

## PAI-1 and kidney fibrosis

Li-Jun Ma, Agnes B. Fogo

*Vanderbilt University Medical Center, Department of Pathology, Nashville, Tennessee*

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## 1. ABSTRACT

Substantial evidence demonstrates a link of increased plasminogen activator inhibitor-1 (PAI-1) and glomerulosclerosis and kidney fibrosis, providing a novel therapeutic option for prevention and treatment of chronic kidney diseases. Several mechanisms contributing to increased PAI-1 will be addressed, including classic key profibrotic factors such as the renin-angiotensin-system (RAS) and transforming growth factor-beta (TGF- $\beta$ ), and novel molecules identified by proteomic analysis, such as thymosin- $\beta$ 4. The fibrotic sequelae caused by increased PAI-1 in kidney depend not only on its classic inhibition of tissue-type and urokinase-type plasminogen activators (tPA and uPA), but also its influence on cell migration.

## 2. INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1) is a 50kd glycoprotein and is the primary physiological inhibitor of tissue-type and urokinase-type plasminogen activators (tPA and uPA) (1). tPA and uPA degrade both fibrin and extracellular matrix (ECM). PAI-1 thus affects both thrombosis and fibrosis. PAI-1 effects extend to cell migration through interactions with its cofactor, vitronectin (also known as protein S) and with the urokinase receptor (uPAR) and its co-receptors (2, 3). In particular, although a unique PAI-1 receptor has not been identified, PAI-1 modifies the function of the uPAR and several of its co-receptors. The normal kidney shows nearly undetectable

PAI-1. Plasma PAI-1 levels increase in several chronic inflammatory states, including chronic kidney disease (CKD) (4-7). Liver and adipose tissue appear to be the primary sources of plasma PAI-1 (8, 9). PAI-1 is increased in several acute and chronic disease states, contributed to both by resident and infiltrating cells, and is linked to worsened fibrosis. Conversely, amelioration of injury is associated with less PAI-1 (10). We will review how PAI-1 actions promote renal fibrosis.

### 3. PAI-1 IN HUMAN CHRONIC KIDNEY DISEASE

In humans, PAI-1 is prominent in atherosclerotic lesions and in sclerotic glomeruli in e.g. arterionephrosclerosis, diabetic nephropathy and chronic allograft nephropathy (CAN). In a study of CAN, there was increased PAI-1 expression compared to transplanted kidneys followed for the same amount of time without CAN. Increased expression was present in tubules, vessels and within glomeruli, in mesangial areas, podocytes and endothelial cells in addition to infiltrating macrophages. Within this group of kidneys with CAN, there was a trend for less overall sclerotic injury to be associated with less PAI-1. These findings support the hypothesis that altered matrix metabolism and macrophage function could be involved in the development of CAN (11). Microdissected glomeruli from kidney transplants were used to study expression of PAI-1 and the thrombin receptor protease activated receptor-1, PAR-1,  $\alpha 2$  type IV collagen and a housekeeping gene. Decreased renal function was correlated with increased mRNA levels of PAI-1 in the biopsy. PAI-1 was also increased along with  $\alpha 2$  type IV collagen mRNA in acute rejection compared to normal kidneys (12). PAI-1 protein in diabetic nephropathy was particularly prominent in Kimmelstiel-Wilson nodules, often associated with fragmented red blood cells in regions of local injury and mesangiolysis (13).

PAI-1 levels are also influenced by PAI-1 genotype. Higher levels are seen with the 4G vs 5G polymorphism, describing the number of guanine bases (4 vs 5) in the promoter at position -675. The 5G, but not 4G, variant binds the E2F transcription repressor, and thus decreases PAI-1 transcription. The PAI-1 4G/4G genotype was linked to increased risk of vascular complications in diabetics, especially when superimposed on the ACE D/D genotype that is associated with increased RAS activity (14). Increased PAI-1 plasma levels and 4G/4G PAI-1 genotype have also been linked to increased risk of chronic allograft dysfunction (15).

Conversely, treatments that inhibit the renin-angiotensin-aldosterone system (RAAS) also decrease PAI-1. Of note, combined ARB and aldosterone inhibition in humans suppressed the enhanced PAI-1 levels that occurred in response to a diuretic, whereas monotherapy with either alone had no effect (16). In a small study of hypertensive human kidney transplant recipients, elevated plasma PAI-1 levels were decreased by treatment with ARB but not by nifedipine (17). Whether this modulation affects graft outcome and tissue fibrosis has not been proven. However, plasma PAI-1 levels did correlate with

rate of GFR decline in a study of renal transplant patients (18).

### 4. PAI-1 IN ANIMAL MODELS OF KIDNEY DISEASE

Animal models support a role for PAI-1 in the pathogenesis of diabetic kidney injury. Of note, rodent models of diabetes do not result in overt diabetic nephropathy. However, in the streptozotocin diabetic model, streptozotocin-treated PAI-1<sup>-/-</sup> mice had reduced albuminuria and fibronectin levels compared to wild-type diabetic mice (19). *db/db* diabetic mice with added PAI-1 deficiency had reduced albuminuria and kidney collagen levels (20). In early streptozotocin-induced nephropathy in rats, spironolactone therapy reduced PAI-1 and transforming growth factor- $\beta$  (TGF- $\beta$ ) expression levels and matrix deposition (21). In another study, PAI-1<sup>-/-</sup> mice were protected from high fat diet-induced diabetic metabolic changes that occurred in wild-type mice (22). Furthermore, PAI-1<sup>-/-</sup> adipocytes were functionally distinct, with altered levels of key potential mediators of the dysmetabolic syndrome, including resistin, peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) and adiponectin (23). These studies suggest complex roles for PAI-1 in the pathogenesis of diabetes and its complications.

