Plasminogen activator inhibitor-1 deficiency retards diabetic nephropathy

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Background. Plasminogen activator inhibitor-1 (PAI-1) is increased in kidneys of humans and animals with diabetic nephropathy and is associated with extracellular matrix (ECM) accumulation. PAI-1 may promote ECM buildup by preventing plasmin and matrix metalloproteinase (MMP) activation. However, the importance and mechanism of PAI-1 action in the pathogenesis of diabetic nephropathy is unknown.

Methods. We investigated the effect of streptozotocin (STZ)-induced diabetes in wild-type (PAI-1+/+) mice and mice null for PAI-1 (PAI-1−/−). After 1 month of diabetes, animals were placed in metabolic cages for 24-hour urine collection. Total RNA was isolated from kidney cortex for reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis, and Western blots were quantitated from cortical protein. Primary mesangial cells were grown from Sprague–Dawley rats and used in signal transduction studies.

Results. Urinary albumin excretion (UAE) in diabetic PAI-1+/+ mice increased >threefold, but remained unchanged in PAI-1−/− mice. Transforming growth factor-β (TGF-β) and fibronectin message and protein levels were lower in diabetic PAI-1−/− vs. PAI-1+/+ mice, suggesting that PAI-1 deficiency impaired TGF-β expression despite diabetes. Indeed, recombinant PAI-1 directly stimulated TGF-β message and protein via mitogen-activated protein kinase (MAPK) signal transduction in cultured mesangial cells. Urokinase plasminogen activator (uPA) inhibited this PAI-1 action in a dose-dependent manner. The inhibitory effect of antibody to uPA receptor (uPAR) on PAI-1–induced TGF-β function suggested that uPAR mediated the cellular effect of PAI-1.

Conclusion. PAI-1 can regulate TGF-β expression by binding to uPAR and activating the extracellular-regulated signal kinase (ERK)/MAPK pathway. Therefore, PAI-1 contributes to diabetic nephropathy by regulating TGF-β and renal ECM production and may be a therapeutic target in diabetic nephropathy.

Plasminogen activator inhibitor-1 (PAI-1) prevents conversion of tissue plasminogen activator and urokinase plasminogen activator (uPA) to plasminogen [1]. Plasmin, the active form of plasminogen, is a broad-spectrum protease that degrades fibrin clots and extracellular matrix (ECM) proteins. As a result, PAI-1 is an important regulator of both fibrinolysis and tissue remodeling. PAI-1 levels are normally tightly regulated to maintain homeostasis of both processes. A deficiency in PAI-1 results in hemorrhage [2–4], while overexpression is prothrombotic contributing to coronary events and deep venous thrombosis [1]. PAI-1 is thought to promote tissue fibrosis by inhibiting plasmin and metalloproteinase ECM degradation [5]. It has been implicated in experimental glomerulonephritis [6], chronic renal transplant rejection [7], and pulmonary fibrosis [8]. Transgenic mice overexpressing PAI-1 develop significantly greater pulmonary fibrosis when administered bleomycin, while similarly treated PAI-1–deficient mice have substantially less fibrosis compared to wild-type mice [9]. PAI-1 deficiency also protects against renal interstitial fibrosis induced by unilateral ureteral obstruction (UUO) [10]. More recently, a mutant human PAI-1 that could bind to vitronectin but not inhibit plasminogen activator–decreased ECM accumulation in experimental glomerulonephritis [11].

In diabetic nephropathy, accumulation of ECM proteins in the mesangium leads to glomerulosclerosis, the hallmark of diabetic nephropathy [12]. In normal human kidney, PAI-1 levels are undetectable, but in diabetes, PAI-1 expression is up-regulated in renal glomeruli and arteries [13]. The increased glomerular PAI-1 accompanies microangiopathic injury and accumulation of ECM in patients with diabetes and may
indicate a worse prognosis [14]. The increase in tissue PAI-1 likely occurs in response to factors inherent to the diabetic environment such as increased transforming growth factor-β (TGF-β), hyperglycemia, very low-density lipoprotein (VLDL) triglycerides, and angiotensin II (Ang II), all of which activate the PAI-1 promoter [15].

