

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. 2013;33:935-942; originally published online March 7, 2013;
doi: 10.1161/ATVBAHA.113.301224

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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World Wide Web at:

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Data Supplement (unedited) at:

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

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Objective—Macrophage (M ϕ) migration rests on the adhesion/detachment between M ϕ surface components and extracellular matrixes, and the contribution of numerous inflammatory disorders. Plasminogen activator inhibitor (PAI)-1, a serine protease inhibitor, influences M ϕ 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 M ϕ migration in the pathogenesis of renal injury.

Approach and Results—M ϕ 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. M ϕ 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 M ϕ 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 M ϕ 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 (M ϕ) migration¹⁰ 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 M ϕ function, such as migration and cytokine secretion. We demonstrate that PAI-1 functions

Received on: October 11, 2012; final version accepted on: February 20, 2013.

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The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.301224/-/DC1>.

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Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.113.301224

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

Materials and Methods

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

Results

TM5275 Inhibits M ϕ Migration Into the Peritoneum

The effect of TM5275 on in vivo M ϕ migration was investigated. M ϕ 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 M ϕ 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 M ϕ migration in a dose-dependent manner in vivo (Figure 1B). To confirm that TM5275 inhibited M ϕ 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 M ϕ accumulation in PAI-1-deficient mice was significantly reduced as compared with wild-type mice, whereas TM5275 failed to inhibit M ϕ infiltration (Figure 1C), indicating that the PAI-1 modulates M ϕ migration.

Is TM5275-mediated inhibition of M ϕ infiltration a direct effect on M ϕ or an indirect effect through other immune cells, such as inflammatory T-cells known to activate M ϕ ?¹³ TM5275 was therefore tested in T-cell-deficient nude mice. It inhibited M ϕ 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 M ϕ migration.

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

PAI-1 Induces M ϕ Migration Via LRP

The benefit of TM5275 on the M ϕ migration was tested in vitro in a modified Boyden chamber with a human acute monocytic leukemia cell line, THP-1, which differentiates into M ϕ -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

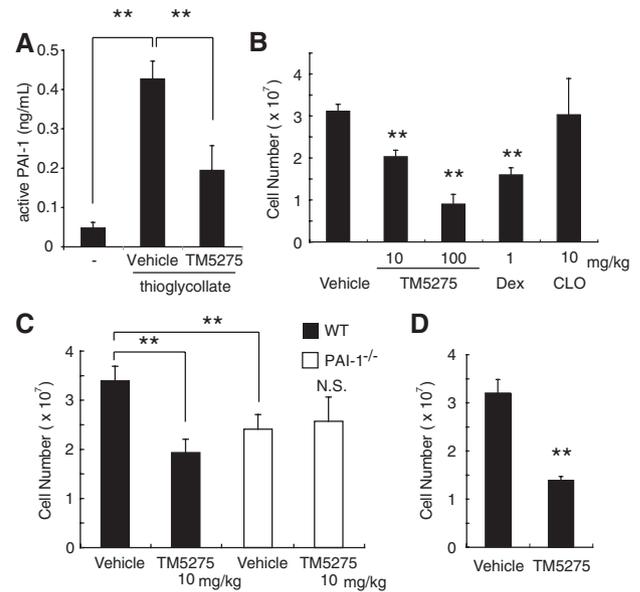


Figure 1. TM5275 inhibits the plasminogen activator inhibitor (PAI)-1-induced macrophage (M ϕ) migration in vivo. **A**, PAI-1 concentration was elevated by thioglycollate. TM5275 significantly decreased intraperitoneal levels of active plasminogen activator inhibitor 1 (PAI-1). **B**, The number of M ϕ migrating into the peritoneal cavity in vivo on stimulation by thioglycollate was assessed. TM5275 inhibited M ϕ migration in a dose-dependent manner. Dexamethasone (Dex) and Clopidogrel (CLO) were used as positive and negative controls, respectively. **C**, The number of M ϕ migrating into the peritoneal cavity in vivo on stimulation by thioglycollate was assessed in wild-type (WT) or PAI-1-deficient mice. TM5275 inhibited M ϕ migration in the WT mice but not in the PAI-1-deficient mice. **D**, The number of M ϕ migrating into the peritoneal cavity in vivo on stimulation by thioglycollate was assessed in T-cell-deficient nude mice. TM5275 inhibited M ϕ 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.