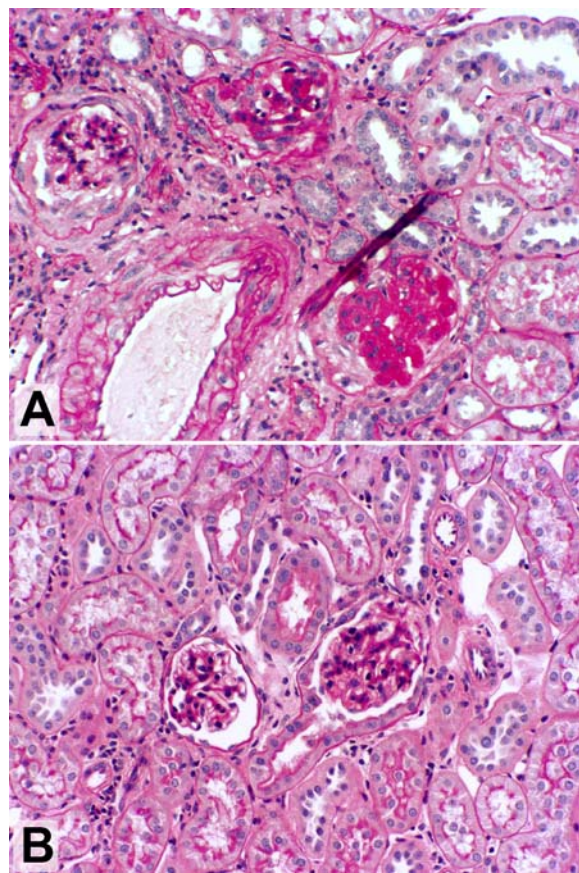
PAI-1 is also increased in many experimental models of non-diabetic glomerulosclerosis, including cyclosporin-induced glomerulosclerosis, radiation nephropathy, various models of FSGS, chronic anti-Thy-1 nephritis, aging and CAN (24, 25).

*In vitro*, angiotensin II directly induced PAI-1 gene expression via the AT1 receptor and aldosterone enhanced angiotensin-induced PAI-1 expression via a glucocorticoid response element in the PAI-1 promoter (26, 27). AT1 receptor blockade but not nonspecific antihypertensive treatment reduced renal PAI-1 expression induced by angiotensin II infusion (28).

Genetic PAI-1 deficiency also ameliorated sclerosis. Sclerosis-prone 129Sv mice bred to generate a PAI-1 null genotype were protected from glomerular scarring and tubulointerstitial fibrosis after 5/6 nephrectomy (Figure 1) (29). *In vitro*, PAI-1<sup>-/-</sup> podocytes synthesized less collagen in response to angiotensin II than wild-type cells (30). PAI-1<sup>-/-</sup> mice also had less vascular sclerosis than wild-type mice in response to angiotensin II and salt-loading or nitric oxide inhibition by L-NAME (31-33). PAI-1<sup>-/-</sup> mice were protected from interstitial fibrosis induced by protein overload (34). Of note, these protective effects of PAI-1 deficiency occurred in several settings without changes in renal protease activity. However, macrophages and myofibroblasts were decreased, suggesting effects on cell migration independent of tPA and uPA modulation by PAI-1.

### 5. ANGIOTENSIN, PAI-1 AND TGF $\beta$ -1- THE TRIPLE THREATS

TGF- $\beta$  is secreted as a complex, composed of three proteins derived from two genes, each encoding a



**Figure 1.** Plasminogen activator inhibitor-1 (PAI-1) deficiency attenuates glomerulosclerosis in mice with remnant kidneys. Ten weeks after sclerosis-prone 129Sv mice underwent 5/6 nephrectomy, glomerulosclerosis and interstitial fibrosis were evident (A). These lesions failed to develop in 129Sv /PAI-1<sup>-/-</sup> mice (B). Magnification, x200 (periodic acid-Schiff). Reproduced with permission from 10.

procytokine. The N-terminal region, latency-associated peptide, LAP, prevents TGF- $\beta$  from binding to its receptor (35). Because angiotensin, PAI-1 and TGF- $\beta$  are often co-expressed in chronic renal disease and can influence expression of the other molecules, it has been challenging to unravel the independent contributions of each of these molecules to sclerosis (36). However, in the anti-Thy-1 model, combined TGF- $\beta$  and angiotensin inhibition reduced PAI-1 expression and glomerular ECM accumulation and was more effective than either therapy alone (37), suggesting complex interactions and interdependent but not identical effects of sclerosis. Activation of TGF- $\beta$  occurs through several mechanisms, including plasmin (38). Other mechanisms for activating TGF- $\beta$  include reactive oxygen species, thrombospondin-1 (TSP-1) and  $\alpha$ v $\beta$  integrin (39). This integrin is expressed in epithelia of the lung, skin and kidney, and induces activation of TGF- $\beta$ 1 by cleaving it from LAP. In the kidney,  $\beta$ 6 is present in the proximal tubule, cortical thick ascending limb, inner and outer medullary collecting ducts

and macula densa (40). Thus, in a bleomycin model of lung epithelial cell injury,  $\beta$ 6<sup>-/-</sup> mice were completely protected against the fibrosis that developed in the wild type (41).

