

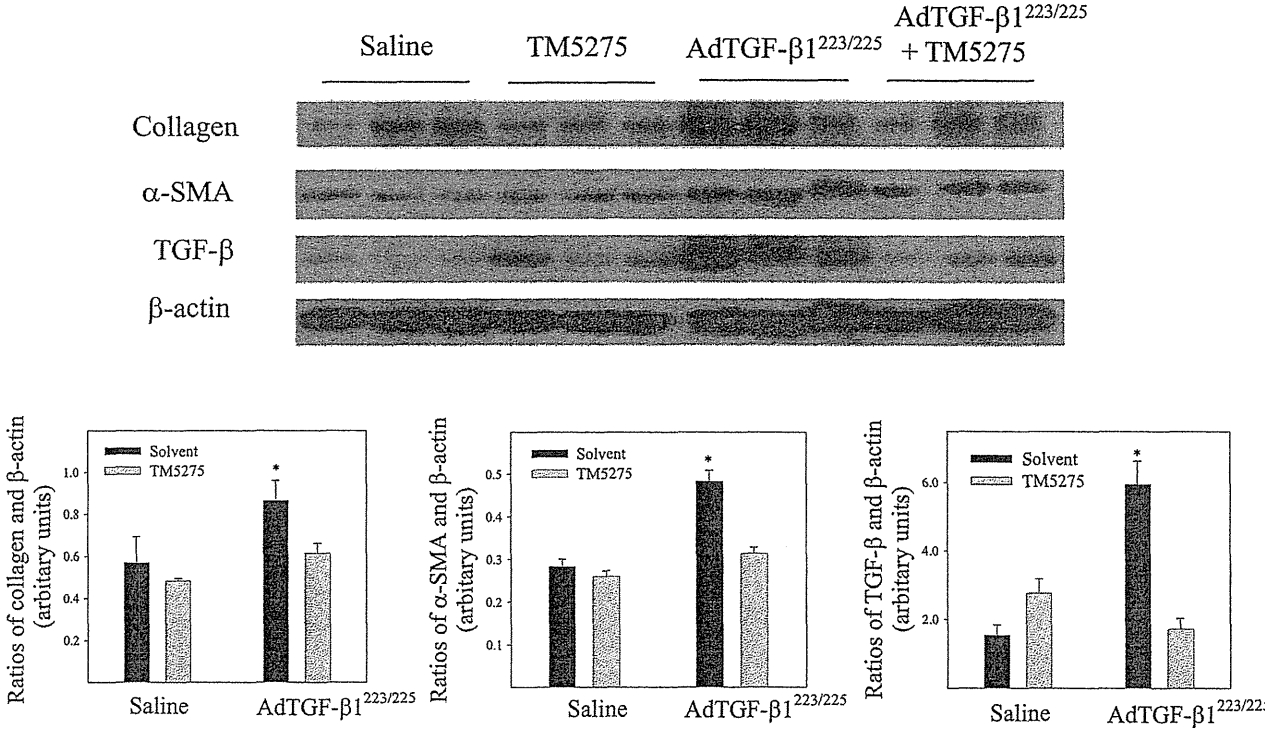


Figure 4. Western blot analyses of effects of TM5275 on the expression of collagen, α-SMA, and TGF-β proteins in lungs of TGF-β1–treated mice. *Top*, representative Western blotting images of collagen, α-SMA, and TGF-β. β-actin was used to show equal protein loading. *Bottom*, summaries of semiquantified 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).

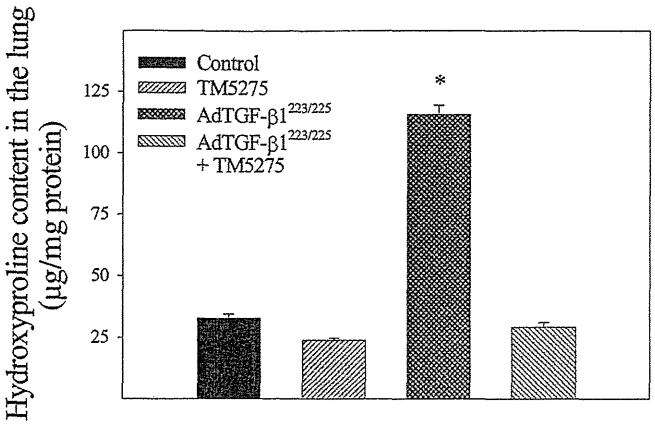


Figure 5. Effect of TM5275 on accumulation of hydroxyproline in lungs of TGF-β1–treated mice. The amount of hydroxyproline was determined by a color reaction assay, as described in MATERIALS AND METHODS. *Significantly different from saline + solvent–treated control group ($P < 0.05$, $n = 3$–6).