To more precisely determine the role of PAI-1 in diabetic nephropathy, we induced diabetes in genetically deficient PAI-1 (PAI-1−/−) mice with streptozotocin (STZ) (Sigma Chemical Co., St. Louis, MO, USA). Lack of PAI-1 prevented albuminuria and accumulation of fibronectin mRNA and protein, a major ECM component. However, renal matrix metalloproteinase (MMP)-9 and MMP-2 activities were not increased in diabetic PAI-1−/− mice. On the other hand, both TGF-β mRNA and protein levels were significantly reduced in diabetic PAI-1−/− mice compared to diabetic wild-type (PAI-1+/+) mice. Consistent with this observation, we found that PAI-1 directly regulates TGF-β expression in mesangial cells by binding to uPA receptor (uPAR) and activating the extracellular-regulated signal kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway. Thus, the lack of PAI-1 likely is associated with a decrease in ECM production contributing to decreased ECM accumulation in diabetes. Targeting PAI-1 may be a useful therapeutic strategy to prevent glomerular fibrosis and proteinuria in diabetic nephropathy.

**METHODS**

**Mouse breeding and measurements**

PAI-1–deficient (PAI-1−/−) mice on a C57B/L6 background (breeding pairs) and wild-type C57B/L6 (PAI-1+/+) mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and placed on a protocol approved by the Animal Research Committee of the University of California, Los Angeles. Mice were ad libitum in isolation-topped cages under a 12-hour light-dark regime. Ten-week-old mice were divided into nondiabetic PAI-1−/− (N = 14), diabetic PAI-1+/+ (N = 15), nondiabetic PAI-1−/− (N = 16), and diabetic PAI-1−/− (N = 13) mice. Diabetes was induced with a single intraperitoneal injection of STZ (Sigma Chemical Co.), 120 to 180 mg/kg body weight to achieve plasma glucose ≥300 mg/dL after 5 to 7 days. Plasma glucose, from retro-orbital blood, was determined by glucose oxidase reaction (Beckman Glucose Analyzer 2; Beckman Instruments, Fullerton, CA, USA). Weekly blood pressures were obtained from trained, prewarmed mice by computerized, noninvasive tail-cuff system (BP 2000 Blood Pressure Analysis System) (Visitech Systems, Apex, NC, USA) [16].

Animals were placed in metabolic cages for 24-hour urine collection. Albumin concentrations were measured using the Albuwell M Assay Kit (Exocel, Philadelphia, PA, USA). Albumin standards and samples were placed in 96-well plates, and washed with rabbit antimurine albumin antibody and antirabbit horseradish peroxidase (HRP) conjugate for color detection at 450 nm absorbance used to calculate albumin concentration.

**Quantitative reverse transcription (RT) real-time polymerase chain reaction (PCR)**

Total RNA (45 μg) isolated from mouse kidney cortex or primary mesangial cells by Trizol method (Invitrogen Life Technologies, Carlsbad, CA, USA) was DNase-treated and (200 ng) reverse transcribed using Applied Biosystems Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with a parallel negative control. Triplicate PCR amplifications of cDNA (5 μg) were performed with the following primers (200 nmol/L) and probes (100 nmol/L) (Perkin-Elmer/Applied Biosystems PE/ABD Primer Express Software): PAI-1 forward primer 5′-TGC ATC GCC TGC CAT TG-3′; PAI-1 reverse primer 5′-GG A CCT TGA GAT AGG ACA GTG CTT-3′; PAI-1 probe 5′-6FAM TGG AGG GTG CCA TGG GCC A TAMRA-3′; fibronectin forward primer 5′-GGA CCT TGA GAT AGG ACA GTG GCA GAA AGA-3′; fibronectin reverse primer 5′-CCG CTG GCC TCC GAA-3′; fibronectin probe 5′-6FAM TCG GAG CCA TTT GTT CCT GCA GTG TAMRA-3′; TGF-β forward primer 5′-CCA TTC ATG ACA TGA ACC GA-3′; TGF-β reverse primer 5′-CAG GTG GTG TGG AAC CCT TTC CA-3′; and TGF-β probe 5′-S6-FAM/CCA-TCC- TTT-CCC-ATA-TCC-TGT-CC/36-TAMTPH/-3′. PCR amplification [including glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] was performed (Perkin-Elmer/ Applied Biosystems 7700 Sequence Detector) with negative RT controls to detect contaminating genomic DNA. The data were analyzed using Perkin-Elmer Applied Biosystems Sequence Detection System software.