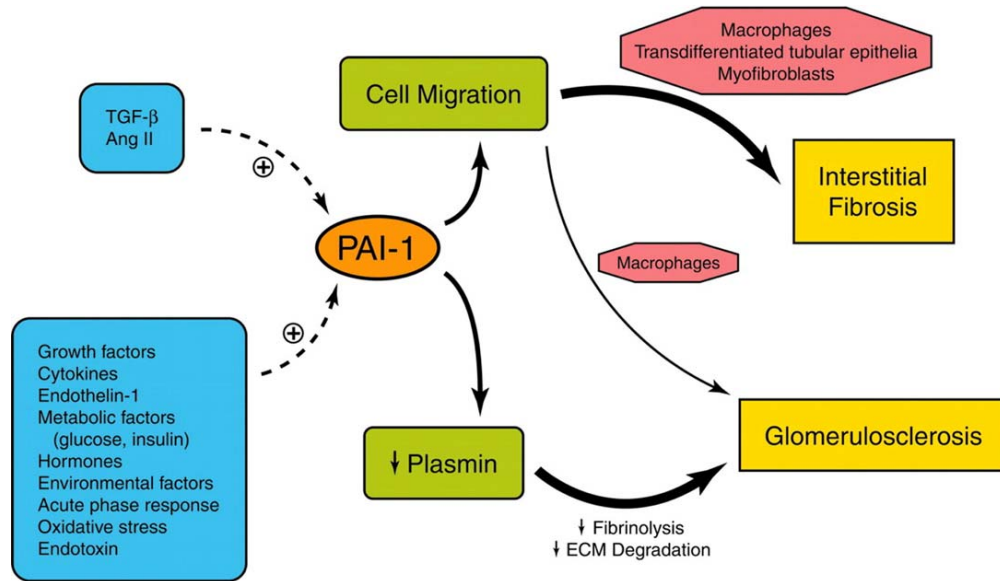
Studies in the  $\beta$ 6 knockout mice provide additional insights on interactions of the RAAS, TGF- $\beta$  and PAI-1. In contrast to wild-type mice,  $\beta$ 6<sup>-/-</sup> mice did not develop fibrosis and active TGF- $\beta$  and PAI-1 were not increased after UUO (41, 42). However, fibrosis could be restored in  $\beta$ 6<sup>-/-</sup> mice by adding angiotensin II or aldosterone infusion to UUO. In these mice, PAI-1 expression and fibrosis, but not TGF- $\beta$ , were increased to wild-type levels. These data support a direct link among angiotensin or aldosterone, PAI-1, and fibrosis, without dependence on TGF- $\beta$  as an intermediary in this setting.

TGF- $\beta$  and PAI-1 interactions have been extensively studied. TGF- $\beta$ -overexpressing mice develop progressive glomerulosclerosis and also have increased PAI-1 (43). PAI-1 deficiency attenuated this TGF- $\beta$ -induced kidney disease, as examined when these TGF- $\beta$ 1 transgenic mice were cross-bred with PAI-1<sup>-/-</sup> mice. The lack of PAI-1 resulted in less mesangial expansion and basement membrane thickening than seen in transgenic TGF- $\beta$  mice with intact PAI-1, with associated decreased collagen accumulation due to decreased expression of both collagen I and collagen III. Interestingly, these results were not correlated with differences in protease activity, suggesting that other activities of PAI-1 resulted in the observed effects (43).

TGF- $\beta$ 1 has numerous effects beyond stimulating ECM synthesis, including effects on cell proliferation and immune modulation. Thus, TGF- $\beta$ 1<sup>-/-</sup> mice have multifocal inflammatory disease, resulting in early death (44, 45). Antagonism of TGF- $\beta$  with a pan-antibody at low dose was protective against development of sclerosis in puromycin aminonucleoside nephropathy. However, higher doses of the anti-TGF- $\beta$  antibody had no effect on glomerulosclerosis, and macrophage infiltration was increased, perhaps reflecting block of TGF- $\beta$  immune modulatory effects (46).

