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Noninhibitory PAI-1 enhances plasmin-mediated matrix degradation both *in vitro* and in experimental nephritis

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Plasminogen activator inhibitor-type 1 (PAI-1) is thought to be profibrotic by inhibiting plasmin generation, thereby decreasing turnover of pathological extracellular matrix (ECM). A mutant, noninhibitory PAI-1 (PAI-1R) was recently shown by us to increase glomerular plasmin generation and reduce disease in anti-thy-1 nephritis. Here, *in vitro* and *in vivo* studies were performed to determine whether enhanced plasmin-dependent ECM degradation underlies the therapeutic effect of PAI-1R. ^{3}H-labeled ECM was produced by rat mesangial cells (MCs). The effect of wild-type PAI-1 (wt-PAI-1) and PAI-1R on ECM degradation by newly plated MCs was measured by the release of ^{3}H into medium. *In vivo*, anti-thy-1 nephritis was assessed in normal, untreated diseased and PAI-1R treated rats with or without the plasmin/plasminogen inhibitor, tranexamic acid (TA). wt-PAI-1 totally inhibited plasmin generation and reduced ECM degradation by 76% when exogenous plasminogen was added. Although PAI-1R alone had no effect, PAI-1R in the presence of wt-PAI-1 reversed the wt-PAI-1 inhibition of ECM degradation in a time- and dose-dependent manner ($P<0.001$). Plasmin activity and zymography were consistent with ECM degradation. Plasmin inhibitors: α2-antiplasmin, aprotinin, and TA completely blocked PAI-1R's ability to normalize ECM degradation ($P<0.001$). Consistent with the *in vitro* results, TA reversed PAI-1R-induced reductions in glomerular fibrin and ECM accumulation. Other measures of disease severity were either unaltered or partially reversed. PAI-1R reduces pathological ECM accumulation, in large part through effectively competing with native PAI-1 thereby restoring plasmin generation and increasing plasmin-dependent degradation of matrix components.

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KEYWORDS: PAI-1; plasmin; fibrosis; kidney; ECM degradation

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Progressive accumulation of extracellular matrix (ECM) in glomeruli and interstitium characterizes chronic renal disease, regardless of the underlying etiology.[1,2] Current data strongly suggest that decreased ECM degradation contributes to matrix accumulation,[3] and that ECM degradation is largely controlled by the plasminogen activator (PA)/plasminogen/plasmin system.[3–5] Plasmin, produced by the action of urokinase type plasminogen activator (uPA) or tissue type plasminogen activator (tPA) on plasminogen, is involved in ECM degradation directly by degrading matrix proteins fibronectin, laminin, proteoglycan, and type IV collagen as well as fibrin,[6–8] and indirectly by converting inactive matrix metalloproteinases to active forms that degrade collagenous proteins.[9–11] Plasminogen activator inhibitor-type 1 (PAI-1), as the principal inhibitor of uPA and tPA, regulates the formation and activity of plasmin. Increased PAI-1 and decreased PA and plasmin have been reported in many experimental and human glomerular diseases characterized by mesangial matrix accumulation.[12] The importance of PAI-1 in normal glomerular mesangial matrix turnover was clearly shown when matrix degradation increased four-fold after a monoclonal antibody to PAI-1 was added to cultured mesangial cells (MCs).[13] That PAI-1 facilitates ECM accumulation *in vivo* was shown in the bleomycin model of pulmonary fibrosis when decreased fibrosis was seen in PAI-1-deficient and increased fibrosis was seen in PAI-1-overexpressing mice.[14]

Based on the physicochemical properties of PAI-1, the mutant PAI-1R was constructed on the background of a stable wild-type (wt) PAI-1 mutant with half-life of 142 h.[15] PAI-1 proteinase inhibition was disabled by the introduction of two Arg residues at positions 333 and 335 (residues P14 and P12 of the reactive center loop).[16] These residues are near the hinge region of the reactive center loop and, upon reaction with a proteinase, normally fold into β-sheet A with their side chains oriented towards the interior of PAI-1. Mutations in this region greatly reduce the rate of conformational rearrangement associated with proteinase inhibition, abolishing the molecules inhibitor capacity.[17] These two substitutions would be expected to greatly retard the insertion of the reactive center loop into β-sheet A upon

interaction with a proteinase and also prevent the mutant from adopting the latent conformation. Since loop insertion also results in loss of vitronectin (Vn) affinity, PAI-1R not only fails to inhibit all proteinases but retains significant Vn affinity.[16] Investigating the therapeutic potential of this mutant, we have recently shown that PAI-1R administration to nephritic rats increases glomerular plasmin generation and reduces disease.[18] The studies reported here were designed to test the hypothesis that the therapeutic effect of PAI-1R involves enhanced plasmin-dependent ECM degradation.

Tranexamic acid (TA) inhibits plasmin proteolysis by blocking lysine binding sites on plasminogen and plasmin. TA blocks plasmin(ogen) binding to its proteolytic substrates[19,20] and interferes with plasminogen binding to cellular receptors, where cell surface-bound uPA efficiently converts it to plasmin.[21] TA has been successfully used in mice with bleomycin-induced pulmonary fibrosis to show that the disease reduction observed in PAI-1 null mice is plasmin-dependent.[22] We use TA here to determine whether the therapeutic effect of PAI-1R is plasmin-dependent.