**Northern blot analysis**

Northern blot analysis was performed on total RNA (20 μg) from mouse kidney cortex which was carefully dissected from the renal capsule and medulla [17]. RNA separated by electrophoresis was transferred to nylon membranes (Hybond N+) (Amersham Biosciences, Buckinghamshire, UK) which were probed with 32P-labeled TGF-β or fibronectin cDNA (generously provided by Matthew Breyer, Vanderbilt University). Signals were detected by autoradiography (Amersham Biosciences) scanned and quantitated using NIH Image 1.60 and Image J version 1.28U scan software.
MMP assays
Protein isolated from mouse renal cortex was homogenized, quantitated, and used in MMP assays (Amer- sham Biosciences). Duplicate standard (0 to 16 ng/mL) and experimental samples were placed in 96-well plates for determination of endogenous and active MMP-9 and MMP-2. The plates were read at 405 nm at time zero and after 4 to 10 hours. Active MMP-9 and MMP-2 activity was determined from standard curves and analyzed by standard Student t test.

Mesangial cell culture
Rat glomeruli were isolated from normal Sprague-Dawley rats (200 to 650 g) (Charles River, Boston, MA, USA). Glomerular cores collected in phosphate-buffered Eagle’s medium (DMEM) containing D-valine (inhibits fibroblast growth), and 1 μmol/L recombinant human insulin (Sigma Chemical Co.). Mesangial cells were grown and characterized by phenotype and immunostaining to verify >95% mesangial cells. The stellate-appearing mesangial cells stained positive for fibronectin and α-smooth muscle actin (α-SMA) (Sigma Chemical Co.), and negative for cytokeratin. We used nonconfluent, serum-starved 0.5% fetal bovine serum (FBS) × 24 hours cells between passages 9 and 10 following a 30-minute pre-treatment.

Cells were acid washed [19, 20] to remove plasminogen and endogenous uPA prior to addition of exogenous uPA or recombinant PAI-1 as follows. Cells were incubated in glycine buffer at pH 4.0 × 3 minutes at 4°C followed by neutralization with Tris buffer at pH 7.4 × 10 minutes. This was followed by addition of exogenous uPA or PAI-1 as described.

Mouse mesangial cells were also obtained from PAI-1−/− mice by the sieving technique described above and treated with varying concentrations of glucose, compared to similarly treated mesangial 13 PAI-1+/− cells (American Type Culture Collection, Manassas, VA, USA).

Western blot analysis
Western blot analysis was done by standard procedures using 40 to 100 μg protein from PAI-1−/− and PAI-1+/+ renal cortex. Membranes were probed with TGF-β antimus- mouse primary antibody (BD Biosciences/Pharmingen, San Diego, CA, USA) (1:100) or fibronectin antimouse monoclonal and rabbit secondary antibody (Chemicon International, Temecula, CA, USA) (1:1000). The signals were scanned and quantitated using the Image J version 1.28 U and NIH Image 1.60 scan software. Protein was also obtained from primary rat mesangial cells treated with recombinant PAI-1 (0 to 30 nmol/L) for 0 to 24 hours or pretreated with the MAPK inhibitor PD98059 to detect TGF-β protein. Protein for phosphorylated and total p44/p42 ERK/MAPK protein expression was detected using phosphorylated MAPK p44/ERK1 and MAPK p42/ERK2 primary antibodies (New England Biolabs, Beverly, MA, USA) (1:1000) and corresponding secondary antibody. Following enhanced chemiluminescence (ECL) detection of proteins, the membranes were stripped and rehybridized with total MAPK p44/ERK1 and MAPK p42/ERK2 antibodies. At least three cell preparations were used.

Statistical analysis
Data are expressed as the mean ± SEM. Statistical analysis was performed using Sigma Plot statistical 5.0 software (SPSS, Chicago, IL, USA). Differences in mean values between groups were analyzed by Student t test.