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-β1$^{223/225}$–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-β1$^{223/225}$, 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

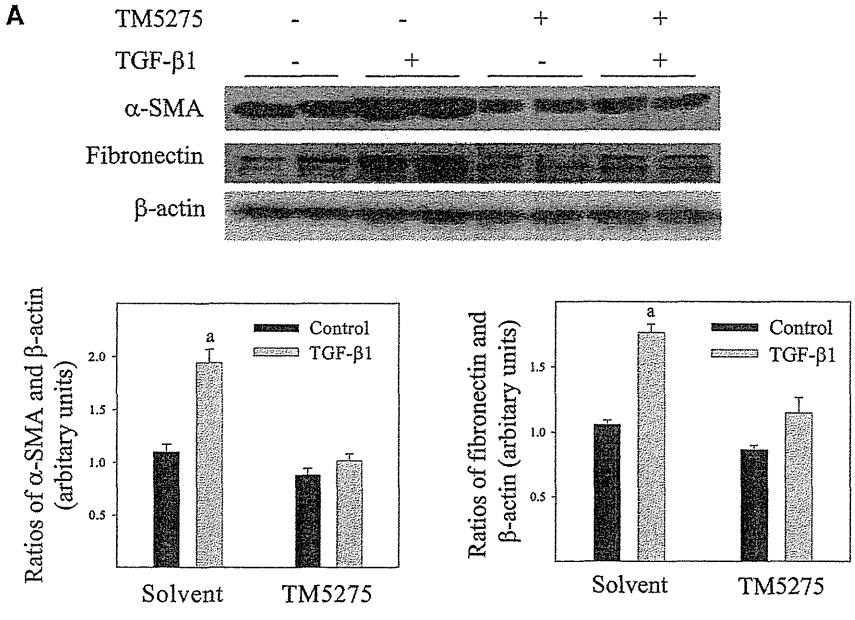
Although the traditional view, supported by our previous studies, suggests that PAI-1 promotes fibrosis by suppressing the degradation of extracellular matrix (ECM) (21, 27), emerging evidence suggests that PAI-1 may exert its profibrogenic effects by preventing apoptosis in (myo)fibroblasts (14, 28). 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.

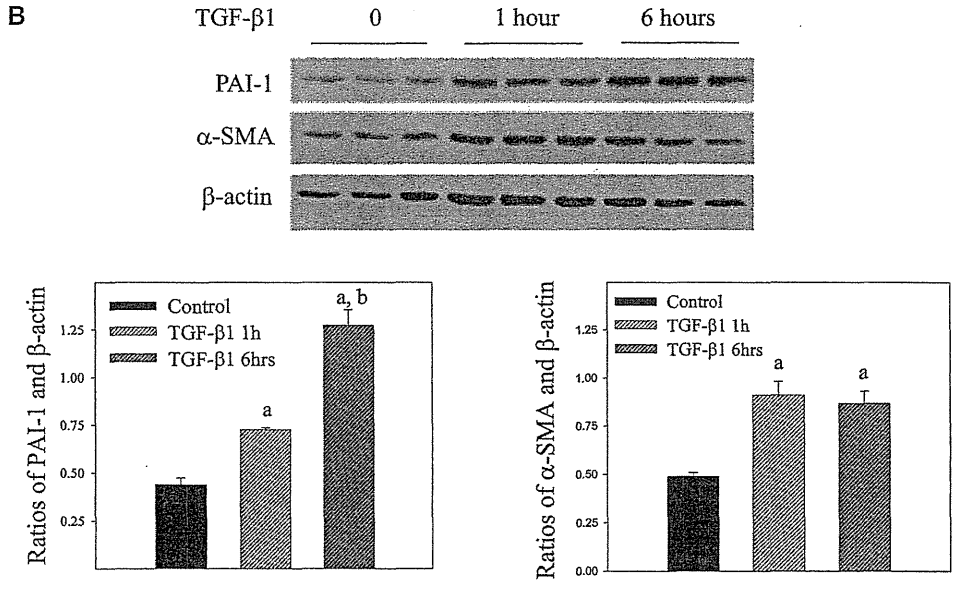
DISCUSSION

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-β1$^{223/225}$. Although we did not monitor fibrotic changes until 7 days after the instillation of AdTGF-β1$^{223/225}$, Sime and colleagues (29), who generated the AdTGF-β1$^{223/225}$ adenovirus, reported that an extensive deposition of ECM proteins occurred, including collagen, elastin, and fibronectin, starting on Day 3 after the instillation of AdTGF-β1$^{223/225}$. 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-β1$^{223/225}$ in this model. Importantly, a comparable amount of total (latent + active) TGF-β1 was detected in BALF after the instillation of AdTGF-β1$^{223/225}$ 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-β1$^{223/225}$, 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





