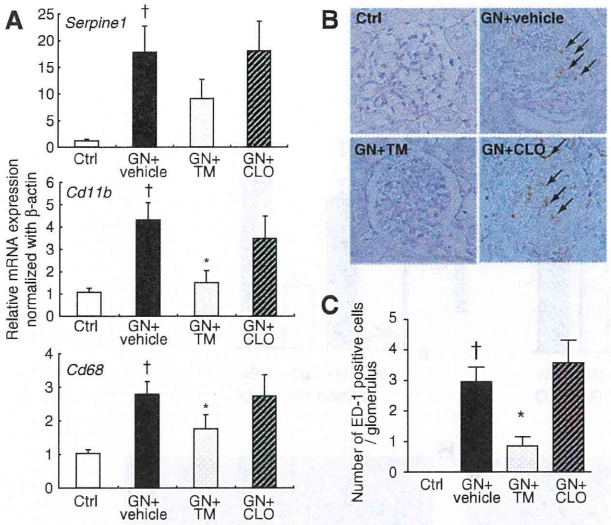

Figure 5. Effects of TM5275 on the macrophage (Mϕ) infiltration in a rat anti–Thy-1 glomerulonephritis model. **A**, Quantitative Real-time-PCR analysis for Serpine1 (plasminogen activator inhibitor 1, [PAI-1]), Cd11b, and Cd68 in the kidney 7 days after induction of glomerulonephritis (GN). **B**, Representative 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 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 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

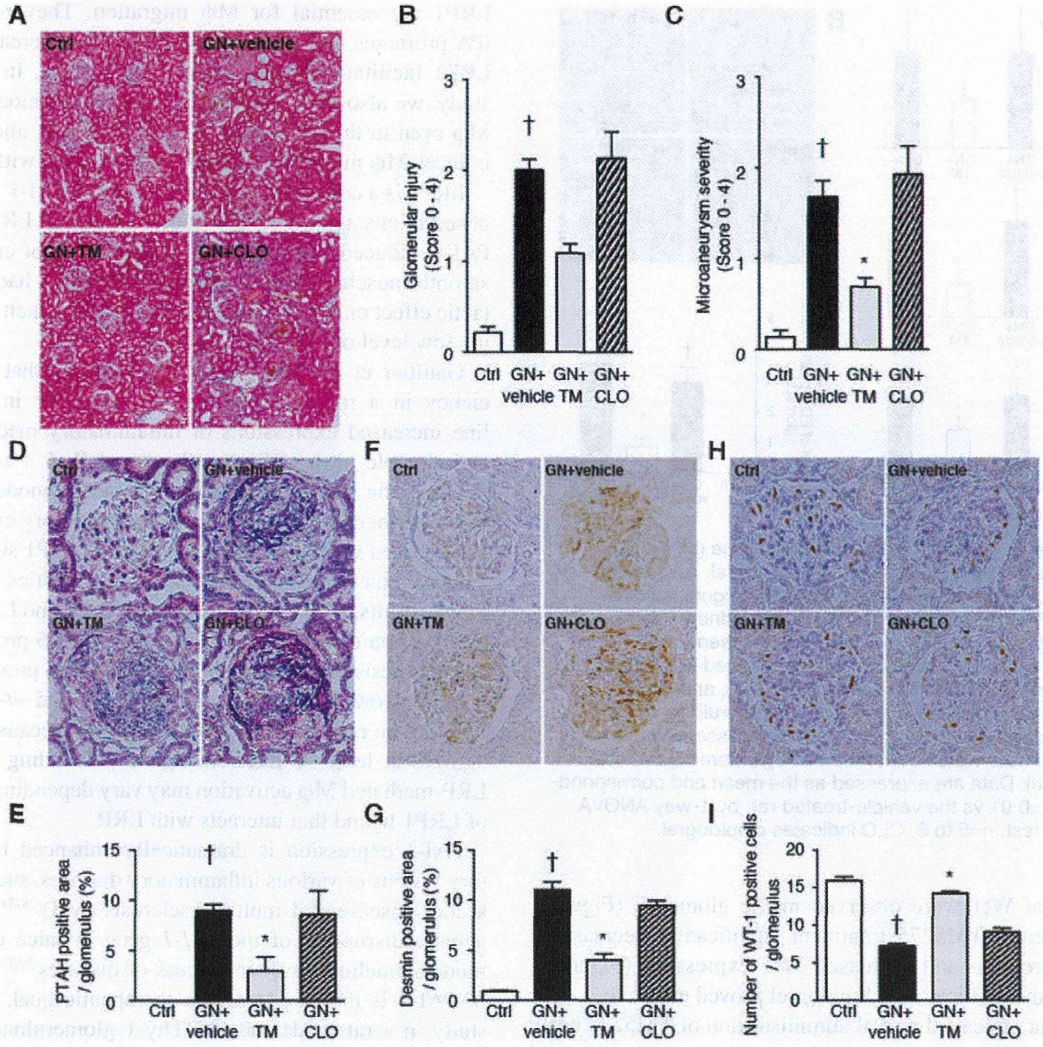

Figure 6. Effects of TM5275 on macrophage (Mϕ) infiltration and kidney injury in glomerulonephritis. Thy1.1 glomerulonephritis (GN) rats were divided into 3 treatment groups: vehicle (GN+vehicle), TM5275 (30 mg/kg per day, PO; GN+TM), and with clopidogrel (CLO; 30 mg/kg per day, PO; GN+CLO). **A**, Representative 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.

