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Novel actions of tissue-type plasminogen activator in chronic kidney disease

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1. ABSTRACT

Tissue-type plasminogen activator (tPA) is traditionally viewed as a simple serine protease whose main function is to convert plasminogen into biologically active plasmin. As a protease, tPA plays a crucial role in regulating blood fibrinolysis, in maintaining the homeostasis of extracellular matrix and in modulating the post-translational activation of growth factors. However, emerging evidence indicates that tPA also functions as a cytokine that transmits its signal across the cell membrane, initiates a diverse array of intracellular signaling, and dictates gene expression in the nuclei. tPA binds to the cell membrane LDL receptor-related protein 1 (LRP-1), triggers its tyrosine phosphorylation. As a cytokine, tPA plays a pivotal role in the pathogenesis of renal interstitial fibrosis through diverse mechanisms. It facilitates tubular epithelial to mesenchymal transition, potentiates myofibroblast activation, and protects renal interstitial fibroblasts/myofibroblasts from apoptosis. Together, growing evidence has implicated tPA as a fibrogenic cytokine that promotes the progression of kidney diseases. These new findings have radically changed our conception of tPA in renal fibrogenesis and represent a paradigm shift towards uncovering its cytokine function.

2. INTRODUCTION

Renal interstitial fibrosis is characterized by florid inflammatory infiltration, massive activation of fibroblasts, excessive production and deposition of extracellular matrix (ECM) components, and decreased ECM degradation. This cascade of destructive events leads to sclerotic alterations that represent the common final pathway of a wide variety of chronic kidney diseases (CKD), regardless of the initial etiologies (1-3). An unbalanced accumulation of ECM components in the injured kidney progressively occupies and eventually replaces the normal renal parenchyma, creating a vicious deterioration cycle that leads to renal functional compromise. The severity of renal interstitial fibrosis is believed to determine the prognosis and faithfully predict the outcome of CKD in both animal models and patients (4).

In normal kidney, the homeostasis of ECM is a tightly regulated and finely tuned process involving multiple proteases and their regulators. Two protease systems, the plasminogen activation system and the matrix metalloproteinase (MMP) family, play an essential role in degrading ECM components. Each of these proteolytic

systems contains their respective endogenous activators and inhibitors, ensuring a delicate balance of the activity of these enzymes by both positive and negative regulation. There are two types of plasminogen activators (PAs): tissue-type PA (tPA) and urokinase-type PA (uPA) (5, 6). Although tPA and uPA display functional overlap in converting plasminogen into plasmin, they may not always work in a reciprocally compensatory manner. Furthermore, they can elicit many biological activities by a mechanism independent of their primary proteolytic function (7-10).

In light of its proteolytic activity, tPA was conventionally thought to be beneficial in the pathogenesis of renal fibrotic lesions, as an elevated tPA would lead to increased matrix degradation with decreased matrix accumulation and deposition (11, 12). However, studies from our laboratory have demonstrated that genetic ablation of tPA actually protects the kidney from developing interstitial fibrosis after obstructive injury (13). This surprising finding suggests that tPA may be detrimental in promoting the progression of kidney fibrosis under certain pathological conditions. Studies over the last several years indicate that tPA is capable of transmitting its signal across the plasma membrane and triggering a diverse array of intracellular signal transduction, leading to specific gene expression in the nuclei (13, 14). In this context, it is tempting to propose that tPA may function as a cytokine that elicits a wide variety of cellular processes that were previously unrecognized. In this article, we will provide a comprehensive review of tPA biology in chronic kidney fibrosis, with emphasis on recent advances in our understanding of its cytokine functions and its intracellular signaling.

3. tPA EXPRESSION IN KIDNEY DISEASES

tPA is a serine protease that converts the proenzyme plasminogen into plasmin, which then degrades the fibrin network of blood clots. Produced as a single polypeptide, tPA is cleaved at a single site in the central region of the molecule, converting it into a two-chain, disulfide-linked form composed of a heavy and a light chain, respectively (15, 16). The light chain of tPA contains the active catalytic site and is highly homologous to other serine proteases (15). In the blood, tPA is mainly derived from vascular endothelial cells. tPA is also widely expressed in neurons and glial cells of the human central nervous system (7).