THP-1 migration induced by the fetal bovine serum, which contained matrix components, such as VN and fibronectin, all factors known to affect M ϕ migration as chemoattractants (Figure 2B). These results suggest that TM5275 inhibits only PAI-1-dependent M ϕ migration.

Several candidate proteins for M ϕ migration, for example, tPA, uPA, VN, and LRP, were investigated to elucidate the molecular mechanism(s) underlying the THP-1-derived M ϕ 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 M ϕ 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 M ϕ migration was confirmed with an LRP1 antagonist,

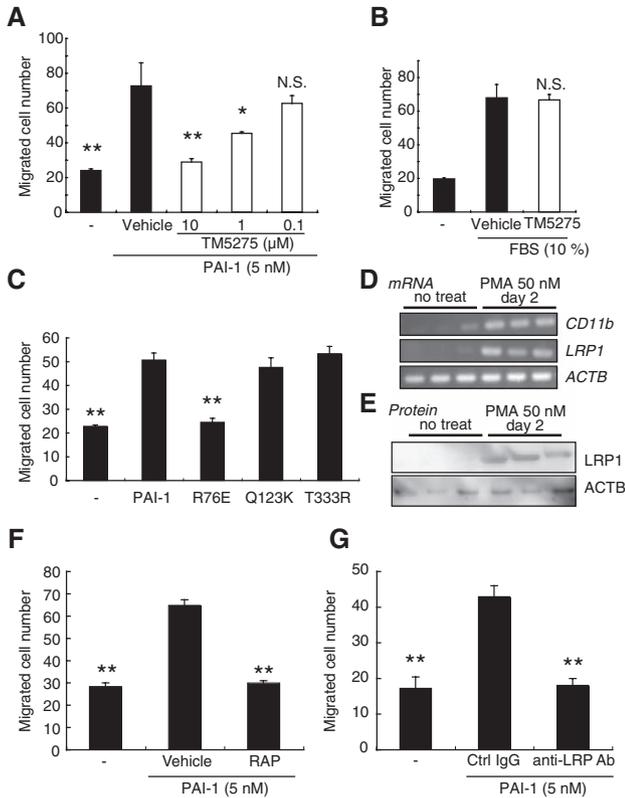


Figure 2. TM5275 inhibits plasminogen activator inhibitor (PAI)-1-induced macrophage (Mφ) migration in vitro. PAI-1-induced cell migration depends on the interaction of PAI-1 with low-density lipoprotein receptor-related protein (LRP), but not with vitronectin (VN), urokinase-type plasminogen activator (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 (CI II) and to cluster IV (CI IV) LRP.^{14,15} LRP1 protein CI II and CI 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 CI II and CI 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

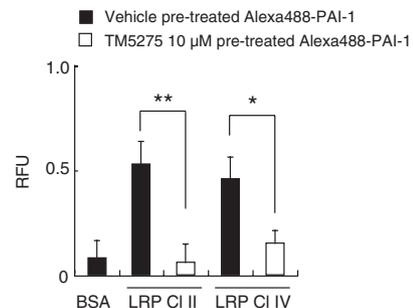


Figure 3. Effects of TM5275 on the interaction between plasminogen activator inhibitor (PAI)-1 and low-density lipoprotein receptor-related protein 1 (LRP1). Recombinant LRP1 cluster II (CI II) and CI IV protein were immobilized on an ELISA plate, and then blocked with 3% BSA for 2 hours at 37°C. The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