RESULTS
Characteristics of wild-type and PAI-1−deficient mice
STZ administration increased plasma glucose >500 mg/dL in PAI-1−deficient (PAI-1−/−) and wild-type (PAI-1+/+) mice 5 to 7 days following STZ injection (Table 1). There was no statistical difference in baseline or final plasma glucose measurements between PAI-1−/− and PAI-1+/+. After 4 weeks, nondiabetic mice tended to increase their body weight, while diabetic PAI-1−/− and PAI-1−/− mice lost 15% and 20% body weight, respectively (P < 0.05). However, both PAI-1+/+ and PAI-1−/− mice had increased kidney weight-to-tibia length ratios (~12%) (P < 0.05) compared to nondiabetic animals. Thus, both PAI-1+/+ and PAI-1−/− mice demonstrated renal hypertrophy with diabetes. In addition, all diabetic animals increased 24-hour urine volume tenfold (P < 0.05), consistent with hyperglycemia. There was no difference in tail-cuff blood pressures in nondiabetic or diabetic mice.

Diabetes increased total urinary albumin excretion (UAE) in PAI-1+/+ but not PAI-1−/− mice
PAI-1 was not expressed in kidneys of PAI-1−/− mice with or without diabetes (Fig. 1A). However, PAI-1 ex- pression increased 1.5-fold (P < 0.05) in diabetic PAI-1+/+ kidneys compared to nondiabetic PAI-1+/+ mice. Baseline UAE between nondiabetic PAI-1−/− (30.5 ± 4.2 μg) and PAI-1+/+ mice (20.3 ± 4.3 μg) was not signifi- cantly different. Diabetic PAI-1+/+ mice increased UAE >threefold compared to nondiabetic PAI-1+/+ mice (62.3 ± 13.9 vs. 20.3 ± 4.3 μg) (P < 0.05) (Fig. 1B).
In contrast, there was essentially no change in UAE in di- abetic PAI-1−/− mice following 4 weeks of diabetes (1.1 fold) (30.5 ± 4.2 vs. 35.0 ± 3.0 μg). Thus, lack of PAI-1 protects against albuminuria induced by diabetes.
PAI-1 deficiency and diabetic nephropathy

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Table 1. Physiologic characteristics of mice

<table>
<thead>
<tr>
<th></th>
<th>PAI-1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>PAI-1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Nondiabetic (N = 14)</td>
<td>Diabetic (N = 15)</td>
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<tr>
<td>Body Weight, initial g</td>
<td>26.2 ± 0.5</td>
<td>25.3 ± 0.4</td>
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<td>Plasma glucose, initial mg/dL</td>
<td>166.7 ± 3.5</td>
<td>514.5 ± 50.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Plasma glucose, final mg/dL</td>
<td>246.9 ± 21</td>
<td>589.2 ± 37.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Urine volume mL</td>
<td>0.98 ± 0.15</td>
<td>1.0 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Blood Pressure mm/Hg</td>
<td>102.9 ± 2.6</td>
<td>96.9 ± 3.8</td>
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<tr>
<td>Kidney weight/tibia length × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>9.5 ± 0.2</td>
<td>10.8 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup>P < 0.05 vs. nondiabetic mice. Values are mean ± SEM.

PAI-1 is plasminogen activator inhibitor-1.
PAI-1<sup>+/+</sup> are wild-type mice, and PAI-1<sup>−/−</sup> are PAI-1 knock-out mice.

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**Fig. 1.** Plasminogen activator inhibitor-1 (PAI-1) expression and total urinary albumin excretion (UAE). (A) Kidney cortex total RNA from PAI-1-deficient (PAI-1<sup>−/−</sup>) and wild-type (PAI-1<sup>+/+</sup>) mice was used to quantitate PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). The fold change in PAI-1 expression is compared to GAPDH (N = 8). (B) After 4 weeks, mice were placed in metabolic cages for 24-hour urine collection. Total UAE (µg) was determined by enzyme-linked immunosorbent assay (ELISA): nondiabetic (Non-DM), PAI-1<sup>+/+</sup> (N = 14), diabetic (DM) PAI-1<sup>+/+</sup> (N = 15), nondiabetic PAI-1<sup>−/−</sup> (N = 16), and DM PAI-1<sup>−/−</sup> (N = 13) mice. Values are expressed as mean ± SEM. *P < 0.05.