In normal kidney, tPA mRNA is expressed in endothelial cells, glomerular podocytes and the epithelial cells of the distal collecting ducts, as demonstrated by *in situ* hybridization (17). After ischemia/reperfusion injury, overall tPA abundance is increased and it is also expressed in the damaged proximal tubules (17). However, controversy exists in this area, as a previous study demonstrated that tPA is down-regulated in a rabbit model of renal isechmia/reperfusion (18). A marked increase in tPA mRNA level in glomerular cells is reported in mice after administration of lipopolysaccharide (LPS); however, it is not accompanied by a concomitant increase in tPA-mediated proteolytic activity (19). Northern blot analysis also reveals that tPA mRNA levels are extremely low in normal mouse kidney, but markedly increased in the obstructed kidney after unilateral ureteral obstruction (UUO) (13). A dramatic increase of tPA protein is also observed by immunofluorescence staining in renal interstitial compartment after obstructive injury, which co-localizes with α-smooth muscle actin (α-SMA), a marker for activated myofibroblasts (20). Clinical studies also show that the circulating levels of tPA are elevated in dialysis patients, compared with healthy controls (21). It is worthwhile to mention that since it is a secreted protein, tPA may elicit its actions in a variety of cells through an autocrine or paracrine fashion, regardless of the source of its production.

4. tPA AS A SERINE PROTEASE

4.1. tPA as a major component of the plasminogen activation system

tPA is best known for its pivotal role in the regulation of blood coagulation and fibrinolysis. Activation of the fibrinolytic system is dependent on the conversion of plasminogen into the biologically active molecule plasmin, which is mediated by tPA and its functional cousin uPA (Figure 1). In plasma, the primary function of plasmin is to degrade fibrin-containing thrombi. Therefore, tPA is used as a thrombolytic agent for acute treatment of ischemic stroke in clinical settings (7). The activities of tPA and uPA are tightly controlled by endogenous physiological inhibitors, plasminogen activator inhibitor-1 (PAI-1) and PAI-2 (Figure 1). Hence, the plasminogen activation process is a delicately balanced system consisting of multiple endogenous activators and inhibitors, in which tPA is an integral component.

4.2. tPA and ECM homeostasis

Apart from its intravascular fibrinolytic action, tPA is widely distributed in many tissues, including the kidney and central nervous system. In the extravascular space, tPA, via its proteolytic product plasmin, plays a fundamental role in regulating ECM degradation and matrix accumulation. As shown in Figure 1, plasmin may directly degrade deposited fibrin or ECM components. Perhaps more importantly, it activates numerous MMPs from their zymogen to active forms, that in turn cleave ECM component substrates (3). Not surprisingly, these plasminogen activation and matrix degradation systems are closely regulated by negative inhibitors at different stages (Figure 1). While PAI-1 and PAI-2 are primary endogenous inhibitors of tPA/uPA activity, plasmin activity is controlled by α_2 macroglobulin (α_2M). Similarly, the activity of MMPs is constrained by a family of endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMP-1-4) (Figure 1). These regulatory components assemble an intricate network of ECM degradation, in which they work in concert to ensure a homeostasis of ECM at normal physiologic conditions.

4.3. tPA and growth factor activation

As a serine protease, tPA has been shown to play a critical role in modulating the post-translational activation of several growth factors such as hepatocyte