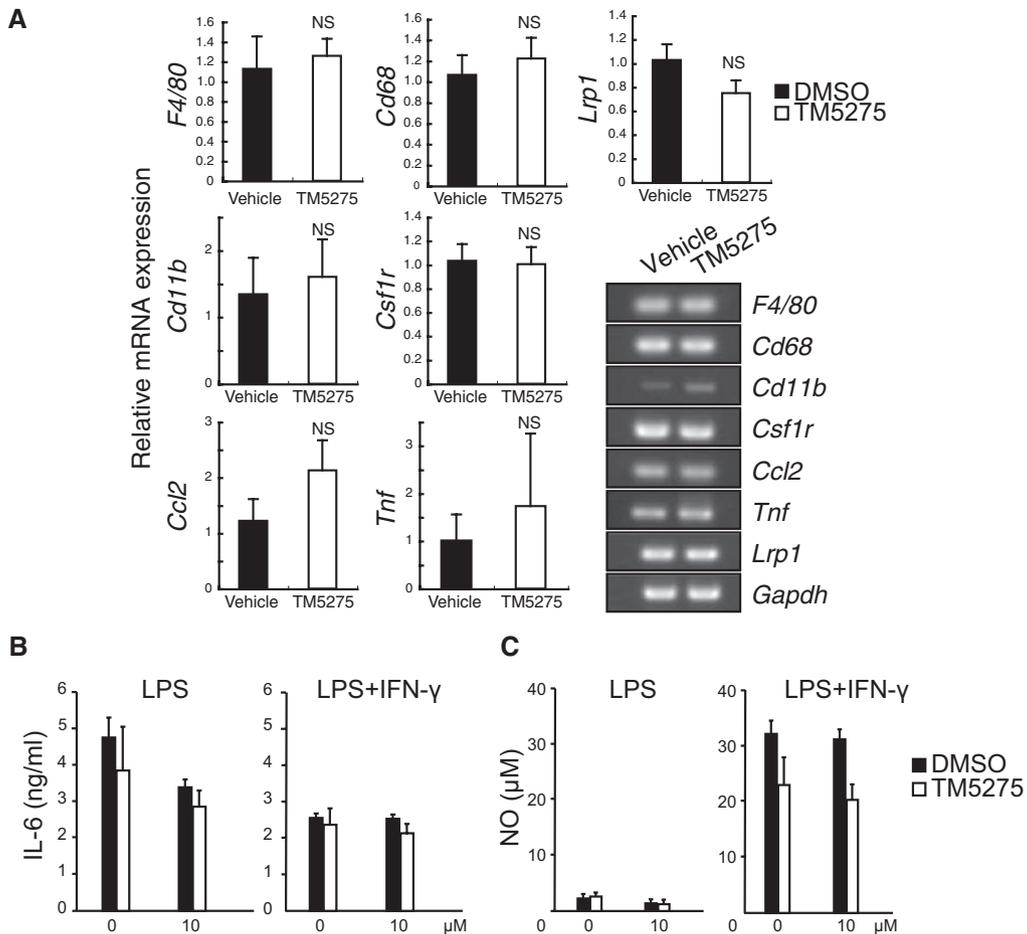


Figure 4. Effects of TM5275 on gene expressions and cytokine secretion in the phorbol 12-myristate 13-acetate, phorbol myristate acetate (PMA)-induced differentiated THP-1 cells. THP-1 cells were treated with 50 nmol/L PMA for 2 days. **A**, Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and quantitative real-time PCR analysis of *F4/80*, *Cd68*, *Cd11b*, *Csf1r*, *Ccl2*, *Tnf*, *Lrp1* 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 M ϕ 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 M ϕ 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 M ϕ infiltration 7 days after disease induction, as determined by M ϕ 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

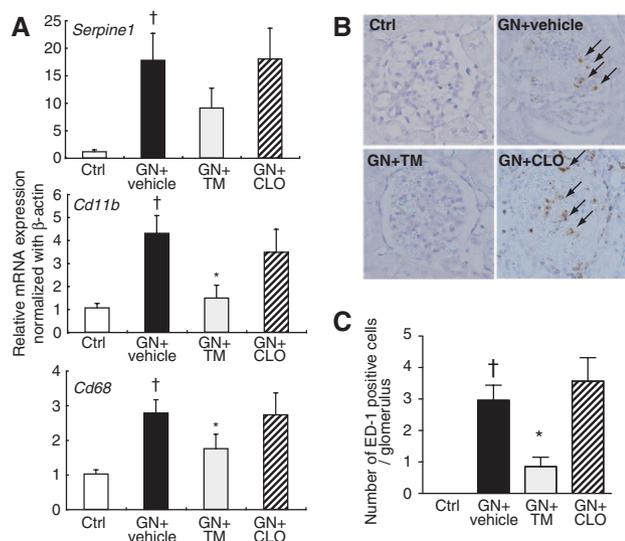


Figure 5. Effects of TM5275 on the macrophage (Mφ) infiltration in a rat anti-Thy-1 glomerulonephritis model. **A**, Quantitative Real-time-PCR analysis for Serpine1 (plasminogen activator inhibitor 1, [PAI-1]), Cd11b, and Cd68 in the kidney 7 days after induction of glomerulonephritis (GN). **B**, Representative 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 al¹⁰ 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 al²⁵ previously demonstrated that LRP1 deficiency in a mouse embryonic fibroblast or in a Mφ cell line increased expressions of inflammatory mediators, such as inducible nitric oxide synthase 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 fibrinolysis³⁹ but also Mφ infiltration⁴⁰ 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