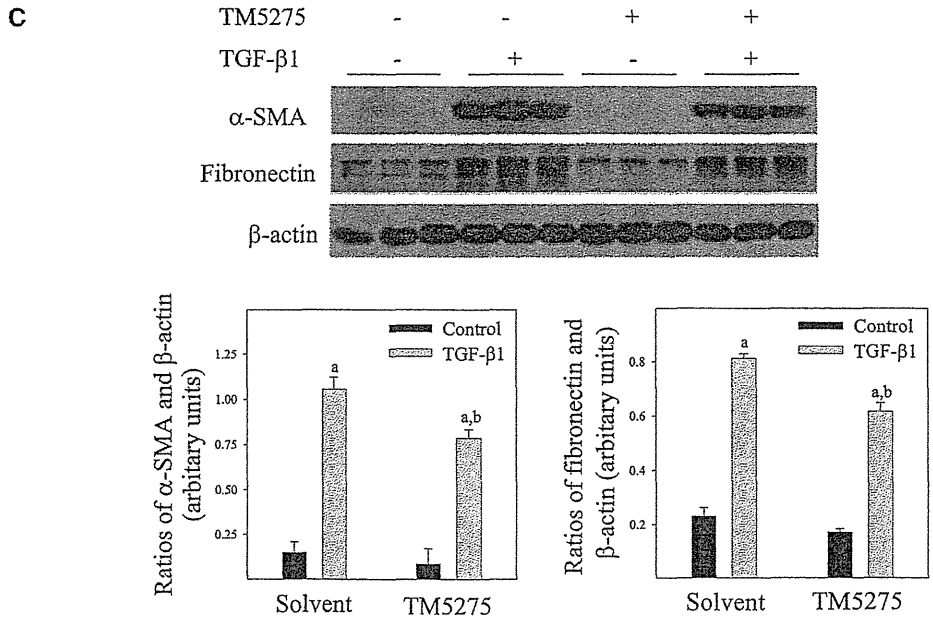


Figure 6. Effects of TM5275 on TGF-β1–induced expression of α-SMA, fibronectin, and PAI-1 in human lung fibroblasts. (*A*) Human lung fibroblasts (CCL-210 cells) were treated with 1 ng/ml of TGF-β1 in the presence or absence of 75 μM of TM5275 for 24 hours. (*B*) CCL-210 cells were treated with 1 ng/nl 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 ($P < 0.05$, $n = 6$).

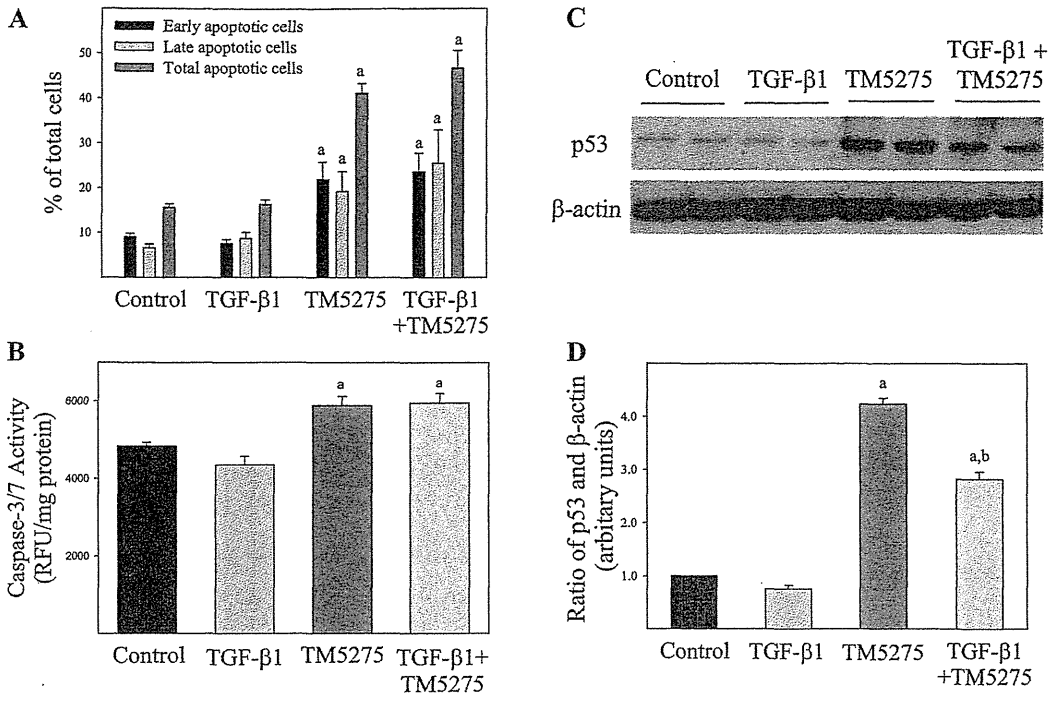


Figure 7. Induction of apoptosis by TM5275 in human lung fibroblasts treated with or without TGF-β1. CCL-210 cells were treated with 1 ng/ml of TGF-β1 in the presence or absence of 75 μM of TM5275 for 24 hours. (*A*) Apoptotic cell death was analyzed by flow cytometry techniques, using an Alexa Fluor 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-β1$^{223/225}$, 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-β1$^{223/225}$) 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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Original Article

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;33:00-00.)

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.