RESULTS

Study 1

Composition of ECM produced by MCs. Immunofluorescent staining revealed that ECM produced by MCs *in vitro* was similar to that *in vivo* and contained type IV collagen, fibronectin, and laminin. Vn was also included in the ECM (Figure 1). Although this Vn could have been produced by MCs, it is likely that it was deposited from the serum present in culture medium during MC matrix production. Without primary antibodies, no staining was seen.

Dependence of ECM degradation on added plasminogen. In the absence of exogenously added plasminogen, no ^{3}H was released into culture medium. In contrast, addition of 4 μg/ml plasminogen (Chromogenix-Instrumentation Laboratory SpA, V le Monza, Milano, Italy) resulted in 82% of total counts being released in 72 h (data not shown). Although plasminogen, the substrate for plasmin generation, is in high concentration in plasma and therefore normally available to MCs *in vivo*, this result indicates that plasminogen is not present in this culture setting and must be added in order to observe matrix degradation.

***Effects of wt-PAI-1 and PAI-1R on degradation of matrix proteins* in vitro.** As shown in Figure 2a, addition of wt-PAI-1 in the presence of exogenous plasminogen for 72 h reduced the release of ^{3}H into medium in a dose-dependent manner ($^*P<0.01$ vs Control). When 1.5×10^{-7} M wt-PAI-1 was added for 24, 48, or 72 h, release of ^{3}H into medium was reduced by 76% ($P<0.001$) (Figure 2b). In striking contrast, addition of PAI-1R at 1.5×10^{-7} M had no effect on the

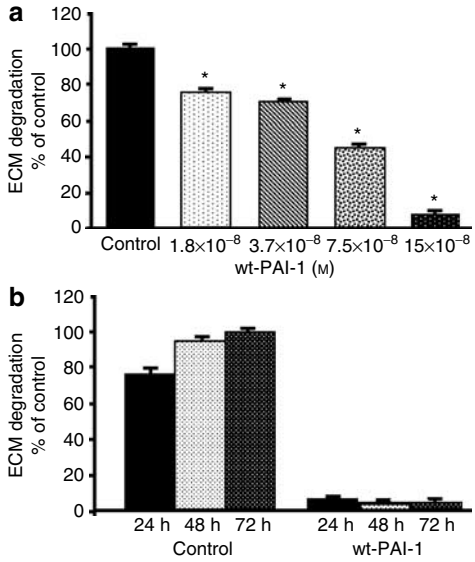


Figure 2 | Dose (a) and time (b) effects of wt-PAI-1 on ECM degradation. $^*P<0.01$ vs control.

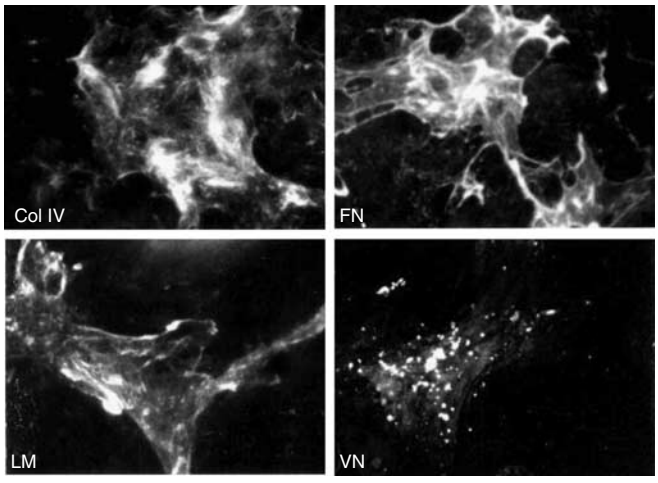


Figure 1 | Immunofluorescent staining for ECM produced *in vitro* by MCs. Original magnification $\times$400. Col IV, Collagen type IV; FN, fibronectin; LM, lamin; VN, vitronectin.

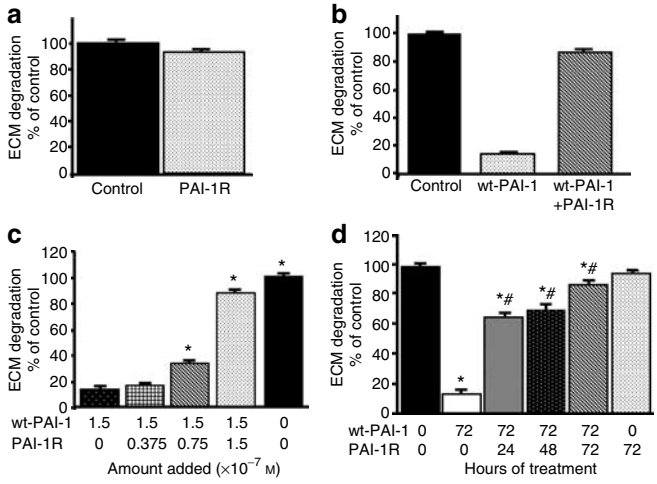


Figure 3 | Effect of PAI-1R alone on ECM degradation (a) and the protective effect of PAI-1R on wt-PAI-1-induced inhibition of ECM degradation (b–d). (**c**) $^*P<0.05$ vs wt-PAI-1 alone treated. (**d**) $^*P<0.05$ vs control, $^\#P<0.05$ vs wt-PAI-1 alone treated for 72 h.