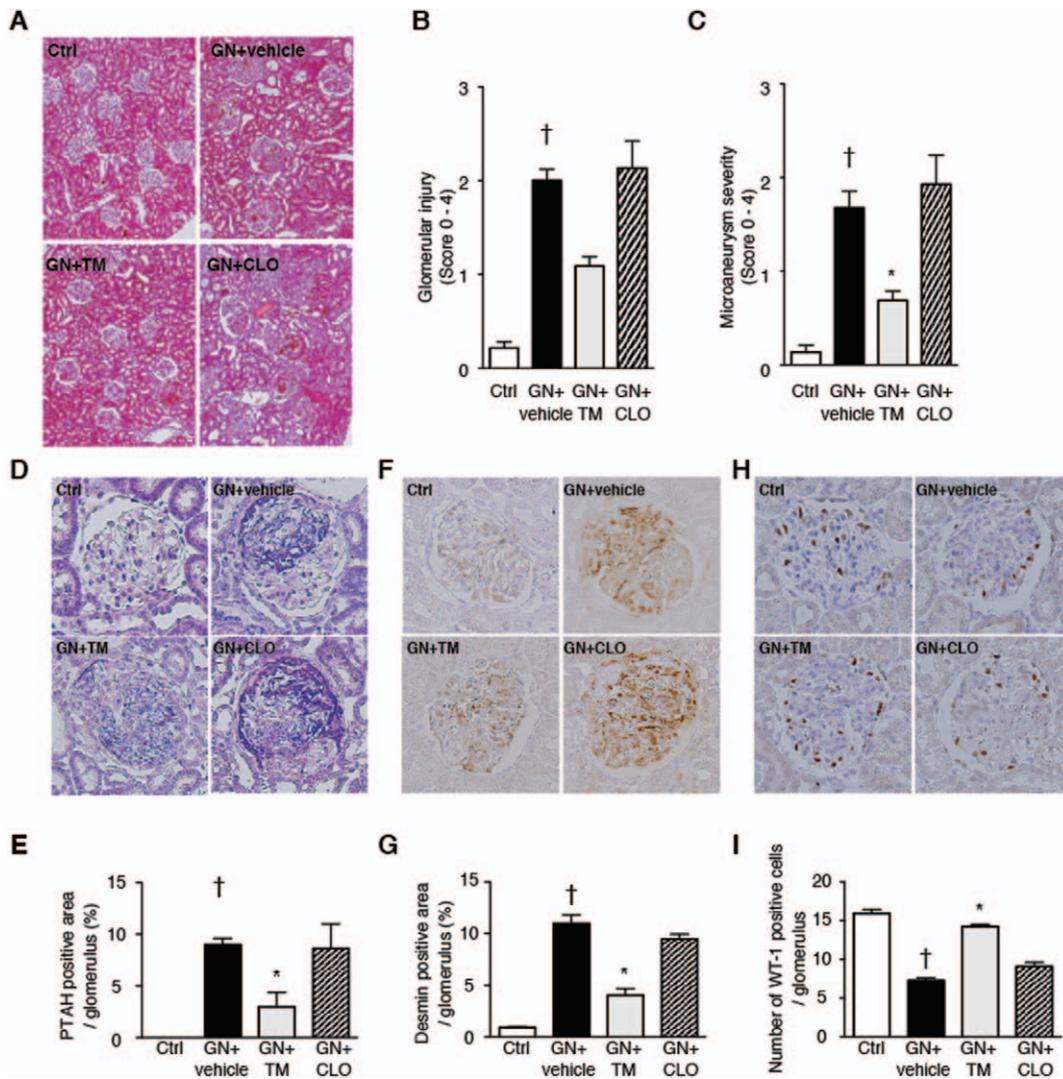


Figure 6. Effects of TM5275 on macrophage (M ϕ) infiltration and kidney injury in glomerulonephritis. Thy1.1 glomerulonephritis (GN) rats were divided into 3 treatment groups: vehicle (GN+vehicle), TM5275 (30 mg/kg per day, PO; GN+TM), and with clopidogrel (CLO; 30 mg/kg per day, PO; GN+CLO). **A**, Representative photomicrographs of Masson trichrome-stained kidney sections 7 days after induction of GN (original magnification $\times 100$). 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 $\times 400$). 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 \pm SEM. $\dagger P < 0.01$ 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 M ϕ infiltration, supporting the contention reviewed by Ha et al⁴² 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 M ϕ 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 M ϕ 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.