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 monocytic 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

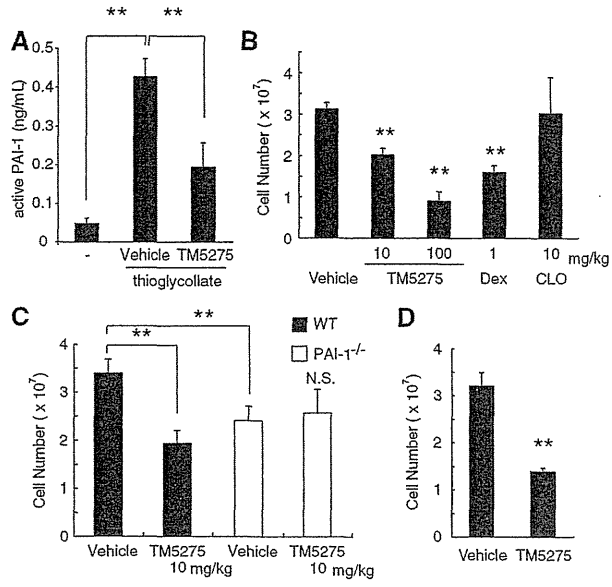

Figure 1. TM5275 inhibits the plasminogen activator inhibitor (PAI)-1–induced macrophage (Mϕ) migration in vivo. **A**, PAI-1 concentration was elevated by thioglycollate. TM5275 significantly decreased intraperitoneal levels of active plasminogen activator inhibitor 1 (PAI-1). **B**, The number of Mϕ 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.

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,

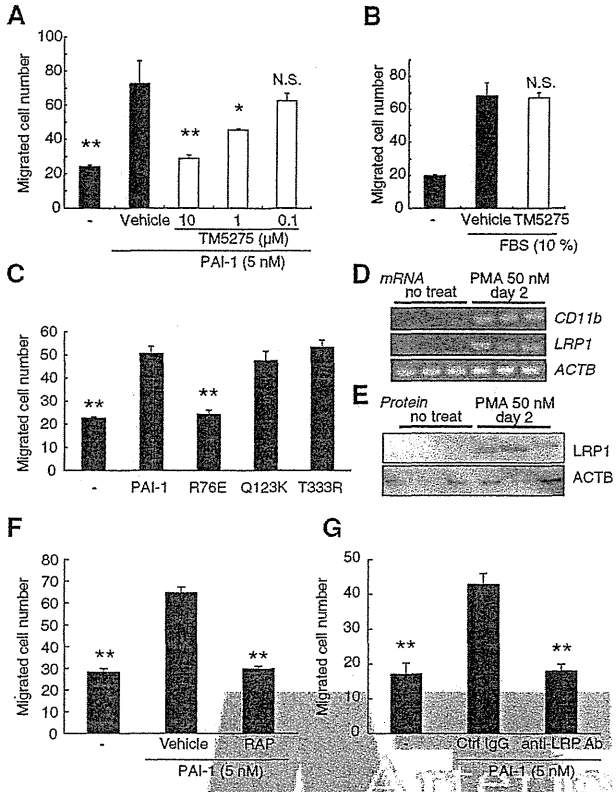

Figure 2. TM5275 inhibits plasminogen activator inhibitor (PAI)-1–induced macrophage (Mϕ) migration in vitro. PAI-1–induced cell migration depends on the interaction of PAI-1 with low-density lipoprotein receptor–related protein (LRP), but not with vitronectin (VN), urokinase-type plasminogen activator (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

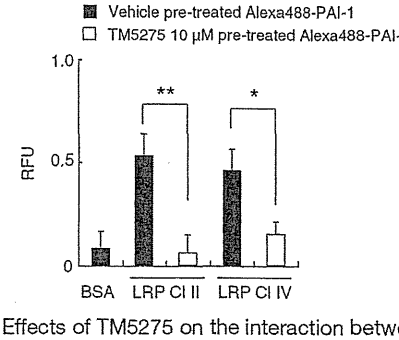

Figure 3. Effects of TM5275 on the interaction between plasminogen activator inhibitor (PAI)-1 and low-density lipoprotein receptor–related protein 1 (LRP1). Recombinant LRP1 cluster II (Cl II) and Cl IV protein were immobilized on a ELISA plate, and then blocked with 3% BSA for 2 hours at 37°C. The 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.