**Fig. 2.** Matrix metalloproteinase (MMP) activity. After 4 weeks, protein obtained from mouse kidney cortex was used for active MMP-9 (A) and MMP-2 (B) concentration. Nondiabetic (Non-DM), plasminogen activator inhibitor-1 (PAI-1) non-DM PAI-1<sup>+/+</sup> (N = 14), diabetic (DM) PAI-1<sup>+/+</sup> (N = 15), non-DM PAI-1<sup>−/−</sup> (N = 16), and DM PAI-1<sup>−/−</sup> (N = 13) mice. Values are expressed as mean ± SEM. *P < 0.05.

**MMP activity is reduced in diabetic PAI-1<sup>−/−</sup> mice**

MMP-2 and MMP-9 are the major glomerular MMPs. Renal levels and activity of MMP-9 are decreased in experimental and human diabetes [21]. Nondiabetic PAI-1<sup>−/−</sup> mice trended toward higher renal MMP-9 activity compared to nondiabetic PAI-1<sup>+/+</sup> mice, which was not statistically significant (Fig. 2A). With diabetes, MMP-9 activity decreased to similar levels in both PAI-1<sup>+/+</sup> and PAI-1<sup>−/−</sup> mice, which was not different between strains. Renal MMP-2 activity was not different in the absence of diabetes between strains (Fig. 2B). However, MMP-2 activity was significantly lower in diabetic PAI-1<sup>−/−</sup> compared to nondiabetic PAI-1<sup>−/−</sup> (P < 0.05) or PAI-1<sup>+/+</sup> mice. These patterns are consistent with observations of different tissue expression patterns and transcriptional regulation of MMP-9 and MMP-2 [22].

**Fibronectin and TGF-β expression is reduced in diabetic PAI-1<sup>−/−</sup> mice**

Fibronectin is the earliest and most abundant ECM protein to increase in kidney and other tissues in diabetes...
[23]. Levels of standardized fibronectin mRNA and protein were 2.6-fold higher (3.7 ± 0.5 vs. 9.5 ± 1.3) and 3.5-fold higher, respectively, in diabetic PAI-1+/+ compared to nondiabetic PAI-1+/+ mice, while basal levels were not different between PAI-1+/+ and PAI-1−/− mice (P < 0.05) (Fig. 3A to C). In contrast, diabetes did not significantly alter fibronectin mRNA expression in PAI-1−/− mice. In fact, fibronectin protein was 50% less in diabetic PAI-1−/− mice than in nondiabetic PAI-1−/− mice (P < 0.05). These results suggest that diabetic PAI-1−/− mice do not accumulate fibronectin.

TGF-β expression and activity increase in experimental and human diabetes. Therefore, we expected equally increased renal TGF-β levels in PAI-1+/+ and PAI-1−/− mice with diabetes. However, we did not observe these predicted changes. Renal TGF-β mRNA levels increased 1.5-fold (P < 0.05) in diabetic PAI-1+/+ mice, but not in PAI-1−/− mice when assessed by either RT-PCR or Northern analysis (Fig. 3D to F). TGF-β protein was also significantly less in diabetic PAI-1−/− (P < 0.05) compared to nondiabetic PAI-1−/− mice (Fig. 3G and H). Thus, diabetic PAI-1−/− had less renal TGF-β mRNA and protein compared to PAI-1+/+ mice, suggesting that PAI-1 may potentially transcriptionally regulate TGF-β.

Additional studies were performed in which primary mesangial cells obtained from PAI-1−/− mice were treated with glucose (0, 4, and 30 mmol/L) for 24 hours in parallel with PAI-1+/+ mouse mesangial cells (mesangial cell 13 stable cell line) at 0, 4, and 30 mmol/L glucose. TGF-β and β-actin protein expression was determined as shown in the representative Western blot and quantitated. (I) At least four separate cell preparations were used. *P < 0.05 compared with control.