Sources of Funding

This work was supported in part by Grant-in-Aid for Scientific Research (A; to T. Miyata), Grant-in-Aid for Research Activity startup (to A. Ichimura) from the Japan Society for the Promotion of Science (JSPS), The Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation (NIBIO; to T. Miyata), Research on New Drug and Medical Device Development toward Recovery of the Disaster-Stricken Area from the Ministry of Health, Labor and Welfare (to T. Miyata), and by Tohoku University's Core Strategy Support Program (to T. Miyata).

Disclosures

None.

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Significance

Macrophage (M ϕ) migration rests on the adhesion/detachment between M ϕ surface components and extracellular matrixes, and the contribution of numerous inflammatory disorders. Plasminogen activator inhibitor 1, a serine protease inhibitor, influences M ϕ 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 M ϕ migration in the pathogenesis of renal injury. M ϕ migration was inhibited both in vitro and in vivo by TM5275. Fed to rats with nephritis, TM5275 significantly decreased M ϕ 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 M ϕ migration.

Figure I

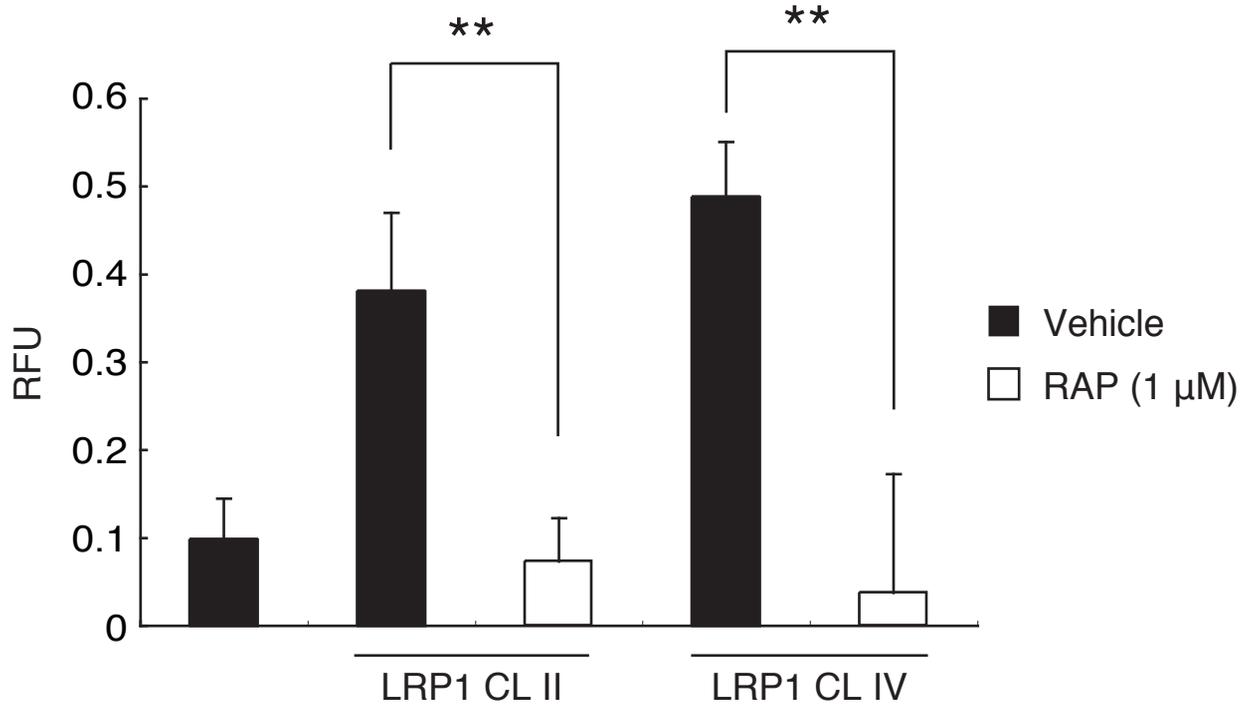


Figure I. Effects of RAP on the interaction between PAI-1 and LRP1

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

Table I. Urinary protein excretion and renal function.