## 6. EFFECTS OF PAI-1 and PAs ON GLOMERULOSCLEROSIS AND FIBROSIS

PAI-1 also impacts matrix accumulation in other organs. In the bleomycin model of pulmonary fibrosis, there was increased fibrosis in mice with over-expression of PAI-1 versus decreased fibrosis in PAI-1<sup>-/-</sup> mice (47). These results were consistent with effects on protease activity, specifically plasmin, because treatment of the PAI-1<sup>-/-</sup> mice with an inhibitor of plasmin formation, tranexamic acid, obviated the protective effect of genetic deficiency of PAI-1 (48). The impact of plasmin on injury was also shown in double tPA/uPA<sup>-/-</sup> mice that developed more severe crescentic glomerulonephritis and had correspondingly less plasmin (49). However, more recent studies point to additional mechanisms whereby PAI-1 can modulate injury (Figure 2). When the UUO model was induced in PAI-1<sup>-/-</sup> mice, there was decreased matrix



**Figure 2.** Schematic summary of the primary effects of PAI-1 in chronic kidney disease (CKD). Renal PAI-1 expression can be induced by a number of factors involved in disease pathogenesis. TGF- $\beta$  and angiotensin II are widely recognized inducers of PAI-1, but many other factors stimulate PAI-1 expression in CKD. In the glomerulus, sclerosis is linked to increased PAI-1 and inhibition of extracellular matrix breakdown. Within the tubulointerstitium, the profibrotic effects of PAI-1 align more closely with its ability to promote migration of monocytes/macrophages, transdifferentiated tubular epithelial cells and (myo)fibroblasts in particular. Reproduced with permission from 10.

accumulation, but plasmin activity was not enhanced in the PAI-1<sup>-/-</sup> mice. In addition to the decreased fibrosis, cell infiltration was also decreased, and conversely, cell infiltration was increased when PAI-1 over-expressing mice were subject to UUO (50, 51). These data suggest direct effects of PAI-1 to enhance infiltration and recruitment of profibrotic cells.

These effects of PAI-1 on cell adhesion and migration are mediated via both protease-dependent and -independent mechanisms. Originally, PAI-1 was thought to be an inhibitor of cell migration. Smooth muscle cell migration is increased in PAI-1<sup>-/-</sup> mice, while PAI-1 overexpression *in vitro* inhibits this process, presumably by vitronectin effects (52, 53). These studies support that PAI-1 can have cell migration-inhibitory effects independent of its proteolytic activity. This effect on cell migration might not only modulate macrophage infiltration but also affect epithelial-mesenchymal transition (EMT), and may possibly relate to effects mediated by complex interactions with vitronectin and maybe other co-receptors for uPAR (54). PAI-1 is bound to the vitronectin in plasma, and when PAI-1 binds to tPA or uPA, this binding is reversed. PAI-1 inhibition of uPA results in inactivation of the uPA-uPAR-integrin complex. The complex is internalized by the low density lipoprotein receptor related protein, LRP. LRP modulates cell migration in several *in vitro* systems (55, 56). PAI-1 activates an LRP-dependent signaling pathway that contributes to PAI-1's pro-migratory effects (57). PAI-1 activates the Jak/Stat signaling pathway via binding to LRP in cultured smooth muscle cells. When PAI-1 was mutated to prevent LRP binding, this signaling pathway was not activated, nor was cell migration enhanced.

Similarly, antibodies or antagonism or genetic deficiency of LRP blocked PAI-1's pro-migratory effects (57). The endocytic receptor LRP together with tPA and PAI-1 also coordinated Mac-1-dependent macrophage migration (58). Genetic inactivation of PAI-1, Mac-1 or LRP abrogated macrophage migration. The dissociation of uPAR and integrins from the matrix, induced by PAI-1 binding to uPA at the cell surface, could thus promote cell migration (59). PAI-1 also binds to vitronectin with much higher affinity than does uPAR. When PAI-1 binds to vitronectin, cell adhesion mediated by integrins is inhibited. This so-called "de-adhesion" property of PAI-1 could contribute to recruitment of macrophages. Whether this also could facilitate EMT remains to be determined. Interesting experiments have further explored these various functions of PAI-1 using a mutant PAI-1 that has normal vitronectin affinity but is not capable of inhibiting tPA or uPA. Using this compound, macrophage infiltration and markers of fibrosis were reduced, supporting a key pathophysiological role for these non-protease functions of PAI-1 (60). Increased PAI-1 is also linked to more aggressive tumor cell metastases, further indicating possible effects on cell migration and even possible direct chemotactic actions of PAI-1 (24). Additional studies in fibrotic models also observed protective effect of decreased PAI-1 independent of its regulatory function on plasmin activity.

In spontaneously hypercholesterolemic rats, treatment with a PPAR $\gamma$  agonist or the ARB candesartan resulted in amelioration of development of proteinuria and fibrosis, with corresponding attenuation of PAI-1 protein level, but no decrease in plasmin activity (61). Further studies on the actions of uPA in the kidney observed no protection against interstitial fibrosis induced by UUO and

there was no compensatory change in renal uPAR mRNA levels, or PAI-1 protein or tPA activity levels. This study also examined activation of hepatocyte growth factor (HGF), as uPA can activate latent HGF, but this was not increased in uPA<sup>-/-</sup> mice. These studies suggest that the profibrotic actions of PAI-1 are not dependent on uPA and that uPA is not the mechanism by which HGF is activated in the kidney. The results are also in contrast to previous reports that show that increased uPA activity could attenuate pulmonary and hepatic fibrosis, suggesting organ-specific pathways of fibrogenesis (see below) (62). Additional studies in plasminogen (PLG)-deficient mice subjected to UUO showed decreased fibrosis in the PLG<sup>-/-</sup> mice and decreased EMT, associated with significant decreased phosphorylated ERK and active TGF- $\beta$ . These *in vivo* observations were examined in further detail *in vitro*, where cultured murine tubular epithelial cells showed increased ERK phosphorylation when exposed to plasmin, with phenotypic transition to become more fibroblast-like, with de novo expression of fibroblast specific protein-1 and alpha-smooth muscle actin. Blocking of protease activated receptor-1 (PAR-1) genetically or with a specific anti-PAR-1 signaling peptide or an ERK kinase inhibitor blocked these *in vitro* EMT events. Conversely, when PAR-1 was overexpressed in these cells, ERK correlation response to plasmin was enhanced. These studies provide strong evidence for a profibrotic role for plasmin mediated through PAR-1-dependent ERK signaling, with important effects to induce EMT (63).