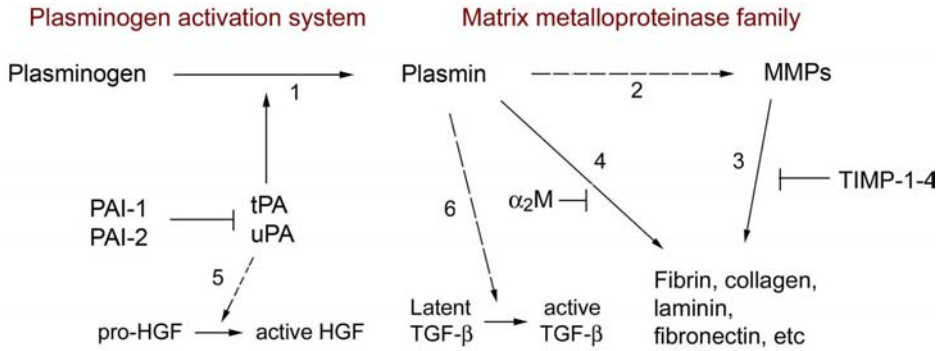

Figure 1. Major protease systems regulate the homeostasis of extracellular matrix in kidney. Two protease systems, the plasminogen activation system and the matrix metalloproteinase family, play an essential role in regulating the homeostasis of extracellular matrix (ECM) in kidney. Tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA) convert plasminogen into plasmin by proteolytic cleavage (1). Plasmin subsequently initiates fibrinolysis or activates matrix metalloproteinases (MMPs) (2), which in turn degrade ECM components (3). Plasmin may also be directly involved in ECM degradation (4). The activities of these proteases are tightly controlled by various endogenous inhibitors. Two plasminogen activator inhibitors (PAI-1 and PAI-2) negatively control the tPA and uPA activities, while α_2 macroglobulin (α_2M) blocks plasmin activity. A family of tissue inhibitors of matrix metalloproteinases (TIMP 1-4) controls the proteolytic activities of MMPs. PAs are also involved in the post-translational activation of anti-fibrotic hepatocyte growth factor (HGF) by proteolytically converting single-chain, pro-HGF to double-chains, active form (5). Plasmin also participates in the post-translational activation of pro-fibrotic TGF-β by promoting the conversion of active TGF-β from its latent form (6).

growth factor (HGF) (directly) or transforming growth factor-β (TGF-β) (indirectly via plasmin) (Figure 1). HGF is a mesenchyme-derived, pleiotropic protein originally characterized as a potent mitogen for hepatocytes (22). HGF is synthesized and secreted as a 728-amino acid, single chain, biologically inactive precursor, and then processed proteolytically to generate a 69 kDa α-chain and a 34 kDa β-chain, active form. tPA and uPA have been implicated as major serine proteases that are responsible for this proteolytic conversion during the post-translational activation of HGF (23). As HGF is an endogenous renoprotective factor that prevents the initiation and progression of chronic kidney fibrosis in a wide variety of animal models (24-26), tPA-mediated HGF activation could be beneficial in ameliorating fibrotic lesions after chronic injury. Consistent with this notion, a recent study demonstrates that an elevated tPA activity due to PAI-1 deficiency in mice results in an increased HGF activation, leading to reduction of liver fibrosis after bile duct ligation (11).

tPA is also implicated indirectly via plasmin in the post-translational activation of TGF-β, a pro-fibrotic cytokine that plays a crucial role in tissue fibrogenesis (27). TGF-β is secreted as a biologically inert, latent complex that has to be dissociated before it can exert its effects. This proteolytic activation of latent TGF-β is associated with the activity of tPA and uPA in rat osteosarcoma cells, and prevented by aprotinin, an inhibitor of plasmin activity. Therefore, it appears that the local TGF-β activity in a particular tissue may be controlled by the PA/plasmin system. Such plasmin-mediated proteolysis in TGF-β activation is operative *in vivo*, since renal activation of TGF-β is dramatically enhanced in the course of glomerulonephritis in the absence of PAI-1 (28). Hence, an increased plasmin activity, due to PAI-1 deficiency, may lead to an uncontrolled activation of TGF-β, thereby resulting in the poor outcome of glomerulonephritis. It should be stressed that the post-translational activation of such important growth factors as HGF and TGF-β is regulated by numerous serine proteases besides tPA. This functional redundancy explains why tPA deficiency sometimes causes no alteration in the activation of plasmin, HGF and TGF-β after kidney injury (13).

Recent studies also suggest that tPA may be of importance in activating heparin-binding epidermal growth factor-like growth factor (hb-EGF) (29). In pancreatic cancer cells, tPA induces a rapid and transient phosphorylation of the EGF receptor (EGFR) and promotes cell proliferation, which can be abolished by specific EGFR kinase inhibitors. The mitogenic activity of tPA is also inhibited by siRNA depletion of EGFR, thus confirming the involvement of this receptor in tPA-triggered signaling (29). These signaling and mitogenic effects of tPA require its proteolytic activity and activation of hb-EGF. Therefore, tPA, via activating hb-EGF, stimulates EGFR signaling and induces cell proliferation by triggering a proteolytic cascade.