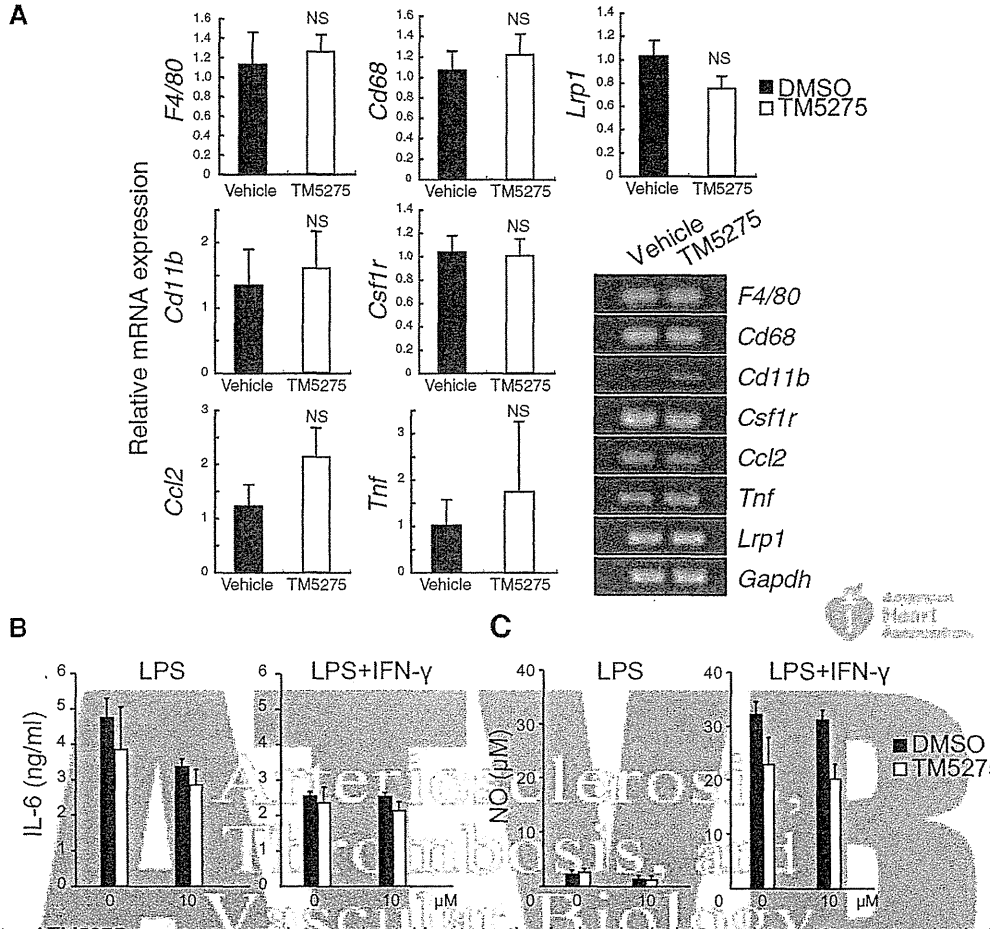

Figure 4. Effects of TM5275 on gene expressions and cytokine secretion in the phorbol 12-myristate 13-acetate, phorbol myristate acetate (PMA)-induced differentiated THP-1 cells. THP-1 cells were treated with 50 nmol/L PMA for 2 days. **A**, Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis 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 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

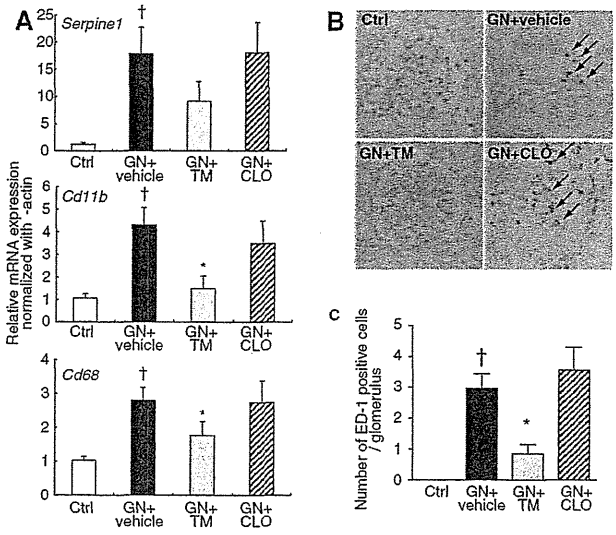

Figure 5. Effects of TM5275 on the macrophage (Mϕ) infiltration in a rat anti–Thy-1 glomerulonephritis model. **A**, Quantitative Real-time-PCR analysis for Serpine1 (plasminogen activator inhibitor 1, [PAI-1]), Cd11b, and Cd68 in the kidney 7 days after induction of glomerulonephritis (GN). **B**, Representative photomicrographs of ED-1 immunohistochemical-stained kidney sections of control, vehicle, TM5275 (30 mg/kg per day), and clopidogrel rats 7 days after induction of anti–Thy-1 glomerulonephritis. Original magnification, ×400. Fifty glomeruli were assessed randomly. ED-1 positive cells were counted in each glomerulus and averaged (**bottom**). Data are expressed as the mean and corresponding SEM. $^{**}P<0.01$ vs the vehicle-treated rat, by 1-way ANOVA, and Dunnett test, n=5 to 6. CLO indicates clopidogrel.

expression of Wt1 were observed in the glomeruli (Figure 6F–6I), whereas TM5275 treatment significantly decreased desmin expression and increased Wt1 expression (Figures 6F–6I). Neither vehicle nor clopidogrel proved effective.

These data indicate that oral administration of TM5275 not only reduces Mϕ infiltration, but also ameliorates microaneurysm formation, mesangial proliferation, endothelial dysfunction, and podocyte injury, all this without adverse effects.