Plasmin also acts as a potent proinflammatory activator of monocyte-derived macrophage and triggers cytokine induction (64). Annexin A2 is a calcium and phospholipid-binding protein. The annexin A2 heterotetramer, composed of annexin A2 and S100A10, is a co-receptor for plasmin and regulates plasmin activity (65-67). Annexin A2 mediates plasmin induced-activation of macrophages via intracellular Janus kinase JAK1/Stat3 signaling (64, 66). Annexin A2 is also present on renal tubular epithelial cells (68). In an acute renal failure model in mice, expression of annexin A2 parallels the time course of tubular injury and recovery (69). Whether annexin II modulates plasmin's effects on EMT and/or fibrosis remains to be determined.

A possible impact of EMT on injury has been shown in elegant studies by Neilson and colleagues *in vivo* in the UUO model (70). Interestingly, tPA<sup>-/-</sup> mice showed a decrease in interstitial collagen after UUO, linked to decreased matrix metalloproteinase-9 (MMP-9) induction and preserved tubular basement membrane integrity (71, 72), events that could prevent EMT. Modulation of the EMT process by tPA is further supported by a recent finding that tPA directly promotes murine myofibroblast activation through LRP-mediated integrin signaling (73). These findings contrast with the protective effects of increased tPA to protect against glomerular matrix accumulation.

These data suggest complex effects of PAI-1 beyond plasmin inactivation that could particularly enhance infiltration of cells into the interstitium, including EMT or

macrophage influx. Whether similar effects of PAI-1 on cell migration in the glomerulus occur has not yet been established.

## 7. POSSIBLE ORGAN-SPECIFIC PAI-1 EFFECTS

The plasmin/PA system has varying effects in different organs. Renal fibrosis after UUO was less severe in plasminogen-deficient mice, associated with lower levels of active TGF- $\beta$  (63, 74). These findings contrast with outcomes in the bleomycin-induced lung injury model, where fibrosis was worse in plasminogen-deficient mice, suggesting that the fibrogenic effects of plasminogen might be organ-specific (75). The contribution of PAI-1 to aldosterone-induced injury also showed organ specific effects. Infusion of aldosterone with salt in the drinking water in wild-type mice induced increased albuminuria and glomerulomegaly due primarily to mesangial expansion, with increased renal collagen content, with corresponding increases in mRNA for collagen I, collagen III, osteopontin, fibronectin and the macrophage marker F4/80 and monocyte chemotactic protein. In contrast, PAI-1<sup>-/-</sup> mice were partially protected from these injuries, with less mesangial matrix expansion and without significant increase in collagen III and with less osteopontin expression. Of interest, TGF- $\beta$  was not increased in this model. A differential effect on kidney versus heart injury was observed in that aldosterone induced similar cardiac hypertrophy in both wild-type and PAI-1<sup>-/-</sup> mice, perhaps related to diverse effects on infiltrating cells in these two organs (76).

Effects of PAI-1 on cardiac injury were examined in a model of uninephrectomy with angiotensin infusion. This infusion caused aortic remodeling with increased medial, adventitial and overall wall thickness, which was attenuated by the novel orally active small molecule PAI-1 inhibitor, PAI-039. The angiotensin II-induced increased aortic osteopontin was significantly decreased by the PAI-1 inhibitor. However, overall cardiac hypertrophy was not affected, and the PAI-1 inhibitor actually increased angiotensin II-induced cardiac fibrosis. Whether these varying effects are species specific or organ specific remains to be determined (31).

## 8. NOVEL MOLECULES AND RAS-PAI-1 INTERACTIONS: THYMOSIN $\beta$ -4 AND Ac-SDKP

Recent studies suggest that thymosin  $\beta$ -4 may be one intermediary linking actions of angiotensin II and PAI-1. Thymosin  $\beta$ -4 is a ubiquitously expressed G-actin binding protein with both nuclear and cytoplasmic locations (77, 78). Thymosin  $\beta$ -4 accelerates dermal wound healing, related to anti-inflammatory activity, increased tissue remodeling and angiogenesis and increased collagen deposition and wound contraction (79). In cultured corneal cells, thymosin  $\beta$ -4 increases TGF- $\beta$  and laminin-5 (80). Thymosin  $\beta$ -4 had direct effects on corneal cell migration, and additional effects that may be in part mediated by induction of TGF- $\beta$ . Interestingly, thymosin  $\beta$ -4 promotes cardiac myocyte survival after ischemia, linked to increased vascular endothelial growth factor (VEGF) expression and



activation of integrin-linked kinase (ILK) (81). Thymosin  $\beta$ -4 plays a key role in cardiac development, stimulates coronary vasculogenesis and angiogenesis, and induces adult epicardial progenitor mobilization and neovascularization (82).