release of ^{3}H (Figure 3a). When both PAI-1R and wt-PAI-1 were added together at 1.5×10^{-7} M for 72 h, the inhibition of release of ^{3}H seen with wt-PAI-1 was significantly reversed by PAI-1R (Figure 3b, $P<0.001$). This effect is dependent on the dose of PAI-1R added (Figure 3c) and becomes significant when PAI-1R is added at half the dose of wt-PAI-1. As shown in Figure 3d, the ability of PAI-1R to reverse wt-PAI-1-induced inhibition of release of ^{3}H is decreased if PAI-1R was added later than wt-PAI-1. A partial but significant ($P<0.05$) reversal was seen if PAI-1R was added for the last 24 or 48 h. Overall, the results indicate that wt-PAI-1 almost completely blocks mesangial matrix degradation while PAI-1R does not. Importantly, mutant PAI-1R can reverse the wt-PAI-1-induced inhibition of degradation.

Corresponding results of plasmin activity and plasmin zymography are shown in Figure 4. 1.5×10^{-7} or 3×10^{-7} M PAI-1R had no effect on plasmin activity in culture supernatant or in the ability of supernatant to degrade casein (Figure 4a and b, third and fourth bars and lanes). In contrast, wt-PAI-1, added at the same concentrations, totally blocks the plasmin activity and degradation of casein seen in the cell-only control (Figure 4a and b, fifth and sixth bars and lanes). The last two lanes of Figure 4a and b indicate that when PAI-1R and wt-PAI-1 are both added at 1.5×10^{-7} or 3×10^{-7} M, plasmin activity and casein degradation are partially reversed toward the cell control value. The ability of PAI-1R to normalize ECM degradation was completely blocked by each of three plasmin inhibitors (α2-antiplasmin (40 μg/ml), aprotinin (68 μg/ml) and TA (50 μg/ml), $P<0.001$) (Figure 5). These results indicate that the *in vitro* PAI-1R effects on matrix degradation are plasmin mediated.

Study 2

In vivo *effect of blocking plasmin/plasminogen with TA on the therapeutic effect of PAI-1R.* *Urinary protein excretion:* Disease-induced increases in urinary protein excretion were reduced 57% by PAI-1R treatment (Figure 6). Administration of TA with PAI-1R did not reverse the therapeutic effect of

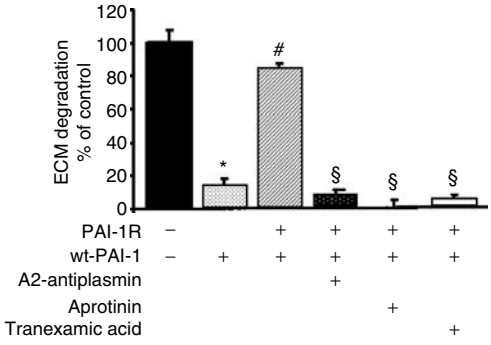


Figure 5 | Effects of plasmin inhibitors on the ability of PAI-1R to normalize the wt-PAI-1-induced inhibition of ECM degradation. *$P<0.05$ vs Control, $^{\#}P<0.05$ vs wt-PAI-1 alone treated. $^{\S}P<0.05$ vs PAI-1R and wt-PAI-1 treated.