4.4. tPA protease activity and renal fibrosis

The proteolytic activity of tPA could be beneficial in slowing the progression of renal fibrosis as it promotes the degradation of excessive ECM components that are a hallmark of the disease. This notion is validated in a mouse model of crescentic glomerulonephritis, in which deficiency of plasminogen or combined deficiency of tPA and uPA is associated with severe functional and histological exacerbation of glomerular injury (12). Deficiency of tPA, the predominant PA expressed in glomeruli, also aggravates glomerular lesions, while uPA

deficiency results in little effect on disease progression. Therefore, tPA is the major protective PA that reduces glomerular fibrin deposition and ameliorates fibrotic lesions after glomerular injury. Consistently, administration of recombinant tPA to rats with experimental glomerulonephritis promotes plasmin generation and matrix degradation in the glomeruli, leading to a decreased matrix accumulation without affecting latent TGF-β activation (30). These studies suggest a renal-protective function of tPA via its proteolytic capacity.

The effect of tPA on renal fibrosis appears to be disease- and tissue compartment-specific, however. In renal tubulointerstitial compartment, the story of tPA tilts in the opposite direction. Renal expression of tPA mRNA and its resultant activity are markedly induced after UUO, a model of interstitial fibrosis characterized by massive myofibroblast activation, inflammatory infiltration and tubular atrophy. One may speculate that tPA deficiency could aggravate renal fibrotic lesions, in view of its proteolytic activity. However, mice with tPA deficiency are protected against the development of renal tubulointerstitial fibrosis after obstructive injury. This surprising outcome is primarily attributed to the fibrogenic cytokine function of tPA (13, 14). Remarkably, because of functional redundancy from uPA, knockout of tPA does not affect renal plasmin generation and activity in this model (13). Interestingly, other studies show that uPA deficiency has no effect on the development and progression of renal interstitial fibrosis after UUO (31), although ablation of the uPA receptor (uPAR) exacerbates renal fibrosis (32).

Another way to establish the relationship between tPA protease activity and renal fibrosis is to investigate the role of plasmin, the major product of tPA protease activity, in renal interstitial fibrogenesis. An earlier study demonstrated that mice with plasminogen deficiency have significantly less collagen accumulation after obstructive injury, compared with their wild-type counterparts (33). This pro-fibrotic effect of plasmin is associated with its ability to promote TGF-β activation, as less renal TGF-β activity is detected in the plasminogen null mice (33). A recent, more comprehensive investigation has confirmed the fibrogenic effect of plasmin (34). Furthermore, reduction of renal fibrosis in plasminogen-deficient mice is accompanied by robust attenuation of epithelial-mesenchymal transition (EMT) (34), as characterized by tubular loss of E-cadherin and acquisition of αSMA (4, 35). Attenuation of EMT and renal fibrosis in plasminogen null mice is associated with a lower level of phosphorylated extracellular signal-regulated kinase-1 and -2 (Erk-1/2) and active TGF-β. This observation is further supported by the fact that direct addition of plasmin into cultured tubular epithelial cells *in vitro* initiates Erk-1/2 phosphorylation and promotes mesenchymal transition in a protease-activated receptor-1 (PAR-1)-dependent manner (34). Therefore, although tPA and plasmin are beneficial in ameliorating fibrotic lesions in glomerular diseases (12), they are actually deleterious in the pathogenesis of renal interstitial fibrosis, primarily by a mechanism independent of their direct proteolytic activity (13, 33, 34).

The role of tPA protease activity in renal fibrosis is not only defined by different disease models, but may be disease stage-dependent as well. Recent studies show that matrix-degrading proteases, such as MMP-2 and MMP-9, act in a paradoxical manner during different stages of renal fibrogenesis (36). A dual role for MMPs in the progression of Alport disease is proposed, with an early pathogenic function and a later protective action (36). Given such complexity, it would not be surprising if the effect of tPA on renal fibrosis is also disease stage-dependent. Thus, the role of tPA protease activity in renal fibrosis appears enormously complicated, and is largely dependent on particular disease models, distinct tissue compartments, and perhaps, specific disease stages.