	Ctrl	GN+vehicle	GN+TM	GN+CLO
Urinary protein day -1 (mg/dL)	23.0 ± 0.0	23.0 ± 0.0	23.0 ± 0.0	26.0 ± 18.8
day 1 (mg/dL)	32.0 ± 17.6	88.3 ± 11.7	72.0 ± 17.1	88.0 ± 42.7
day 3 (mg/dL)	29.0 ± 18.6	151.7 ± 25.2 †	100.0 ± 0.0 *	160.0 ± 36.7
day 5 (mg/dL)	12.0 ± 5.6	183.3 ± 44.1 †	116.0 ± 36.1 *	206.0 ± 44.0
Body Weight (g)	281.6 ± 2.7	278.7 ± 2.5	280.2 ± 5.6	267.8 ± 3.7
BUN (mg/dL)	17.2 ± 0.9	23.4 ± 3.5	17.1 ± 0.7	28.74 ± 5.5
Creatinine (mg/dL)	0.27 ± 0.03	0.18 ± 0.03	0.26 ± 0.02	0.52 ± 0.13

Body weight at day6. BUN; blood urea nitrogen. Creatinine; serum creatinine. Values are given as the mean ± s.e.m.

Table II. Sequences of primers used for the qRT-PCR (5' to 3')

Gene	Sense primer	Antisense primer
<i>Human Serpine 1</i>	GATGGCTCAGACCAACAAGTTCAA	TGGTAGGGCAGTTCCAGGATG
<i>Human Cd11b</i>	ATAGTGACATTGCCTTCTTG	ATCTTGGGTTAGGGTTGTTC
<i>Human Cd68</i>	AGTGGACATTCTCGGCTCAG	ATGATGAGAGGCAGCAAGAT
<i>Human F4/80</i>	TCGGACGGAATACTTAGACA	TCAGAGGTGGTCAAGGGAGC
<i>Human Tnf</i>	CTCCAGGCGGTGCTTGTTC	GGCTTGCTCACTCGGGGTTCCG
<i>Human Il6</i>	CACAGACAGCCACTCACCTC	TCCAAAAGACCAGTGATGAT
<i>Human Csf1r</i>	CATCCTCAGCACCAACAACG	GATAGTCCTGGCTCTGAATG
<i>Human Ccl2</i>	CCTTCTGTGCCTGCTGCTCA	ACTTGCTGCTGGTGATTCTT
<i>Human Lrp</i>	GGGCTCTGGTGGTGGATGTG	AATGTAGTCCTCGCGGGCGT
<i>Human Actb</i>	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
<i>Rat CD68</i>	AAACAGGACCGACATCAGAG	ATTGCTGGAGAAAGAACTAT
<i>Rat Cd11b</i>	GACATCCCTTCCTTCAACAG	GATGAGAGCCAAGAGCACCA
<i>Rat Actb</i>	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

Materials and Methods

Animals. The studies were performed in 8-week-old C57Bl6/J Jcl female mice and 6-week-old male Sprague-Dawley rats obtained from SLC (Shizuoka Japan). 8-week-old female Balb/c nude mice were obtained from CLEA Japan (Tokyo, Japan). Mice and rats were housed under a 12hr light-dark cycle and given regular chow, MF (Oriental Yeast Co., Ltd.). Homozygous PAI-1 deficient mice and their littermate (wild-type) mice were previously described¹. All animal experiments conformed to the National Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Committee at Tohoku University.

Materials. PMA was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque (Kyoto, Japan). Recombinant human PAI-1 and RAP was obtained from Millipore (MA, USA). Recombinant human mutant PAI-1 (Q123K, R76E, T333R) and Alexa fluor -labelled PAI-1 were obtained from Molecular Innovations (MI, USA). Human recombinants LRP1 cluster II and cluster IV were obtained from R&D systems (MN, USA). All other materials were from standard sources and of the highest purity available commercially. The media containing recombinant PAI-1 were investigated by a kit (Toxicolor® system, Seikagaku Corp., Tokyo, Japan) detecting endotoxin levels; they were endotoxin-free (<0.01 EU/ml of endotoxin).

Cell culture conditions. The human monocytic cell line THP-1 was cultured in RPMI 1640 (GIBCO, Paisley, UK) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) under 5 % CO₂.

PAI-1 inhibitor. The recently described PAI-1 inhibitor TM5275, 5-chloro-2-((2-(4-(diphenylmethyl) piperazin-1-yl)-2-oxoethoxy acetyl)amino)benzoate,

was used. TM5275 inhibits the PAI-1 activity with a half-maximal inhibition (IC₅₀) value of 6.95 mM, as measured by assay of tPA-dependent hydrolysis of a peptide substrate. In vitro, TM5275 (up to 100 mM) does not interfere with other serpin/serine protease systems such as alpha1-antitrypsin/trypsin and alpha2-antiplasmin/plasmin. Therefore, its PAI-1-inhibitory activity appears to be specific. Preincubation of PAI-1 with TM5275 abolishes detection of the covalent PAI-1-tPA complex by SDS-PAGE². TM5275 (50 mg/kg), given by gavage in rats, yields calculated plasma T_{max} , C_{max} , and $T_{1/2}$ of 2 h, 34 μ mol/L, and 2.5 hr, respectively².