In contrast to these potentially beneficial effects after dermal, corneal and cardiac injury, thymosin  $\beta$ -4 appears to potentiate glomerulosclerosis. We performed proteomic analysis of isolated laser-capture microdissected glomeruli from frozen sections. Thymosin  $\beta$ -4 was increased in 5/6 nephrectomy rats, markedly in sclerotic glomeruli, but also in nonsclerotic glomeruli, compared to normal (83). Immunohistochemistry showed that increased glomerular thymosin  $\beta$ -4 was contributed to by glomerular endothelial cell (GEN) staining. We further investigated the functional significance of up-regulated thymosin  $\beta$ -4, by manipulating its expression *in vitro*. Knock-down of thymosin  $\beta$ -4 by siRNA in cultured GEN prevented the increased PAI-1 expression seen in response to angiotensin II in normal GEN with intact thymosin  $\beta$ -4 (83). This data supports that thymosin  $\beta$ -4 is not merely a marker of sclerosis, but promotes matrix accumulation within glomeruli.

Our new preliminary data show that thymosin  $\beta$ -4 is also up-regulated in the fibrotic interstitium in the UUO model in a variety of cells, including myofibroblasts, distal tubules, macrophages and also occasional peritubular capillary and proximal tubular cells (84). We also found that integrin  $\beta$ 6-/- mice with UUO that were protected from fibrosis had little thymosin  $\beta$ -4 expression. In contrast, when addition of AngII infusion in these mice restored fibrosis, thymosin  $\beta$ -4 was markedly upregulated to levels similar to that seen in wild-type mice after UUO with consequent fibrosis (85).

Thymosin  $\beta$ -4 is degraded by endoproteinases or prolyl oligopeptidase (POP) to Ac-SDKP which also has manifold actions. Ac-SDKP is further degraded to an inactive metabolite by ACE. Thus, ACEI increases Ac-SDKP plasma levels four- to five-fold (86, 87). Conversely, administration of a monoclonal antibody to Ac-SDKP blocked beneficial effects of ACEI or infused Ac-SDKP, resulting in worse cardiac fibrosis and hypertension in response to AngII (88). Ac-SDKP inhibited TGF- $\beta$ 1 induction of increased PAI-1 and  $\alpha$ 2 type I collagen *in vitro* in mesangial cells, linked to inhibition of Smad2 phosphorylation (89). Ac-SDKP enhanced corneal endothelial cell proliferation both *in vitro* and *in vivo* (90), and exogenous Ac-SDKP infusion improved fibrosis in a rat anti-GBM nephritis model (91) and ameliorated both renal insufficiency and mesangial expansion in the *db/db* mouse model of diabetes (92). Further, Ac-SDKP blocked collagen synthesis in a cardiac fibrosis model (93, 94). Our preliminary data suggest synergistic beneficial effects of ARB and exogenous Ac-SDKP on injury in UUO, with decreased thymosin  $\beta$ -4 and PAI-1 (95).

These data support that the balance of thymosin  $\beta$ -4 and Ac-SDKP is important in modulating fibrotic vs

antifibrotic responses, and that beneficial actions of ACEI are at least in part contributed to by enhanced Ac-SDKP.

## 9. REGRESSION OF RENAL FIBROSIS

The RAS is a central target for treatment of progressive renal diseases. ACEI or ARB are effective in treatment of CKD, perhaps even better than other antihypertensives. The apparent superior efficacy of ACEI was first linked to decreased intraglomerular hypertension and hyperfiltration, due to preferential decrease of efferent arteriolar resistance. Evidence then emerged that additional non-hemodynamic effects of angiotensin II are also pivotal for progressive renal injury, including induction of aldosterone, increased ECM synthesis, immune modulation, induction of other growth factors such as platelet-derived growth factor B (PDGF-B), TGF- $\beta$ , connective tissue growth factor (a downstream mediator of TGF- $\beta$  actions), and increased PAI-1 (96). Angiotensin can directly induce PAI-1. The RAS also modulates the inflammatory response, at least in part by affecting both lymphocytes and macrophages, which express RAS components, including angiotensinogen and angiotensin receptors.

Recent experimental and clinical data have explored the potential reversibility of glomerulosclerosis, and examined possible mechanisms of this glomerular remodeling (96, 97). In studies in the rat 5/6 nephrectomy model induced by renal artery branch ligation, we started treatment with high or normal dose ACEI at 8 weeks after injury, a point of established sclerosis as verified by renal biopsy. Higher doses of ACEI were more effective than normal antihypertensive doses of ACEI in ameliorating development of glomerulosclerosis over the next 4 weeks, although both glomerular and systemic hemodynamic effects were similar with both doses (96). In some rats, regression of glomerulosclerosis occurred in response to high dose ACEI, as shown by less severe sclerosis at autopsy than at biopsy 4 weeks earlier at initiation of treatment (96). Similar results are seen in aging rats, where existing aortic and glomerular sclerosis are reversed by high dose ARB and are associated with decreased PAI-1 levels (98).