Discussion

In this study, we demonstrate that PAI-1 attracts Mϕ and functions thus as a chemotactic factor. First, in the peritoneum of mice given thioglycollate, PAI-1 is involved in vivo in Mϕ migration, as shown by a specific small molecule PAI-1 inhibitor or in PAI-1 knockout mice. At least, T-cells are not required for this effect. Second, PAI-1 is essential for the in vitro Mϕ migration, assessed in a modified Boyden chamber with a human monocytic cell line THP-1. We confirmed that the effect of PAI-1 on Mϕ migration is direct, and that PAI-1–induced cell migration hinges on its interaction with LRP1, whereas VN, uPA, or tPA is not necessarily required. The effect of PAI-1 on Mϕ migration is direct. TM5275 effectively inhibits Mϕ migration by preventing the interaction between PAI-1 and LRP1, whereas it does not affect the LPS or LPS/interferon-γ–induced cytokine secretion and NO synthesis.

Previous studies suggested links of Mϕ migration to thrombotic sites and tPA-mediated fibrinolysis.[10,22,23] Cao et al[10] demonstrated that tPA, Macrophage-1 antigen (Mac-1), and LRP1 are essential for Mϕ migration. They reported that tPA promotes Mac-1–mediated adhesion, whereas PAI-1 and LRP1 facilitate Mϕ detachment from fibrin. In the present study, we also show that PAI-1 acts as a chemoattractant for Mϕ even in the absence of VN, uPA, or tPA, and that PAI-1 induces Mϕ migration through its interaction with LRP.

LRP1 is a cell surface molecule binding PAI-1. Our present observations agree with previous studies that LRP1 mediates PAI-1–induced cell migration in other type of cells, such as smooth muscle cells and microglia.[9,24] PAI-1 had no chemotactic effect on nonstimulated THP-1 cells, which are expressing low level of LRP1.

Gaultier et al[25] previously demonstrated that LRP1 deficiency in a mouse embryonic fibroblast or in a Mϕ cell line increased expressions of inflammatory mediators, such as iNOS and IL-6. Furthermore, a Mϕ-specific deletion of LRP1 in a mouse model of atherosclerosis increased production of inflammatory cytokines and exacerbated vascular lesions.[26] A lack of LRP1 signals in Mϕ enhances therefore their inflammatory activities. By contrast in our results, an in vitro blockade of PAI-1 and LRP1 interaction on Mϕ did not affect LPS-induced IL-6 production and NO synthesis. In addition, LPS-induced IL-6 production from bone marrow–derived Mϕ of PAI-1 +/+ and −/− mice were found to be comparable (data not shown). Because LRP1 recognizes at least 30 different ligands, including PAI-1,[27] the LRP-mediated Mϕ activation may vary depending on the type of LRP1 ligand that interacts with LRP.

PAI-1 expression is dramatically enhanced in inflammatory lesions of various inflammatory diseases, such as chronic kidney disease and multiple sclerosis (MS).[28–31] In fact, the genetic disruption of the *PAI-1* gene in mice experimental models ameliorates their process of diseases.[29,32,33] Inhibition of PAI-1 is thus a potentially therapeutic goal. Our present study in a rat model of anti–Thy-1 glomerulonephritis provides a clue in this direction. TM5275 treatment significantly reduced glomerular ED-1 positive cells, raised by the anti–Thy-1 antibody treatment, reduced proteinuria, and ameliorated histological injuries, such as microaneurysm formation, mesangial proliferation, endothelial dysfunction, and podocyte injury. Of note, these benefits accrue from TM5275, but not clopidogrel, administration.

Although healthy human kidneys do not express PAI-1, kidney disease leads to expression of PAI-1 in both glomeruli and tubulointerstitium.[34–37] Overexpression of PAI-1 exacerbates renal fibrosis in obstructed kidneys,[38] whereas the specific disruption of the PAI-1 gene ameliorated the injury.[32] Interestingly, the attenuation of renal fibrosis in PAI-1–deficient mice with obstructive nephropathy was associated with a substantial delay in the recruitment of Mϕ in the kidney.[32] Conversely, mice that overexpress PAI-1 exhibit increased renal damage, accompanied by increased infiltration of Mϕ[38]. Indeed, not only coagulation and fibrinolysis[39] but also Mϕ infiltration[40] into the kidney itself would be a key for induction of renal damage. In agreement with our present finding, expression of a mutant, noninhibitory form of PAI-1 decreased matrix accumulation in the anti–Thy-1–induced nephritis.[41] These observations, together with our study