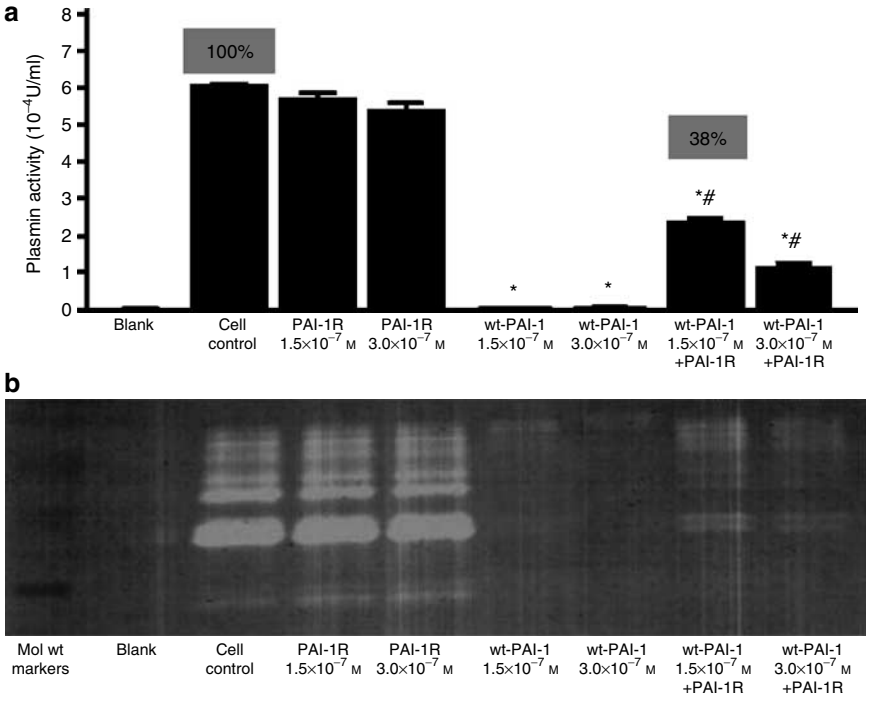


Figure 4 | Plasmin activity in cultured supernatant after 72 h treatment was determined by a (a) chromogenic substrate assay and (b) 4–16% Zymogram. In the Zymogram assay, equal amount of culture supernatant was separated by 4–16% Zymogram (blue casein) gel. The gels were then incubated in the NOVEX zymogram renaturing buffer twice for 30 min at room temperature, and then incubated at 37°C overnight in NOVEX zymogram developing buffer. The gel was photographed by a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). *$P<0.05$ vs Control, $^{\#}P<0.05$ vs wt-PAI-1 treated.

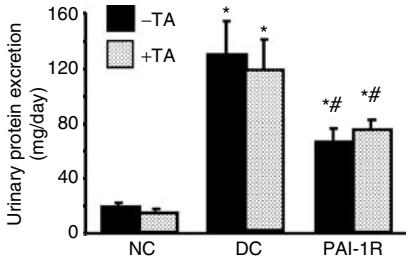
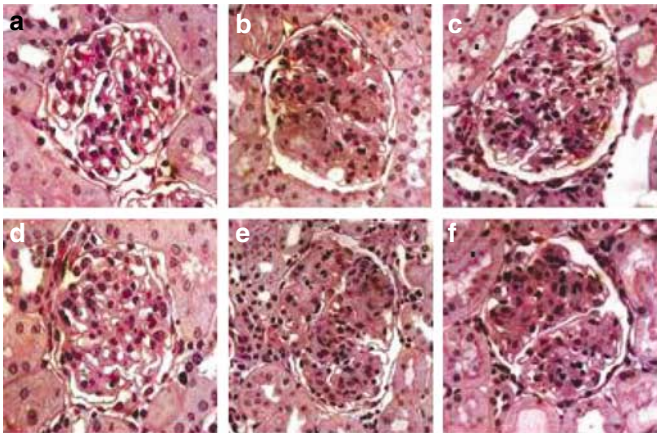


Figure 6 | Effect of TA on 24-h urinary protein excretion from d5 to d6. $^{*}P<0.05$ vs normal control (NC). $^{\#}P<0.05$ vs disease control (DC).



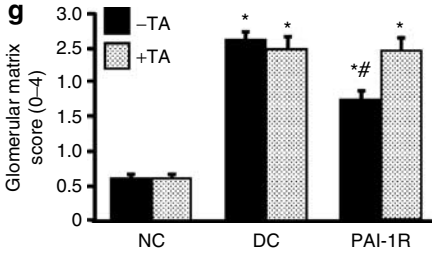


Figure 7 | Glomerular histology. Representative photomicrographs of glomeruli from normal control rats treated (**d**) with or (**a**) without TA, disease control rats treated (**e**) with or (**b**) without TA, and PAI-1R treated nephritic rats treated (**f**) with or (**c**) without TA at d6. (**g**) Graphic representation of PAS staining scores of each group. A total of 30 glomeruli per animal were scored for PAS staining, scores were averaged for each animal, and then for each group. $^{*}P<0.05$ vs normal control (NC). $^{\#}P<0.05$ vs disease control (DC).

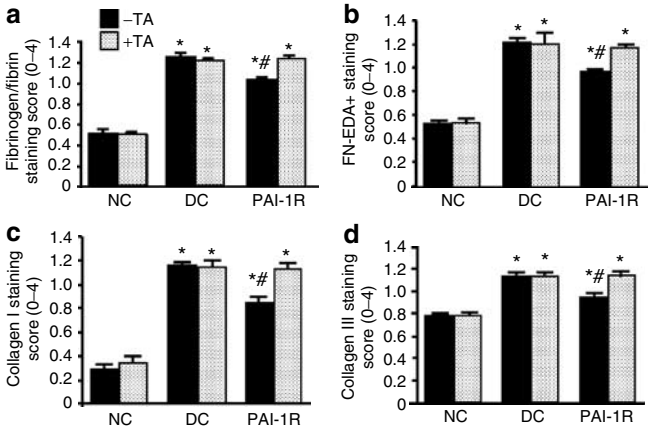


Figure 8 | Immunofluorescent staining score for ECM proteins at d6. FITC-conjugated rabbit anti-human fibrinogen/fibrin (DAKO, Carpinteria, CA, USA) was used directly. Monoclonal mouse anti-cellular fibronectin EDA^{+} (Harlan Sera-Lab Belton, England), goat anti-human type I collagen and goat anti-human type III collagen (Southern Biotechnology Associates, Birmingham, AL, USA) were used as primary antibodies with FITC-conjugated rat F (ab′)$_2$ anti-mouse immunoglobulin G (H + l) (Jackson Immunoresearch laboratories, West Grove, PA, USA) and FITC-conjugated rabbit anti-goat immunoglobulin G (DAKO) as secondary antibodies. A total of 20 glomeruli per animal were scored for immunofluorescent staining, scores were averaged for each animal, and then for each group. $^{*}P<0.05$ vs normal control (NC). $^{\#}P<0.05$ vs disease control (DC).