**Recombinant PAI-1 stimulated TGF-β production by MAPK signal transduction**

To determine whether PAI-1 might have a direct effect on TGF-β levels, we incubated primary mesangial cells with recombinant PAI-1 (0 to 30 nmol/L) and examined TGF-β protein expression between 0 and 24 hours. PAI-1 increased TGF-β protein in a dose-dependent manner up to 40 minutes (Fig. 4A and B), to approximately three-fold at 30 nmol/L PAI-1. The increase continued up to
Fig. 3. (continued)
Recombinant PAI-1 stimulated TGF-β via a uPAR mechanism

In several cell types, PAI-1 interacts with uPA bound to its cell surface receptor uPAR, which also interacts with integrins [1, 24]. When the uPA•PAI-1•uPAR complex is formed, the integrin is inactivated so that the complex is internalized and promotes downstream intracellular signaling of ERK/MAPK [25]. PAI-1 can also independently recruit uPAR without uPA to potentially activate intracellular signaling pathways [26]. When added to cultured mesangial cells which were acid-washed (to remove plasminogen and endogenous uPA), recombinant PAI-1 or uPA independently stimulated TGF-β expression ($P < 0.05$) (Fig. 5A and B). However, blockade of uPA binding with CD87, an anti-uPAR antibody which blocks the amino terminal domain of uPA that binds uPAR and prevents uPA/PAI-1 internalization [27, 28], abolished PAI-1–induced TGF-β regulation. Excess uPA (50 nmol/L) significantly reduced PAI-1–induced TGF-β expression in a dose-dependent (5 to 50 nmol/L) manner (Fig. 5C and D). These data indicate that stimulation of TGF-β by PAI-1 is mediated by uPAR.

DISCUSSION

The availability of mice genetically lacking PAI-1 allowed us to elucidate the role of this profibrotic factor in diabetic nephropathy and to define a potential new mechanism that mediates glomerular fibrosis. STZ-induced diabetic PAI-1$^{-/-}$ mice did not develop albuminuria, while PAI-1$^{+/+}$ mice demonstrated a $>threefold$ increase in UAE. The PAI-1$^{-/-}$ mice also had no increase in renal fibronectin mRNA or protein levels compared to PAI-1$^{+/+}$ mice. This apparent decreased ECM accumulation in PAI-1$^{-/-}$ mice could not be explained by increased MMP activity, since MMP-9 and MMP-2 activities were not increased in diabetic PAI-1$^{-/-}$ compared to PAI-1$^{+/+}$ mice. Therefore, decreased production, possibly associated with degradation of ECM, likely occurred in PAI-1$^{-/-}$ mice. Indeed, PAI-1$^{-/-}$ mice had decreased renal TGF-β mRNA expression and protein levels compared to diabetic PAI-1$^{+/+}$ mice, which likely contributed to the decreased renal fibronectin message and protein that accompanied attenuated albuminuria. These observations were supported by in vitro studies in which recombinant PAI-1 caused a dose-dependent increase in TGF-β protein levels in cultured mesangial cells. Taken together, these data suggest that PAI-1 plays a critical role in diabetic nephropathy by regulating TGF-β expression and protein, which is an important determinant of both ECM production and degradation.

STZ-induced diabetes in rats and mice results in early renal changes similar to that seen in humans with diabetes. These changes include increased albumin excretion and renal TGF-β expression, leading to enhanced production of fibronectin and other ECM proteins [29, 30].
Fig. 4. Recombinant plasminogen activator inhibitor-1 (PAI-1) stimulated transforming growth factor-β (TGF-β) expression by extracellular-regulated signal kinase (ERK)/mitogen-activated protein kinase (MAPK) signal transduction. Primary rat mesangial cells from Sprague-Dawley rats were serum-starved and treated with varying concentrations of recombinant rat PAI-1. (A) Representative Western blot of TGF-β and β-actin expression. (B) Graphic representation of fold change in signals from Western blot. (C) Primary rat mesangial cells were untreated, treated with recombinant rat PAI-1 or pretreated with the MAPK inhibitor, PD98059. Quantitative reverse transcription-polymerase chain reaction (RT-PCR). (D) Representative Western blot of TGF-β protein. (E) Graphic representation of Western blot analysis of TGF-β to β-actin protein. (F) Representative Western blot of phosphorylated and total p42/ERK and p44/ERK, corresponding to protein bands 44 and 42 kD bands, respectively. (G) Graphic representation of ratio of phosphorylated to total p42/ERK, p44/ERK. Values are mean ± SEM. *P < 0.05 compared to control. Each experiment was performed at least three times.