5. tPA AS A CYTOKINE

Mounting evidence demonstrates that tPA also possesses various functions that are discrete from its proteolytic activity in a variety of physiological and pathological settings (7, 37). For instance, tPA has been shown to induce opening of the blood-brain barrier (9), modulates neuron and renal interstitial fibroblast apoptosis (10, 38), increases endothelial cell and pancreatic cancer cell proliferation through a plasmin-independent mechanism (39, 40) and amplifies neurotoxicity after hemoglobin exposure (41, 42). This wide spectrum of biological actions portends the ability of tPA to activate intracellular signal pathways that lead to modulation of specific gene expression. We recently showed that tPA binds to a cell membrane receptor, triggers a cascade of signal transduction, and controls MMP-9 gene expression in the nuclei (14). This mode of action elicited by tPA resembles that of classic cytokines, signaling proteins that transduce extracellular cues into cellular responses across the plasma membrane in either autocrine and/or paracrine fashions (43, 44).

5.1. Structural similarity to cytokine

Structurally, tPA belongs to a family of the so-called kringle-containing proteins that includes uPA, plasminogen, prothrombin, HGF and macrophage stimulating protein (MSP) (45-47). By definition, all proteins from this family contain at least one characteristic kringle domain in their N-terminus (α-chain), while their C-terminus (β-chain) often harbors a serine protease domain. The kringle domain is a looped, disulfide-linked structure consisting of approximately 78-amino acid residues (Figure 2), and is present in various numbers ranging from one in uPA to five in plasminogen. Functionally, the kringle domains are thought to play a critical role in the protein-protein interactions necessary for the biologic functions of their resident proteins. It has been shown that the second kringle domain in tPA harbors the binding sites for fibrin (16). In addition, receptor binding domains in tPA are located in the kringle-containing subunit, indicating that it plays a crucial role in receptor recognition.

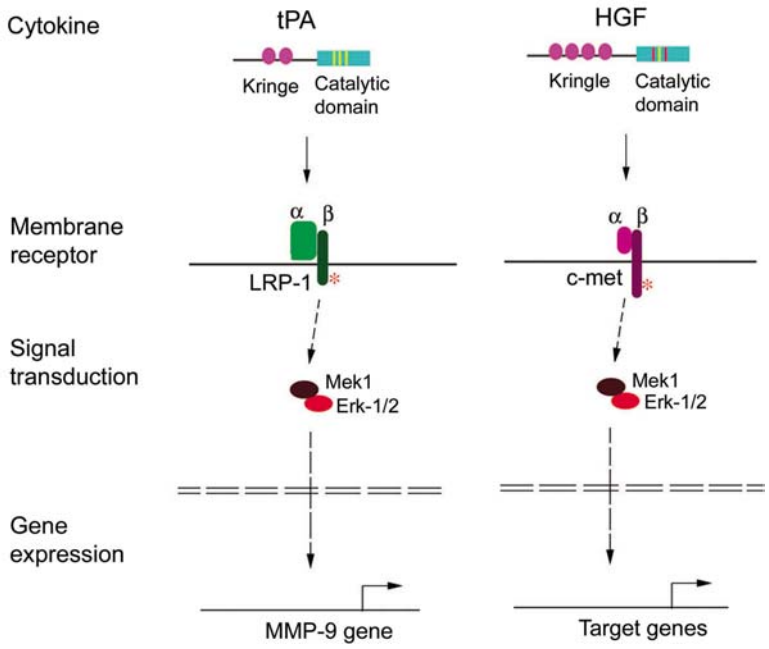

Figure 2. Schematic model depicts the similarity of the signal transduction pathways elicited by tPA and the classic cytokine HGF. tPA, a kringle-containing protein with structural similarity to HGF, acts as a cytokine that binds to the cell membrane receptor LRP-1, induces its tyrosine phosphorylation, activates signal transducers such as Mek1/Erk-1/2, and ultimately leads to specific gene expression in the nuclei. This signaling scheme is similar to that utilized by classic cytokines such as HGF, which transmit an extracellular cue across the plasma membrane to control nuclear gene expression. Asterisks indicate tyrosine phosphorylation sites. Reproduced with permission from (14).