More recent studies by the groups of Ritz, Remuzzi, Zatz, Chatziantoniou, and further studies by our group, have further explored the mechanisms and potential for regression of glomerulosclerosis (99-103). Although glomerular pressure again was lowered to a similar extent by an extremely high dose and a lower dose of the ARB losartan, the higher dose was more effective and decreased renal inflammation and restored glomerular and interstitial injury to pretreatment levels (104). We also showed that high dose ARB was particularly effective in achieving regression, numerically even better than ACEI (103). Of interest, inhibition of aldosterone by spironolactone also decreased PAI-1 levels and induced regression of sclerosis in some animals (105, 106). The group of Ritz investigated regression, comparing injury in subgroups of rats sacrificed at different times after subtotal nephrectomy induced by cautery without ligation, a model with less severe

hypertension and sclerosis than the ligation model. Glomerulosclerosis, vascular lesions and tubulointerstitial fibrosis were less in rats sacrificed at 12 weeks after 5/6 nephrectomy after 4 weeks of ACEI than in those sacrificed at 8 weeks (99). The efficacy of combination therapy with an ACEI, ARB and statin on sclerosis and regression beyond monotherapy with any one of these drugs was shown by Remuzzi's group (101), supporting that a multipronged therapeutic approach can achieve better results than targeting of a single pathway.

The molecular composition of sclerotic glomeruli differs from that of the normal mesangial matrix and appears to be more resilient to proteolytic degradation, perhaps explaining why glomerulosclerosis regression is not achieved in all 5/6 nephrectomized rats treated with high dose RAS blockers. Nonetheless, a recent mouse UUO study reported convincing evidence of interstitial matrix remodeling when UUO was released after 7 days (54).

Although regression of sclerosis was achieved in the above studies using angiotensin inhibition, regression did not occur in all animals. Further, glomerular structure did not completely return to normal, suggesting that additional mechanisms such as reactive oxygen species, abnormal cell growth, etc. promoting sclerosis were still active, or that inherent limits of regenerative capacity of remaining glomerular components exist.

### 9.1. ECM modulation by RAS and aldosterone

The mechanisms of the observed regression in the above studies have been partially elucidated. The balance of ECM synthesis and proteases and their inhibitors is key in determining whether ECM accumulation occurs. Key modulators of ECM turnover in the glomerulus include MMPs 2 and 9 and tissue inhibitors of matrix metalloproteases (TIMPs). Key factors that promote ECM synthesis include TGF- $\beta$ 1. In our studies of regression, high-dose angiotensin inhibition did not increase MMP-2 or -9 messenger RNA or activity; these were actually reduced. TGF- $\beta$ 1 messenger RNA was also not changed (103). Regression of sclerosis induced by angiotensin inhibition was, however, tightly linked to decreases in PAI-1 and TIMP-1 (103, 106). Kidney plasmin activity was markedly decreased in untreated 5/6 nephrectomy rats, but was restored towards normal when regression was achieved by high dose ARB. Thus, ARB decreased TIMP-1 and PAI-1, and increased plasmin, with net effect to induce regression of sclerosis. The decreased PAI-1 may also have contributed to less inflammatory cell infiltrate. Inhibition of aldosterone also resulted in regression in some rats, an effect that was enhanced by blood pressure control with nonspecific antihypertensives (106). This effect was again linked to decreased PAI-1.

### 9.2. Cell responses mediated by RAS and aldosterone

The complex interactions of cells within the glomerulus determine the potential for regression of sclerosis. Mesangial cells readily proliferate and can be replenished after injury. However, the interaction between podocytes and endothelium is quite complex. The podocyte normally secretes growth factors, including VEGF-A and

angiopoietin-1, that are necessary for normal glomerular endothelial cell survival and function (107, 108). The mechanism whereby such mediators from the podocytes can flow in a countercurrent fashion across the GBM to the endothelium could relate to the recently described subpodocyte space, cave-like domains under podocytes overlying the GBM with limited egress, which could locally alter transcapillary pressures and flow (109).

Glomerular VEGF and capillary density were decreased in the rat remnant kidney model of glomerulosclerosis and, conversely, treatment with exogenous VEGF ameliorated development of glomerulosclerosis and tubulointerstitial fibrosis (110). In studies of regression induced by ACEI, podocyte number was not changed but the volume of the podocytes was increased. Interestingly, this regression induced by ACEI also decreased mesangial and endothelial cell proliferation in this model with a corresponding reduction in glomerular volume and capillary number (100). In our recent preliminary confocal Z-section studies in the remnant kidney ligation model, we observed decreased capillary branching in untreated sclerotic rats, with increased capillary branching and length occurring when regression of sclerosis was induced by angiotensin inhibition. This observation indicates that increased capillary growth occurs and contributes to induction of regression, i.e. more open capillary loops and less sclerosis (111). In vitro, we showed that ARBs may affect injured podocytes, restoring their synthesis of the key angiogenic factors VEGF and angiopoietin-1, and thus promoting capillary growth (112). The endothelial cell growth in response to podocyte-conditioned media was blocked by antibodies inhibiting these angiogenic proteins. These data imply that angiotensin inhibition could contribute to regression by restoring the injured podocyte's ability to induce capillary growth (112).