PAI-1R on protein excretion. TA did not affect the urinary protein excretion in normal and disease control rats.

Matrix accumulation. periodic acid Schiff (*PAS) staining*: Representative glomeruli stained with PAS are shown in Figure 7. Glomeruli from the disease control rats showed marked accumulation of ECM at d6 (Figure 7b) compared with normal glomeruli (Figure 7a). Treatment of nephritic rats with PAI-1R resulted in a significant reduction in mesangial ECM accumulation in glomeruli (Figure 7c), which was reversed by administration of TA (Figure 7f). TA administration had no effect on matrix accumulation in either normal or disease control animals (Figures 7d and e). Quantitative analysis of PAS scoring presented graphically in Figure 7g indicates that administration of TA completely blocks the therapeutic effect of PAI-1R on matrix accumulation.

Immunofluorescent staining: Specific staining of glomerular proteins revealed that PAI-1R treatment significantly reduced the disease-induced increases in fibrinogen/fibrin, fibronectin (FN) EDA+, collagen I, and collagen III deposition by 31, 37, 36, and 52%, respectively (Figure 8). These effects were reversed by administration of TA. No differences in staining were seen in normal or disease control animals untreated or treated with TA.

Glomerular transforming growth factor β1 (TGFβ1) and fibronectin production: Glomeruli isolated from d6 diseased animals showed a 10.4-fold increase in fibronectin production (Figure 9a) and a 4.7-fold increase in TGFβ1 production compared to normal glomeruli (Figure 9b). FN and TGFβ1 production were reduced with PAI-1R treatment by 49% ($P<0.001$) and 33% ($P<0.05$), respectively (Figure 9). Administration of TA with PAI-1R had no effect on either FN or TGFβ1 production.

TGFβ1, PAI-1, fibronectin, and type I collagen mRNA levels: As shown in Figure 10, PAI-1R administration had no effect on the disease-induced overexpression of TGFβ1 and PAI-1 mRNAs. In contrast, PAI-1R treatment significantly reduced the levels of FN-EDA+ and type 1 collagen mRNAs by 26% ($P<0.001$) and 44% ($P<0.001$), respectively. TA administration had no effect on expression of these mRNAs in any group.

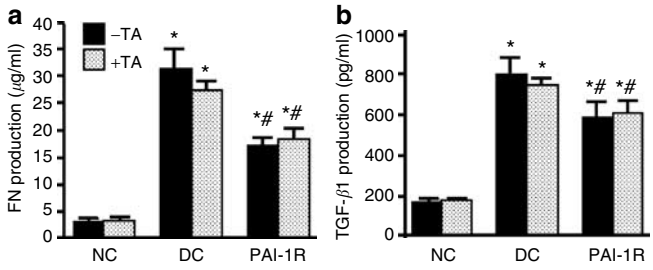


Figure 9 | Effect of TA on glomerular production of FN and TGF-β1 at d6. *$P<0.05$ vs normal control (NC). #$P<0.05$ vs disease control (DC).

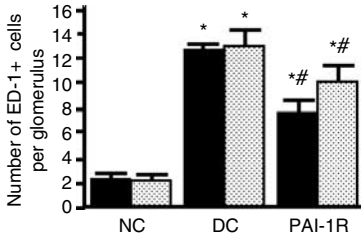


Figure 11 | Number of monocytes/macrophages infiltrating glomeruli in anti-thy-1 nephritis at d6. *$P<0.05$ vs normal control (NC). #$P<0.05$ vs disease control (DC).