Within the kringle-containing protein family, both HGF and MSP are well-characterized cytokines that execute diverse cellular processes such as cell proliferation, survival and differentiation (22, 48). In these proteins, the serine protease-like domain exists in the C-terminus; however, it is proteolytically inactive, because of the substitution of two essential residues in the active catalytic triad of the serine protease-like domain. When site-directed mutation is used to restore a functional catalytic triad in the HGF molecule, there is virtually no effect on its ability to promote cell growth and other cytokine functions (49), suggesting that protease activities of the members of this protein family do not negate their cytokine functions. In view of these structural features, tPA is considered to be a hybrid molecule, possessing both proteolytic activity and cytokine function in a non-mutually exclusive manner. It is therefore not completely surprising that tPA, as a kringle-containing protein with structural similarity to HGF and MSP, is also able to function as a cytokine, initiating intracellular signaling and dictating specific gene expression. It is of interest to point out that another member of this kringle-containing protein family, plasminogen/plasmin, has also been shown recently to bind to the cell membrane receptor PAR-1, triggering intracellular signaling and promoting tubular epithelial cell phenotypic conversion (34).

5.2. Cell membrane receptors for tPA

Unlike its closely-related protein uPA, tPA does not have a dedicated, specific receptor thus far. However, extensive studies have pointed to potential candidates that act functionally and biologically as tPA receptors by initiating intracellular signaling and eliciting downstream cellular responses. At present there are at least two known receptors for tPA. One, the most well-studied, is known as the low density lipoprotein (LDL) receptor-related protein 1 (LRP-1), which was originally identified as a receptor for tPA on hepatocytes (50); the other is annexin II, which was initially found on microglia (51).

LRP-1, also known as α2-macroglobulin receptor (α_2M_R) (50) or type V TGF-β receptor (TβR-V) (52), is a member of the LDL receptor family that is implicated in lipoprotein metabolism and in the homeostasis of proteinases and proteinase inhibitors (53). After being synthesized as a single chain molecule, LRP-1 is subsequently processed by furin into a 515-kDa α subunit, and an 85-kDa β subunit that harbors the transmembrane segment and a cytoplasmic tail with two NPxY motifs and numerous tyrosine residues (53-55). The α and β subunits are non-covalently associated with each other on the cell surface. LRP-1 is present on most cells and recognizes more than 30 structurally distinct ligands with high affinities, including lipoproteins, proteinases, proteinase-inhibitor complexes, matrix proteins and growth factors such as connective tissue growth factor (CTGF). Although originally considered as a scavenger receptor, increasing evidence indicates that LRP-1 has the potential to mediate intracellular signaling (53, 54). There are four putative ligand-binding domains in the extracellular region of LRP-1; each of them contains two to eleven individual cysteine-rich repeats (53). The binding