### 9.3. Angiotensin receptors: AT1 vs AT2

Angiotensin transduces effects via two major angiotensin receptors: the angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors. The AT1 receptor mediates classic angiotensin actions, including vasoconstriction, growth and matrix synthesis, whereas the AT2 receptor counteracts these effects, mediating vasodilation and apoptosis (113). Additional novel RAS actions are contributed to by ACE2, angiotensin metabolites and the renin receptor (114-116).

AT2 receptor expression can be altered after injury. Thus, the AT2 receptor was upregulated after angiotensin infusion (117), and in response to acute injury (118). AT2-induced apoptosis could minimize injury by removal of injured cells before activation of profibrotic chemokines and cytokines occurs. AT2 receptor-mediated vasodilation occurs due to nitric oxide, or indirectly via the bradykinin B2 receptor (119). Not all AT2 actions appear to be beneficial. The AT2 receptor also increases the chemokine RANTES in response to angiotensin II, a potential profibrotic mechanism (120, 121).

The net effect of the AT2 receptor in interstitial fibrosis was examined in AT2 $^{-/-}$  mice with UUO. AT2 $^{-/-}$

mice developed worse fibrosis than wild-type counterparts after UUO, linked to marked decrease in apoptosis of interstitial and tubular epithelial cells (122). Similar observations have been made in the hamster cardiomyopathic model of spontaneous cardiac fibrosis, where blockade of the AT1 receptor resulted in less cardiac fibrosis. In contrast, antagonism of the AT2 receptor in these hamsters resulted in worse injury, linked to inhibition of apoptosis in cardiac neomyocytes in parallel in vitro experiments (123). Further, compared to wild-type, mice with overexpression of the AT2 receptor (AT2 Tg) developed less glomerular injury, less proteinuria, less PDGF-B and TGF- $\beta$  expression and more nitric oxide after subtotal nephrectomy. The protective effect of AT2 overexpression was blocked by treatment with an AT2 receptor antagonist, supporting that the decreased injury was AT2-mediated (124). Our recent study investigated the effects of the AT2 receptor in existing glomerulosclerosis. AT2 receptor antagonism was started at 8 weeks after subtotal nephrectomy, a time point by which moderate glomerulosclerosis had developed. AT2 receptor antagonist treatment resulted in worsened glomerulosclerosis over the next 4 weeks compared to AT1 receptor blocker treatment, where regression occurred. Importantly, adding AT2 receptor blocker to concomitant AT1 receptor blocker prevented the beneficial effects of the ARB and worsened sclerosis (125). The protective mechanisms of AT2 receptor in this model were linked to shifts in apoptosis/proliferation and change in fibrinolytic/proteolytic effects, with increased PAI-1 in rats receiving the AT2 receptor antagonist. In contrast, sclerosis was decreased when AT2 was blocked before sclerosis occurred, i.e. at the onset of injury in the rat radiation nephropathy model or the time of renal ablation in the subtotal nephrectomy model (126). These results suggest that mechanisms activated by AT2 may have varying effects depending on stage and type of injury. The results further suggest that efficacy of AT1 receptor blockers could in part be due to increased available angiotensin II to bind to and activate the AT2 receptor.

## 10. RAS, PAI-1 AND DYSMETABOLIC SYNDROME

The RAS and PAI-1 also have broad-ranging effects on risk for cardiovascular and renal disease by modulating obesity and dysmetabolic syndrome. Data from our group and others have recently shown that pharmacological blockade or genetic deletion of AT1 attenuates development of high fat diet-induced obesity, and decreases expression of key markers of dysmetabolic syndrome, associated with decreased PAI-1 (22, 127). Protection against adverse effects of high fat diet, such as increased adiposity, hyperglycemia, hyperinsulinemia and altered balance of key-adipogenic molecules, was also seen in PAI-1 deficient mice (22). Interestingly, weight loss from either gastric restrictive surgery or exercise and diet is associated with a significant decrease in adipose tissue macrophage number, reduction of inflammatory state and normalization of inflammatory mediators within the adipose tissue (including macrophage inhibitory factor, PAI-1, and other acute phase proteins) (128, 129).

## 11. SUMMARY AND PERSPECTIVE

PAI-1 is a multifunctional glycoprotein with complex actions in the kidney with net effect to increase ECM accumulation. Increased local PAI-1 is tightly linked to fibrosis and glomerulosclerosis. Conversely, inhibition of PAI-1 prevents CKD progression and may even facilitate regression of glomerulosclerosis, likely due to effects on proteolysis. In contrast, in the renal interstitium, PAI-1 interactions with vitronectin appear key and facilitate cell migration and potentially EMT. Additional complex interactions with e.g. angiotensin, aldosterone, TGF- $\beta$  and thymosin  $\beta$ -4 are emerging, which may vary in heart, aorta and kidney. Further understanding of local actions of PAI-1 and its interactions with other modulators of fibrosis may direct specific effective approaches for treatment of progressive kidney disease.

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**Send correspondence to:** Agnes B. Fogo, MCN C3310, Dept of Pathology, Vanderbilt University Medical Center, Nashville, TN 37232, Tel: 615-322-3070, Fax 615-343-7023, E-mail: agnes.fogo@vanderbilt.edu

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