In vivo Mf migration assay. The PAI-1 inhibitor TM5275 was resuspended in 200 mL of 0.5% carboxymethylcellulose (MP Biomedicals) and administered orally (10 or 100 mg/kg body weight) daily to mice from day -1 to 4 after thioglycollate broth (BD, Sparks, MD) injection. Mice were injected *i.p.* with 1 mL of 5% sterile thioglycollate broth at day 0. After 4 days, the Mf numbers in the peritoneal lavage were determined by hemocytometer. Giemsa staining confirmed that Mf represented >90% of the cells harvested 4 days after thioglycollate broth injection. Control mice received vehicle only (200 mL of 0.5% carboxymethylcellulose).

In vitro Mf migration assay. Chemotaxis assays were performed as previously described³, with a modified Boyden chamber. Briefly, THP-1 cells were treated with PMA for 2 days. Cells were collected with 0.05 % Trypsin-EDTA and washed with RPMI1640 medium. ~30,000 cells were pre-incubated with the molecules to be tested at 37 °C for 30 min, and added to the upper well of Boyden chambers (Corning Inc, Corning, NY). Cells were then incubated with the indicated amounts of PAI-1 in the RPMI1640 medium for 24 h at 37 °C. The filters were washed, and the contents of the upper surface of the inserts were removed by cotton swabs. The invading cells at the

bottom surface of the inserts were stained with DiffQuick (Sysmex Corporation, Kobe, Japan) and counted in four random high power fields/insert.

Cytokine analysis Before stimulation, THP-1 cells were differentiated for 48 h in the presence of 50 ng/ml PMA, washed three times, and rested overnight. THP-1 cells (1.5×10^5 /well) were incubated with 10 mM of TM5275 or DMSO only for 30 min before stimulation with *P. gingivalis* or *E. coli* LPS (100 ng/ml, Wako Pure Chemical Industries, Osaka, Japan) or *P. gingivalis* or *E. coli* LPS (100 ng/ml) and IFN-g (20 ng/ml, PeproTech, Rocky Hill, NJ) for 48 hr. Cytokine levels were quantitated using Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) and hIL-6 ELISA Ready-Set-Go kit (eBiosciences, San. Diego, CA, USA), according to the protocol suggested by the manufacturer.

Immunoblot analysis. Immunoblot analysis were performed as described previously⁴⁻⁶. Anti-LRP1 (Fitzgerald Industries, International; Action, MA) and anti-b-actin (Sigma-Aldrich) antibodies were used as the primary antibody.

Gene expression analysis. Total RNA was extracted from tissue or cells using ISOGEN (Nippon Gene). Quantitative reverse transcription polymerase-chain reaction (RT-PCR) analysis were performed as described previously⁶. The sequences of the primers are shown in Table S1. Real time RT-PCR was performed on a LightCycler rapid thermal cycler system using a LightCycler 480 SYBR Green I Master (Roche Applied Science) according to the manufacturer's instructions. Data were analyzed by using the comparative Ct method as means of relative quantification, normalized to an endogenous reference (b-actin, Actb) and relative to a calibrator (normalized Ct value obtained from control mice) and expressed as 2^{-DDCt} .

Rat anti-Thy-1 glomerulonephritis model. Glomerulonephritis (GN) was induced by intravenously injection of the monoclonal anti-thy1.1 antibody ER4G (1.0 mg/kg body weight) on day 0. GN rats were divided into 4 groups (n=5-6) as follows: administration of PAI-1 inhibitor, TM5275 (30mg/kg/day), was given orally during the whole observation period (from day -2 to day 7; GN+TM) or clopidogrel (30 mg/kg/day, given during the whole observation period; GN+CLO) or only 0.5% CMC which is vehicle of the drugs every day (GN+vehicle). As a normal control, another group of 5 rats received the same volume of PBS intravenously at Day 0 and orally 0.5% CMC during the whole observation period (control). Urinary excretion of protein was measured with dipstick (Terumo Co., Tokyo, Japan) at day -1, 1, 3 and 5. All rats were anesthetized and blood sample was drawn from the lower abdominal aorta 7 days after the induction of GN. After perfusion with saline, the kidneys were collected.