In man, the resulting increased mesangial volume corresponds to albuminuria and loss of glomerular filtering capacity, ultimately progressing to end-stage renal disease (ESRD) [31]. Inhibition of TGF-β with either blocking antibodies or decorin, a natural TGF-β inhibitor, arrests the process in animals [32]. These observations support a pathophysiologic role for TGF-β in diabetic nephropathy, although other factors may be involved. Thus, understanding the mechanisms that regulate TGF-β is key in prevention and treatment of diabetic nephropathy. Administration of angiotensin converting enzyme (ACE) inhibitors or Ang II type 1 (AT1) receptor blockers (ARBs) attenuate the progression of diabetic nephropathy in humans and are associated with both decreased urinary TGF-β [33, 34] and tissue PAI-1 [35, 36]. Ang II is a potent regulator of PAI-1 in mesangial cells and PAI-1 may be more sensitive to Ang II stimulation than TGF-β (Nicholas, Hsueh unpublished observations). The present investigation identifies PAI-1 as a potentially important regulator of TGF-β in the glomerulus. Hyperglycemia is known to stimulate TGF-β production in mesangial cells. Further, PAI-1 may mediate, at least in part, the glycemic effect on TGF-β, since mesangial cells obtained from PAI-1−/− mice showed no regulation of TGF-β expression in response to hyperglycemia compared to PAI-1+/+ mouse cells. Indeed, hyperglycemic response elements have been demonstrated on the PAI-1 promoter [37].

Our studies suggest that the mechanism by which mice null for PAI-1 are protected against diabetic nephropathy
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Involves regulation of TGF-β, as a more prominent mechanism than increased plasmin and MMP activation potentially associated with lack of PAI-1. MMP-9 and MMP-2 are the major glomerular MMPs [38, 39]. MMP-9 is activated by plasmin and transcriptionally regulated by both TGF-β and hyperglycemia. MMP-2 is not regulated by plasmin but may be activated by membrane-type MMPs, particularly membrane type 1-MMP [21, 40–43]. Although the physiologic regulation of membrane type 1-MMP is not fully characterized, hyperglycemia may down-regulate its activity [44]. Our results were surprising in that, neither MMP-9 nor MMP-2 activities were increased in diabetic PAI-1−/− vs. PAI-1+/+ mice; in fact MMP-2 was decreased. Decreased TGF-β expression could account for decreased MMP-9 and hyperglycemia for the decreased MMP-2 [39]. Despite the cause, lack of increased renal MMP activity argues against accelerated degradation of ECM as the major mechanism for attenuation of ECM accumulation in PAI-1−/− mice.

Similar to results of our study, PAI-1−/− mice subjected to UUO developed less renal interstitial fibrosis compared to PAI-1+/+ mice [10]. Kidneys of PAI-1−/− mice had decreased TGF-β and collagen expression without increases in uPA or plasmin activity compared to PAI-1+/+ mice. Since plasmin activates TGF-β membrane-release [45], the lack of increased TGF-β is consistent with a lack of increased plasmin that might be anticipated in PAI-1−/− mice [10]. Increased interstitial macrophages and myofibroblasts in the fibrotic kidneys of PAI-1−/+, but not PAI-1−/−, mice, was thought to account for decreased TGF-β in PAI-1−/− mice. Decreased fibrosis in this model was attributed to decreased PAI-1-induced cell chemotaxis. In the immune anti-Thy-1 model of glomerulonephritis, which demonstrates glomerular expansion and macrophage/monocyte infiltration but no interstitial fibrosis, the administration of a mutant PAI-1 resulted in increased plasmin activity with subsequent reduced glomerular matrix accumulation within 6 days compared to untreated animals. However, TGF-β mRNA and protein were reduced with subsequent reduction in fibronectin and collagen 1 mRNA levels. Decreased TGF-β was attributed to decreased infiltration of inflammatory cells, but [11] changes in MMP expression and activity were not investigated. Recent investigation suggests that glomerular and interstitial macrophage accumulation and activation accompany prolonged hyperglycemia, and both correlate with renal fibrosis and albuminuria in the db/db mouse, a model of diabetic nephropathy [46]. Thus, it is possible that decreased TGF-β in diabetic PAI-1−/− mice results from decreased inflammatory events in PAI-1−/− mice. However, it is unclear whether glomerular inflammation plays a major role in the STZ model of diabetic nephropathy.