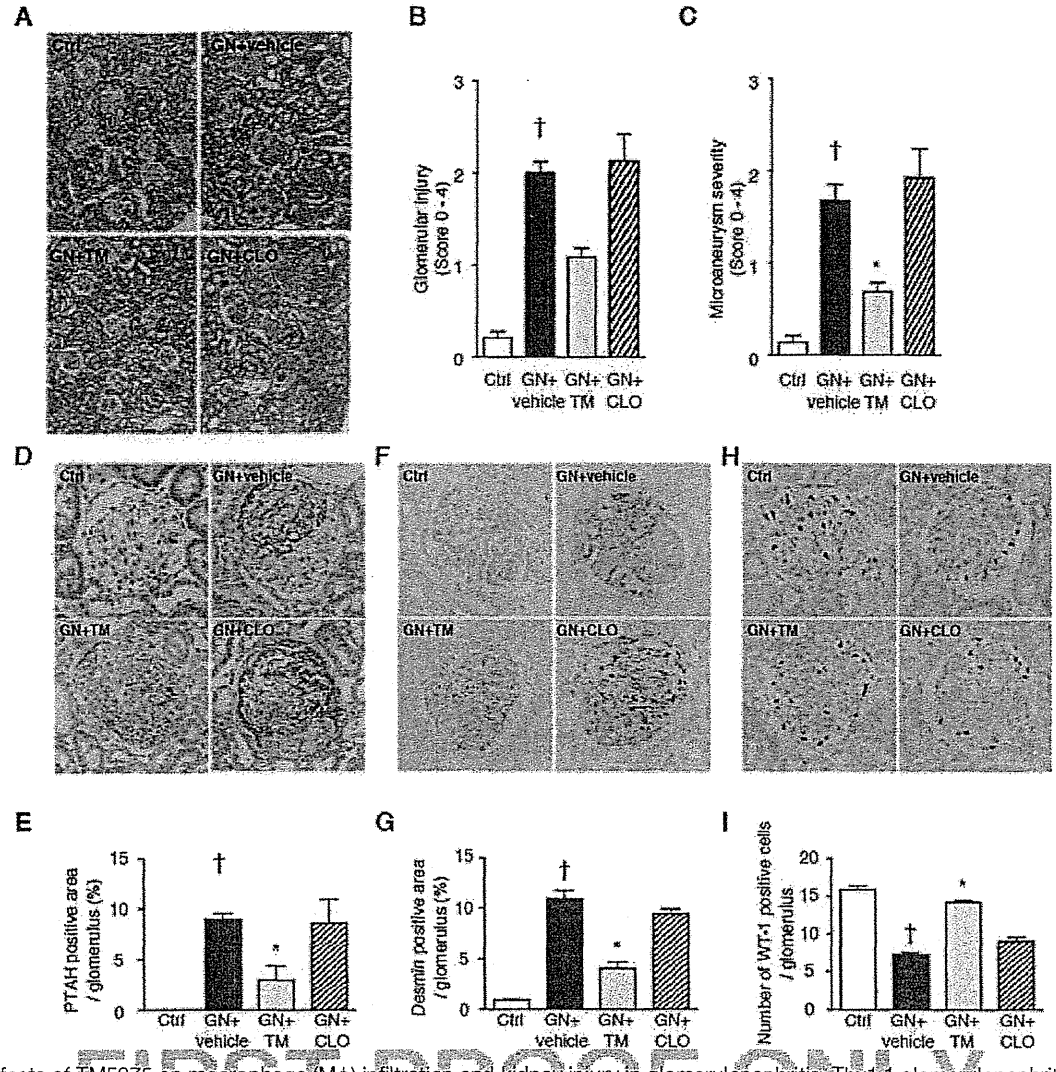

Figure 6. Effects of TM5275 on macrophage (Mϕ) infiltration and kidney injury in glomerulonephritis. Thy1.1 glomerulonephritis (GN) rats were divided into 3 treatment groups: vehicle (GN+vehicle), TM5275 (30 mg/kg per day, PO; GN+TM), and with clopidogrel (CLO; 30 mg/kg per day, PO; GN+CLO). **A**, Representative photomicrographs of Masson trichrome-stained kidney sections 7 days after induction of GN (original magnification ×100). Pathological score (**B**) and microaneurysm severity (**C**) were assessed by the area occupied in the glomerulus as follows: Score 1, 0% to 25%; Score 2, 25% to 50%; Score 3, 50% to 75%; Score 4, 75% to 100%. At least 50 glomeruli were randomly assessed. Representative photomicrographs of phosphotungstic acid-hematoxylin (PTAH; **D**), desmin (**F**), and Wt1 (**H**) stained kidney sections 7 days after induction of GN (original magnification ×400). Fifty glomeruli were observed randomly, and the area of fibrin deposition (**E**) by imaging analyses software, ImageJ ver. 1.440, desmin positive area (**G**), and the number of wild-type (WT)-1 positive cells (**I**) was assessed in each glomerulus and averaged. Data are expressed as the mean±SEM. †$P<0.01$ vs control, *$P<0.05$ vs GN+vehicle.

suggest that PAI-1 is involved in chronic kidney diseases at least in part through the involvement of PAI-1 and Mϕ infiltration, supporting the contention reviewed by Ha et al[42] that inhibition of PAI-1 could be an important therapeutic target in chronic kidney diseases. Other pleiotropic benefits of PAI-1 inhibition, such as antithrombosis, fibrinolysis, antifibrosis, and endothelial remodeling, delineated in the review of Ha et al,[42] should also be considered.