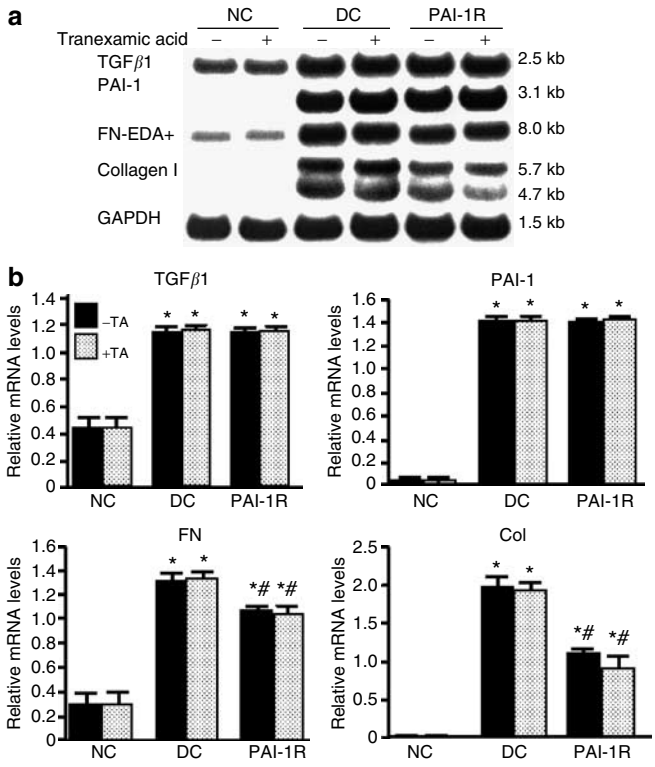


Figure 10 | Effect of TA on glomerular mRNA expression of TGF-β1, PAI-1, fibronectin, and collagen I harvested 6 days after induction of glomerulonephritis. Representative Northern blot is shown in (**a**) and relative glomerular mRNA expression was shown in (**b**). *$P<0.05$ vs normal control (NC). #$P<0.05$ vs disease control (DC).

Monocyte/macrophage infiltration: The number of monocyte/macrophage cells increased from 2.3 ± 0.32 in normal animals to 12.8 ± 0.4 in disease control animals ($P<0.001$, Figure 11). PAI-1R administration reduced this number by 48%, to 7.7 ± 0.9 ($P<0.001$). Co-administration of PAI-1R and TA partially, but not completely, reversed the elevated number of monocyte/macrophage cells. TA alone had no effect on cell number in either normal or disease control animals.

DISCUSSION

In order to study ECM degradation under well-defined conditions, we developed an *in vitro* system utilizing MCs cultured on extracellular matrices produced by MCs themselves, which contain similar matrix components to those seen *in vivo*. Utilizing this system, we confirmed that ECM degradation by cultured rat MCs is dependent upon added plasminogen and is mediated by plasmin. The fact that addition of uPA or tPA was not required for matrix degradation confirm other *in vitro* studies showing that MCs produce uPA and tPA. These data indicate that ECM degradation by cultured rat MCs is dependent on the presence of a functioning PA/plasminogen/plasmin system.

As the primary *in vivo* inhibitor of both PAs, PAI-1 tightly regulates this system and increased PAI-1 levels can dramatically reduce plasmin generation. Addition of PAI-1R, a mutant that binds to Vn but does not inhibit PAs, did not affect plasmin activity or matrix degradation in this system. This is probably because rat MCs in culture produce only about 1×10^{-9} M PAI-1 after 72 h of incubation (unpublished observation) so there would be very little endogenous PAI-1 with which PAI-1R might compete. Therefore, exogenous wt-PAI-1 was added to simulate the disease condition where glomeruli produce approximately 25-fold greater PAI-1 than normal glomeruli.[23] Addition of 1.5×10^{-7} M exogenous wt-PAI-1 dramatically inhibited plasmin activity and ECM degradation, an effect lasting at least 72 h. It is likely that Vn binding stabilizes the wt-PAI-1 in the active conformation maintaining inhibition for at least 72 h.

When PAI-1R and wt-PAI-1 were added together, the inhibition of ECM degradation seen with wt-PAI-1 was reversed in a time- and dose-dependent manner. In addition, the plasmin activity and casein-degrading capacity of culture supernatant were partially restored. These data suggest that PAI-1R successfully competes with wt-PAI-1 for Vn binding sites. When wt-PAI-1 binds a protease, it loses its affinity for Vn and is internalized and degraded. PAI-1R, by virtue of its inability to undergo the reactive center loop conformational change remains bound to Vn longer than wt-PAI-1. In this way, PAI-1R restores plasmin generation, which increases plasmin-dependent degradation of ECM components. That three inhibitors of plasmin, α2-antiplasmin, aprotinin, and TA, completely blocked PAI-1R's ability to normalize ECM degradation further confirms the plasmin dependence of ECM degradation in cultured MCs. Interestingly, while PAI-1R reverses the wt-PAI-1 effect on degradation almost completely, PAI-1R in the presence of wt-PAI-1 restores

plasmin activity to only 38% of that seen with PAI-1R alone (Figure 4). This result suggests that in this system a 38% restoration of plasmin activity by PAI-1R is sufficient to almost completely reverse the inhibition of matrix degradation by wt-PAI-1.

Next, we asked whether enhanced plasmin-dependent matrix degradation is involved in the therapeutic effects of PAI-1R in anti-thy-1 nephritis. The plasmin inhibitor TA was used to see if the therapeutic effect of PAI-1R was reversed when both PAI-1R and TA were given, a finding that would indicate plasmin dependence of the therapeutic effect. Administration of TA to PAI-1R-treated nephritic rats resulted in an interesting picture in that the therapeutic actions of PAI-1R fall into three categories with respect to plasmin-dependence. First, the PAI-1R-induced effect on pathological matrix accumulation, as indicated by PAS score, FN, Collagen I, and Collagen III staining, was completely reversed by TA treatment indicating plasmin dependence of these therapeutic actions. In contrast, TA had no effect on other measures of disease including urinary protein, FN, and TGFβ1 production by nephritic glomeruli in culture or FN and Collagen I mRNA levels. Therefore, these are plasmin-independent therapeutic effects of PAI-1R. A third category of effect was seen with the number of ED-1+ cells per glomerulus. Co-administration of PAI-1R and TA partially, but not completely, reversed the infiltration of monocyte/macrophage cells suggesting a combination of plasmin-independent and plasmin-dependent mechanisms.