Histology. Part of the kidneys from individual rats was immersed and fixed overnight in 10% neutral-buffered formalin. Three-micrometer sections of paraffin-embedded tissue were stained with masson trichrome (MT) for aneurysm and collagen scoring, with periodic acid-Schiff (PAS) for matrix expansion, with phosphotungstic acid hematoxylin (PTAH) for fibrin deposition, and with hematoxylin-eosin (HE). The area of microaneurysm occupying each glomerulus and glomerular sclerosis⁷ were scored as 1 (0-25%), 2 (25-50%), 3 (50-75%), or 4 (75-100%) as shown in Fig. 6A-D, respectively. The area of mesangial matrix and fibrin deposition occupying each glomerulus was assessed by ImageJTM software (version 1.440, National Institute of Health, Bethesda, MD, USA). The number of nuclei per glomerular cross section was counted in 50 glomeruli and averaged. All microscopic examinations were performed in 50 randomly selected glomeruli from each rat by two independent observers in blinded manner.

Immunohistochemistry Indirect immunoperoxidase staining with an anti-CD68 monoclonal antibody was used to detect monocytes and Mf (1:400, ED1; Abd Serotec, Kidlington, Oxford, UK). Briefly, sections were autoclaved in 0.01 M citrate buffer (pH 6.0) at 120 °C for 5 min or were trypsinized with 0.1% trypsin, 0.1% CaCl₂, in 0.05 M Tris buffer (pH 7.6) at 37 °C for 10min in order to retrieve antigen, or were digested with Proteinase K (Proteinase K Ready-to-use; Dako, Glostrup, Denmark) in order to improve the accessibility of antigen, and then immersed in 50% methanol containing 0.3% H₂O₂ to quench endogenous peroxidase activity. Signals were amplified with the immunohistochemical staining system (Histofine simple stain MAX-PO; Nichirei Bioscience Inc., Tokyo, Japan or EnVisionTM+Mouse/HRP; DAKO Co.), visualized by DAB, and counterstained with hematoxylin.

In each slide, the number of ED1-positive cells was assessed in glomeruli. Positive areas of desmin in glomeruli were evaluated by ImageJTM software. All microscopic examinations were performed in 50 randomly selected glomeruli from each rat by two independent observers in blinded manner.

Effect of TM5275 on direct binding between PAI-1 and LRP1 Cluster II, or Cluster IV. Direct binding analysis was performed as previously described⁸. Briefly, 100 ng of LRP, cluster II, or cluster IV was immobilized for 16 h at 4 °C in microtiter wells in 50 mM NaHCO₃ (pH 8.6) in a volume of 50 ml. Subsequently, wells were blocked for 1 h at 37 °C with 3% (w/v) BSA in modified HBST buffer in a volume of 300 ml, washed with modified HBST buffer. The wells were then washed and incubated for 1 h at 37 °C in modified HBST buffer with Alexa fluor 488-labelled PAI-1, with or without pre-treatment of 10 mM TM5275 for 30 min at 37 °C. Bound proteins were measured using the SpectraMax Gemini XS (Molecular Devices, Inc., Sunnyvale, CA) as fluorescent intensity. The data were analyzed with Softmax software, and each data

point presented was the average of a triplicate determination. As controls, direct Alexa-488 labelled PAI-1 binding to immobilized BSA was measured.

ELISA. Blood was collected into tubes containing 0.1 volume of 3.8% sodium citrate for plasma, or tubes containing gel/clot activator (VENOJECT II, Terumo Co., Tokyo, Japan) for serum. Plasma levels of active PAI-1 were measured using the ELISA kit (Molecular Innovations). Serum levels of creatinine and BUN were measured with FUJI DRY-CHEM 3500 (FUJIFILM Co., Tokyo, Japan).

Statistical analysis. The level of significance for the difference between data sets was assessed using the Student's t-test. Analysis of variance followed by Tukey's test was used for multiple comparisons. Proteinuria excretion and microaneurysm severity were analyzed by two-way analysis of variance. Differences between multiple groups were analyzed by one-way analysis of variance or Kruskal-Wallis test in the case of a non-Gaussian distribution, followed by the Bonferroni, Dunn's or Tukey's post-hoc test for comparison between treatment groups. All statistical analyses were performed using the Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). Data were expressed as means \pm standard error. $P < 0.05$ was considered to be statistically significant.

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