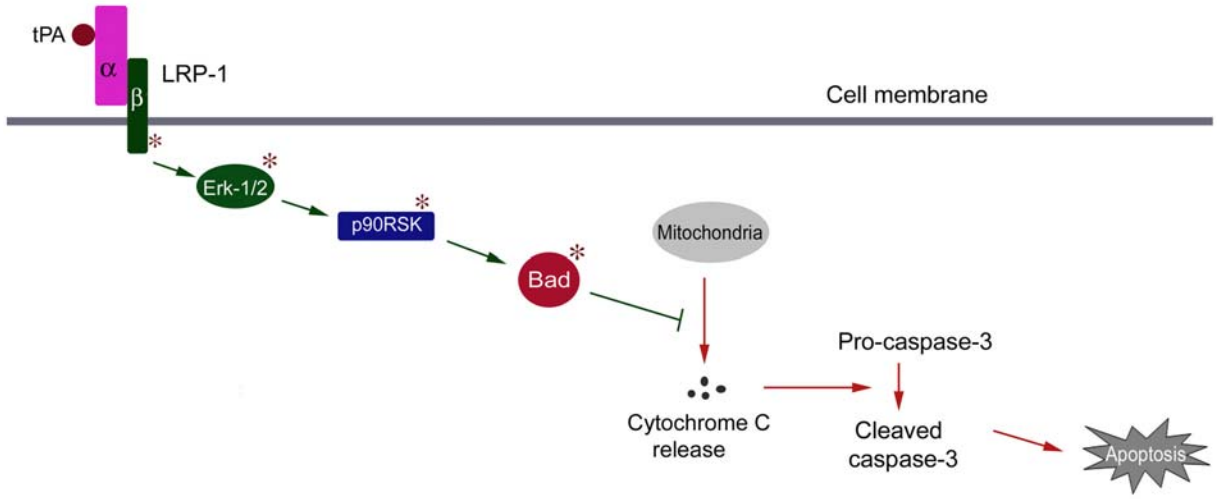

Figure 3. Schematic illustration of the cytoprotective signaling elicited by tPA in renal interstitial fibroblasts. After binding to its membrane receptor LRP-1, tPA induces its tyrosine phosphorylation, which in turn stimulates Erk-1/2 and p90RSK phosphorylation, leading to Bad phosphorylation and its subsequent inactivation. Bad inactivation blocks the cytochrome C release from mitochondria after death stimuli and protects the cells from apoptotic death. Asterisks denote tyrosine or serine/threonine phosphorylation. Modified from (38).

sites for tPA have been mapped to domains II and IV in LRP-1 molecule (53, 56). tPA can bind to annexin II as well, with annexin II providing a platform that facilitates the cleavage of plasminogen to plasmin by tPA. Another function of annexin II is the participation in the clearance and recycling of components within the coagulation-fibrinolysis system. The role of annexin II in renal fibrosis is still obscure, although it appears to have the potential for signal transduction (40, 51, 57).

5.3. tPA-mediated intracellular signaling

The ability of tPA to bind to the cell membrane receptor and initiate intracellular signaling challenges the dogma that tPA is a simple serine protease. We have shown that tPA directly induces MMP-9 gene expression by triggering intracellular signaling in renal interstitial fibroblast cells (14), providing clear evidence for tPA-mediated regulation of a particular gene. Similar to other cytokines such as HGF, the signaling circuit for tPA is comprised of several essential components including a secreted ligand (tPA), the transmembrane receptor containing domains on both sides of the plasma membrane (LRP-1), intracellular signaling mediators (Mek1 and Erk-1/2), and target genes (MMP-9) (Figure 2). These readily fulfill the basic principle of cytokine signaling, which places tPA on the long list of the signaling proteins that transmit their signal across the plasma membrane to elicit a wide range of cellular activities and to mediate inter-cellular communications.

The intracellular signaling pathways activated by tPA are beginning to emerge, although much remains to be elucidated. We show that one of the earliest events after tPA stimulation in renal fibroblasts is the induction of tyrosine phosphorylation on the LRP-1 β-subunit, which takes place as early as 0.5 to 2 minutes. This tyrosine phosphorylation of LRP-1, triggered by tPA, is robust, rapid and transient (14, 20). One downstream event after LRP-1 phosphorylation is activation of the mitogen-activated protein (MAP) kinase pathway, with rapid phosphorylation of Mek1 and Erk-1/2 upon tPA stimulation. It has been found that MAP kinase activation is essential for mediating MMP-9 gene induction by tPA (14). Furthermore, activated Erk-1/2 can phosphorylate p90RSK, which in turn leads to phosphorylation of the pro-apoptotic protein Bad and its subsequent inactivation (Figure 3).

LRP-1 phosphorylation on tyrosine residues may also lead to the recruitment of β1 integrin by facilitating LRP-1/β1 integrin complex formation (20). As shown in Figure 4, tPA triggers LRP-1 tyrosine phosphorylation and induces a complex formation between LRP-1 and β1 integrin by recruiting pre-existing β1 integrin in the plasma membrane. This leads to β1 integrin "clustering", a common mode of integrin activation, which in turn activates its major downstream effector, the intergrin-linked kinase (ILK). ILK works in concert with TGF-β1 signaling and synergistically promotes interstitial myofibroblast activation, as evidenced by an increased expression of α-smooth muscle actin (α-SMA) and type I collagen. Taken together, tPA, via binding to the plasma membrane receptor LRP-1 and induction of its tyrosine phosphorylation, activates MAP kinase and integrin signaling, two major intracellular signal pathways that are essential for mediating a range of cellular functions.