These data are very interesting when compared with those we obtained by treating nephritic rats with tPA.[24] t-PA enhances plasmin generation and subsequent matrix degradation without altering any disease markers except those of matrix accumulation suggesting a pure plasmin-dependent effect of tPA. The current data indicate that PAI-1R treatment also enhances plasmin generation but, because other measures of disease were altered, suggests that PAI-1R has other actions in addition to enhancing plasmin generation.

No effect of TA was detected in normal animals suggesting that blocking the normal actions of plasminogen/plasmin did not affect the variables measured here in the 5 days of treatment. TA also had no measurable effect in disease control animals. This is probably because the dramatically increased PAI-1 in disease control kidney already maximally blocks plasmin generation from plasminogen.

Combined with the *in vitro* data that PAI-1R enhances plasmin-mediated degradation of matrix proteins, these *in vivo* data support the hypothesis that part of the therapeutic effect of PAI-1R in renal fibrosis is to enhance plasmin-dependent ECM degradation. These data further support the notion that PAI-1R has multiple actions on important measures of disease severity. However, because it is the accumulation of matrix that ultimately obliterates organ function, we conclude that PAI-1R actions to enhance matrix degradation are key to its antifibrotic effect, at least in this model of disease. It will be interesting to see if plasmin-independent actions of PAI-1R on disease severity, particularly macrophage/monocyte infiltration, produce an effect on ECM accumulation in longer-term models of renal fibrosis.

In summary, these *in vitro* and *in vivo* observations provide convincing evidence that PAI-1R reduces pathological ECM accumulation in anti-thy-1 nephritis through effectively competing with native PAI-1 for Vn binding sites, thereby restoring plasmin generation and increasing plasmin-dependent degradation of matrix components.

MATERIALS AND METHODS

Study 1. *In vitro* studies of the effects of mutant PAI-1R on ECM degradation by MCs

Materials. Unless otherwise indicated, materials, chemicals, and culture media were purchased from Sigma-Aldrich (St Louis, MO, USA). Wild-type PAI-1 (wt-PAI-1) was produced as an active form in *Escherichia coli*.[25]

Production of radioactive matrices. Rat MC matrices were obtained as described previously.[26,27] Briefly, MCs were seeded into six-well plates at 2×10^5 cells/well. Ascorbic acid (25 μg/ml) was added to the culture on the second day and daily thereafter. The medium was changed twice weekly. L-(2,3-^{3}H)-proline was added to the culture medium at 0.5 μCi/ml on days 3, 7, and 10 after seeding. At 2 weeks after seeding of the cells, cultures were washed with phosphate-buffer saline and the cells were lysed by treatment with 2 ml 2.5 mM NH_4OH, 0.1% Triton X-100 for 2 min. The matrices were then washed extensively with distilled water and kept covered with distilled water under sterile conditions at 4°C until further use.

Characterization of matrix produced by MCs in culture. ECM was produced by MCs without added L-(2,3-^{3}H)-proline. Rabbit anti-human type IV collagen antibody (Rockland), monoclonal mouse anticellular fibronectin extra domain positive (EDA^+) (Harlan Sera-Lab Belton, England), rabbit anti-mouse laminin (Cappel, Aurola, OH, USA), and rabbit anti-mouse Vn antibody (kindly provided by Emile de Heer, Department of Nephrology and Pathology, Leiden University Medical Center, Leiden, The Netherlands) were used as the primary antibodies for detection of matrix components produced by MCs *in vitro*. Fluorescence *in situ* hybridization-conjugated swine anti-rabbit immunoglobulin G (DAKO, Corp.) and fluorescein isothiocyanate (FITC)-conjugated rat $F(ab')_2$ anti-mouse immunoglobulin G (H+l) (Jackson ImmunoResearch Laboratories Inc.) were used as the secondary antibodies. Adding either phosphate-buffer saline or nonimmunized normal serum instead of primary antibodies serve as control.

Degradation of matrices by MCs. Labeled matrices were washed three times with 2 ml of serum-free RPMI-1640 medium before addition of MCs in 15% fetal calf serum RPMI-1640 medium. Plates were incubated for 24 h to allow the MCs to attach and to recover from plating. After 24 h, the medium was removed, cells were washed three times with 2 ml of serum-free RPMI-1640 medium to remove proteolytic enzyme inhibitors potentially present in the serum and incubated for 72 h in 2 ml of serum-free RPMI-1640 containing 0.2% lactalbumin hydrolyzate (RPMI-LH).[26] Exogenously added agents were dissolved in RPMI-LH at the concentrations indicated below. At the end of the incubation, the radioactivity in culture supernatant, representing digested matrix, was measured in a scintillation counter. The matrix remaining on the plate was digested with 2 N NaOH and its radioactivity measured. Background values obtained with medium (RPMI-LH) in the absence of cells were subtracted from these values. The sum of counts in the supernatant and residual undigested matrix was

considered 100%. Percent matrix degradation was calculated as supernatant counts divided by total counts × 100. A plasmin-specific chromogenic substrate, Chromozym PL (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used to measure plasmin activity as described.[18] A measure of 80 μl of cultured supernatant and 20 μl 3 mM Chromozyme PL (Diapharma Group Inc. West Chester, OH, USA) were added per well. The rate of increase in absorbance, corresponding to plasmin activity, was measured over a 2 h interval. A standard curve was generated with serial dilutions of porcine plasmin. Results were expressed as 10^{-4} U/ml.