Another mechanism may involve PAI-1 stimulation of intracellular ERK/MAPK signaling by binding to uPA.

**Fig. 5.** Mechanism of plasminogen activator inhibitor-1 (PAI-1) induction of transforming growth factor-β (TGF-β) expression. Primary rat mesangial cells were untreated (lane 1), treated with recombinant PAI-1 (lane 2), urokinase plasminogen activator (uPA) (lane 3), or pretreated with anti-uPA receptor (uPAR) (lane 4) or uPA followed by PAI-1 (lane 5). (A) Representative Western blot of TGF-β expression. Anti-uPAR, inhibited PAI-1-stimulated TGF-β expression (lane 4). Excess uPA prevented PAI-1-stimulated TGF-β expression. (B) Graphic representation of TGF-β/β-actin protein ratio of at least four separate experiments. *P < 0.05 compared to untreated (lane 1). #P < 0.05 compared to PAI-1-stimulated TGF-β expression (lane 2). (C) Rat mesangial cells were incubated with recombinant PAI-1 and uPA Representative Western blot. (D) Graphic representation of three separate experiments. *P < 0.05 compared to PAI-1 alone. Each experiment was performed at least three times.
complexed to uPAR, which is independent of its enzymatic action to convert plasminogen to plasmin [25, 47, 48]. Mesangial cells express uPAR and, thus, can potentially respond to PAI-1. We found that PAI-1 stimulation of TGF-β in mesangial cells was attenuated by (I) blocking antibody of uPA binding to uPAR, (2) uPA, and (3) inhibition of MAPK activity demonstrating a role for uPAR and MAPK. In diabetic kidney cortex, uPAR, PAI-1, and TGF-β expression is significantly up-regulated [49, 50]. Previous studies demonstrated that uPA/PAI-1/uPAR complexes with integrins, which are then inactivated [1]. Our data suggest that uPAR bound to either uPA or PAI-1 alone increases mesangial TGF-β expression; however, uPA inhibited PAI-1–induced TGF-β expression possibly because the complex enhances degradation of integrins that are also necessary for TGF-β regulation [51]. Further studies are necessary to define these mechanisms. Nevertheless, these observations provide evidence that PAI-1 directly regulates TGF-β expression and hence, ECM production through uPAR binding and subsequent activation of ERK/MAPK signaling. Thus, in diabetic PAI-1−/− mice, decreased renal TGF-β is likely due to the lack of PAI-1, although as discussed above, other potential mechanisms may exist.

Plasmin can stimulate TGF-β, but the present investigation underscores a critical role of PAI-1 in diabetic glomerulosclerosis and uncovers a new, previously undefined mechanism of PAI-1 in fibrosis. PAI-1 itself can stimulate TGF-β to ultimately regulate ECM production. Thus, in the diabetic milieu of hyperglycemia, increased Ang II, hypertension (mesangial stretch) and other factors, two vicious cycles of reciprocal stimulation between PAI-1 and TGF-β perpetuate the fibrotic response. These factors stimulate both TGF-β and PAI-1 which transcriptionally regulate each other, creating a potentially escalating cycle to enhance accumulation of ECM. Both cycles appear to be MAPK-dependent since this signaling pathway regulates transcription of both PAI-1 and TGF-β. Inhibition of PAI-1 and TGF-β expression is important to attenuate glomerular fibrosis, either by treating each of the factors in the diabetic milieu independently (such as aggressive glucose control, ACE inhibitors, ARBs, or blood pressure lowering) or perhaps collectively by inhibiting MAPK signaling [52]. Inhibitors of PAI-1 binding to uPAR may also be a fruitful approach in treatment and prevention of diabetic nephropathy.

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