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 (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

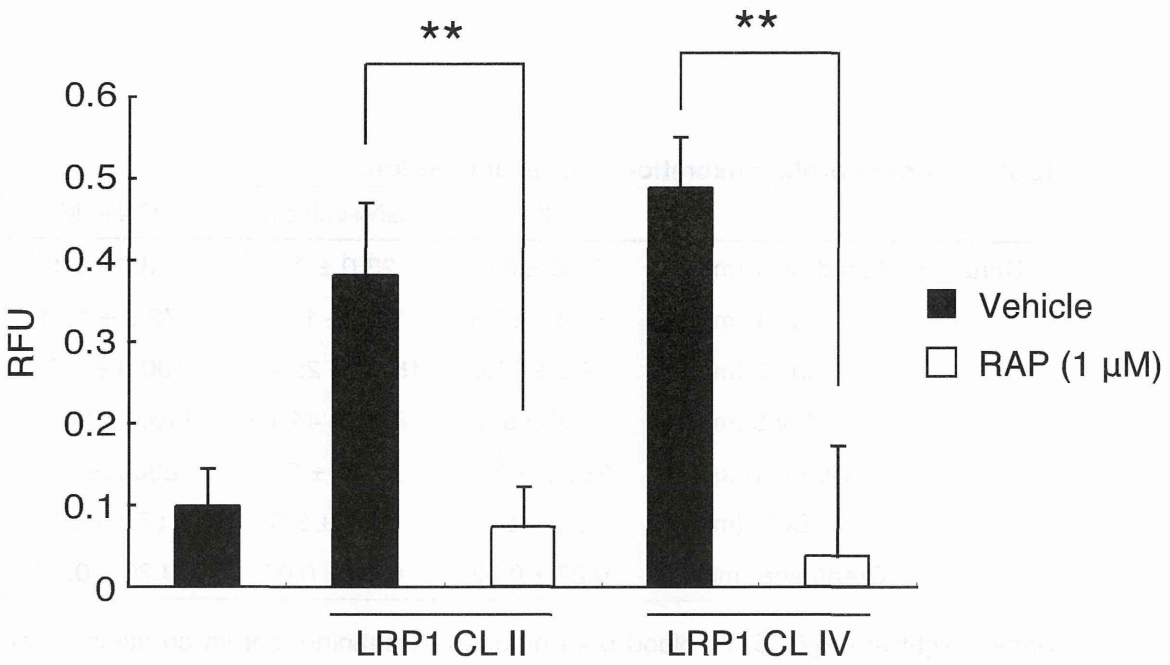

Figure I. Effects of RAP on the interaction between PAI-1 and LRP1

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

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/dL9	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
Human Serpine 1	GATGGCTCAGACCAACAAGTTCAA	TGGTAGGGCAGTTCCAGGATG
Human Cd11b	ATAGTGACATTGCCTTCTTG	ATCTTGGGTTAGGGTTGTTC
Human Cd68	AGTGGACATTCTCGGCTCAG	ATGATGAGAGGCAGCAAGAT
Human F4/80	TCGGACGGAATACTTAGACA	TCAGAGGTGGTCAAGGGAGC
Human Tnf	CTCCAGGCGGTGCTTGTTCC	GGCTTGTCACTCGGGGTTCG
Human Il6	CACAGACAGCCACTCACCTC	TCCAAAAGACCAGTGATGAT
Human Csf1r	CATCCTCAGCACCAACAACG	GATAGTCCTGGCTCTGAATG
Human Ccl2	CCTTCTGTGCCTGCTGCTCA	ACTTGCTGCTGGTGATTCTT
Human Lrp	GGGCTCTGGTGGTGGATGTG	AATGTAGTCCTCGCGGGCGT
Human Actb	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
Rat CD68	AAACAGGACCGACATCAGAG	ATTGCTGGAGAAAGAACTAT
Rat Cd11b	GACATCCCTTCCTTCAACAG	GATGAGAGCCAAGAGCACCA
Rat Actb	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 described[1]. 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 % CO_2.

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-PAGE[2]. TM5275 (50 mg/kg), given by gavage in rats, yields calculated plasma T_{max}, C_{max}, and $T_{½}$ of 2 h, 34 μmol/L, and 2.5 hr, respectively[2].

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 described[3], 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 previously[4-6]. 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 previously[6]. 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 sclerosis[7] 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 ImageJ™ 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% H_2O_2 to quench endogenous peroxidase activity. Signals were amplified with the immunohistochemical staining system (Histofine simple stain MAX-PO; Nichirei Bioscience Inc., Tokyo, Japan or EnVision™+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 ImageJ™ 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 described[8]. Briefly, 100 ng of LRP, cluster II, or cluster IV was immobilized for 16 h at 4 °C in microtiter wells in 50 mM $NaHCO_3$ (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 ± standard error. $P<0.05$ was considered to be statistically significant.

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