Involvement of PAI-1 is also implicated in neuroinflammation in MS.[30,31] In experimental allergic encephalomyelitis, an animal model of MS, levels of PAI-1 are significantly increased in acute MS lesions. In addition, elevated serum and tissue levels of PAI-1 have been found in MS patients.[31] Of note, high levels of PAI-1 during disease relapse are associated with an increase in Mϕ infiltration.[43] The tPA-deficient mice suffer an early onset and more severe form of disease that is associated with high levels of PAI-1.[44] By contrast in the PAI-1–deficient mice, the disease incidence and clinical severity were reduced.[33] The absence of inflammatory cells in the brain from the PAI-1–deficient mice mirrored the clinical picture. Clinical benefit of PAI-1 inhibitors in MS is of interest but remains elusive.

Small molecule PAI-1 inhibitors may thus herald a novel class of anti-inflammatory agents, preventing interaction of PAI-1 and LRP1, and thus targeting Mϕ migration. Recent studies in mice deficient of or overexpressing the PAI-1 gene implicate that in addition to its original action on fibrinolysis, PAI-1 has broad biopathophysiological functions, such

as fibrosis, cell regeneration, and inflammation.[5,6] Small molecule PAI-1 inhibitors might thus further prove useful as tools not only to unravel these disorders but also to open new therapeutic avenues.

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Disclosures

None.

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Significance

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 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 plasminogen activator inhibitor 1 inhibitor (TM5275) to investigate the role of plasminogen activator inhibitor 1 in Mϕ migration in the pathogenesis of renal injury. Mϕ migration was inhibited both in vitro and in vivo by TM5275. Fed to rats with nephritis, TM5275 significantly decreased Mϕ accumulation and ameliorated the progression of renal injury. These findings suggest that a small molecule plasminogen activator inhibitor 1 inhibitor represents a novel class of anti-inflammatory agents targeting Mϕ migration.

Figure I

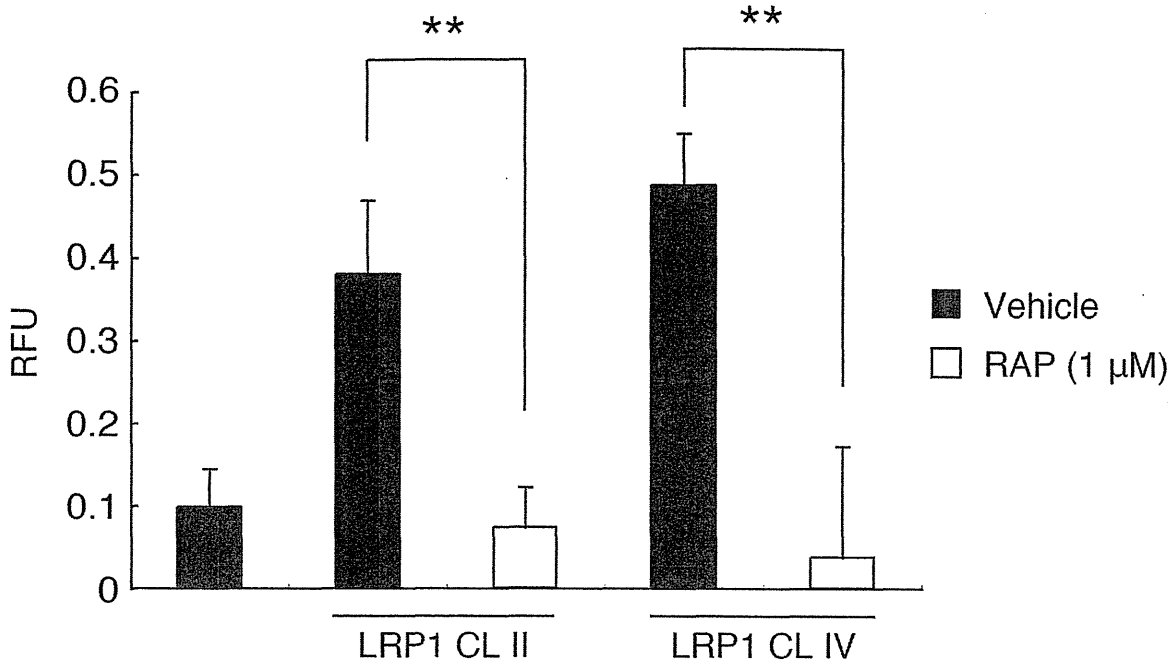

Figure I. Effects of RAP on the interaction between PAI-1 and LRP1

Recombinant lipoprotein receptor related protein-1 (LRP1) Cl II and Cl IV protein were immobilized on a ELISA plate, and then blocked with 3% BSA for 2 hr at 37 °C. Then, the plate was incubated with or without RAP (1 mM) at 37°C for 1 hr. The plate was incubated with Alexa fluor488-labeled recombinant PAI-1 protein. The direct binding between PAI-1 and LRP1 was measured as the fluorescent intensity.
Data are shown as the means and corresponding s.e.m. **P < 0.01 or by one-way ANOVA and Dunnett's test, n=10. *LRP1*, lipoprotein receptor related protein-1. *RAP*, Receptor associated protein