Zymography. To further determine plasmin activity in cultured supernatant, 30 μl of each supernatant was separated by a 4–16% Tris-Glycine gel with blue-stained β-casein incorporated as a substrate for plasmin (NOVEX, San Diego, CA, USA). Plasmin activity was seen as clear bands against a dark blue background where plasmin had digested casein.

Study 2. Plasmin dependence of the therapeutic effect of PAI-1R in experimental glomerulonephritis

Animals. Male Sprague–Dawley rats (180–200 g) obtained from the SASCO colony of Charles River Laboratories (Wilmington, MA, USA) were used. Animal housing and care were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985. Glomerulonephritis was induced by tail vein injection of the monoclonal anti-thy-1 antibody OX-7 (NCCC; Biovest International, Inc., Minneapolis, MN, USA) as previously described.[28] Normal control animals were injected with the same volume of phosphate-buffer saline.

Experimental design. Initial experiments revealed that a dose of TA equivalent to that used in mice[22] produced diarrhea and wasting in rats. Thus, a pilot experiment was carried out to determine an effective and safe dose of TA in normal rats. TA (18 mg/day) was administered to four groups of three rats by continuous subcutaneous infusion via osmotic pumps (model 2 ml1; ALZA Corp., Palo Alto, CA, USA) implanted under the dorsal skin of rats. In addition, three doses of TA from 0.02 to 20 g/l were given in drinking water to supplement the parenteral dose. All rats were killed at day 4. The results indicated that 18 mg/day in osmotic pumps plus 2 g/l in drinking water produced no diarrhea or wasting, and this dose was used in further experiments. Eight rats/group were assigned to groups including normal controls, normal rats treated with TA, disease controls, diseased rats treated with TA, diseased rats treated with PAI-1R, and diseased rats treated with PAI-1R and TA. PAI-1R was administered intraperitoneally twice a day from day 1 to d5 at a dose of 1 mg/kg body weight. TA was given from d1 to d5. At 24-h urinary protein excretion was measured by the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories Inc., Hercules, CA, USA) on urine collected from rats housed in metabolic cages from d5 to d6.

On day 6, animals were anesthetized, 5–10 ml blood was drawn from the lower abdominal aorta and kidneys were perfused with 30 ml ice-cold phosphate-buffer saline. For histological examination, cortical tissue was snap frozen for frozen sectioning or fixed in 10% neutral-buffered formalin for PAS staining. Glomeruli were isolated by graded sieving as described previously.[29] Analysis of glomerular production of FN and TGF-β1 were carried out by enzyme-linked immunosorbent assay as described.[18]

Histological analyses. All microscopic examinations were performed in a blinded fashion. Three *micrometer* sections of paraffin-embedded tissues were stained with PAS. Glomerular matrix expansion was evaluated in 30 glomeruli from each rat where the percentage of mesangial matrix occupying each glomerulus was rated on a 0–4 scale where 0 = 0, 1 = 25, 2 = 50, 3 = 75, or 4 = 100% as previously described.[30] Immunofluorescent staining for matrix proteins was performed on frozen sections as described.[18] Monocyte/macrophage infiltration into glomeruli was assessed using FITC-conjugated mouse anti-rat ED-1 antibody (Serotec, Oxford, UK). Intraglomerular deposition of fibrinogen/fibrin, Fn-EDA+, collagen I and collagen III was quantified by scoring 20 randomly selected glomeruli on a 0–4 scale as described above. The number of monocyte/macrophage cells per glomerulus was counted in 20 glomeruli selected randomly per section.

RNA preparation and northern hybridization. Total RNA was extracted immediately from isolated glomeruli using Trizol™ Reagent (GibcoBRL, Gaithersburg, MD, USA), according to the manufacturer's instructions. RNA from each group was pooled and Northern analysis was performed as previously described.[18] Three blots per probe were performed. Autoradiographic films were scanned on a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For quantitative densitometric measurements of Northern blots, all the signals were normalized in comparison with glyceraldehyde-3-phosphate dehydrogenase levels used for equal loading.

Statistics and calculation of percentage reduction in disease severity. Data are expressed as mean ± s.e.m. Statistical significance between groups was analyzed by Student's *t*-test or Welch's *t*-test. $P < 0.05$ was considered statistically significant. The disease-induced increase in a variable was defined as the mean value for the disease control group minus the mean value of the normal control group (100%). The percent reduction in disease severity in a PAI-1R-treated group was calculated as follows:

$$\{1\text{-}(\text{PAI-1R-treated group mean} - \text{normal control group mean})/(\text{disease control group mean} - \text{normal control group mean})\} \times 100.$$

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