6. tPA CYTOKINE FUNCTION AND RENAL FIBROSIS

Interstitial fibrosis is generally preceded by infiltration of inflammatory cells and by a massive activation and expansion of interstitial fibroblasts and myofibroblasts (2, 58, 59). Emerging evidence

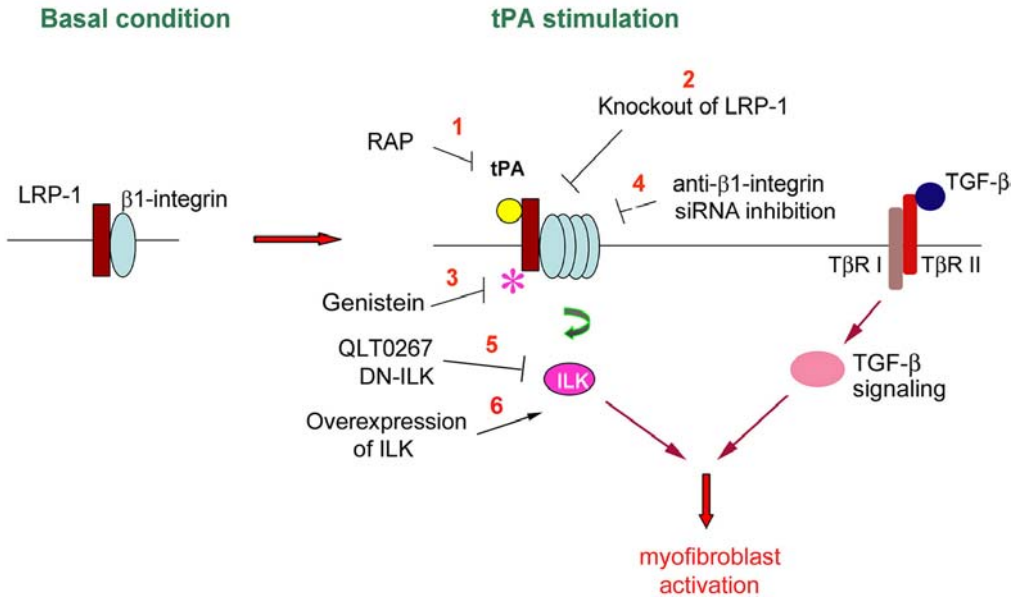

Figure 4. tPA promotes myofibroblast activation through LRP-1-mediated recruitment of integrin signaling. tPA binds to the membrane receptor LRP-1, induces its tyrosine phosphorylation, which then leads to an increased recruitment of β1 integrin and activation of downstream ILK. This results in myofibroblast activation. Blockade of each and every step within this signaling circuit by multiple strategies, such as blocking tPA binding to LRP-1 by the receptor-associated protein (RAP) (1), knockout of LRP-1 (2), inhibition of tyrosine phosphorylation with genistein (3), blockade of β1 integrin signaling by neutralizing antibody or siRNA knockdown of its expression (4), or inhibition of downstream ILK signaling by either a small molecule inhibitor (QLT0267) or disruption of the β1 integrin/ILK engagement by dominant-negative ILK (5), abolishes the fibrogenic effect of tPA on myofibroblast activation. Ectopic expression of ILK mimics tPA and promotes the TGF-β-mediated myofibroblast activation (6). It remains to be elucidated how ILK and TGF-β signaling are integrated, leading to synergistic promotion of myofibroblast activation. Asterisks denote tyrosine phosphorylation. Modified from (20).

suggests that tPA plays a pivotal role in mediating these processes via its cytokine functions. It is of interest to stress that the proteolytic activity of tPA appears functionally redundant, because its deficiency in mice does not result in any alteration in plasmin, HGF and TGF-β1 activation after ureteral obstruction (13). However, tPA ablation largely abolishes MMP-9 induction, indicating that the cytokine function of tPA is irreplaceable *in vivo*.

6.1. tPA facilitates tubular EMT by impairing TBM through induction of MMP-9 gene expression

Activated fibroblasts and myofibroblasts are the principal matrix-producing effector cells; the size of this cell population in fibrotic kidneys is believed to be a determining factor dictating the progression and prognosis of a wide variety of chronic kidney diseases (58, 60, 61). In normal kidneys, interstitial fibroblasts are scarce in number and often quiescent in nature. After chronic injury, these cells become phenotypically activated, with an increased proliferative capacity, *de novo* expression of the myofibroblast hallmark, α-SMA, and active production of ECM components (2, 58, 62-65). Renal interstitial fibroblasts/myofibroblasts appear to come from three major sources: 1) activation of resident interstitial fibroblasts, 2) phenotypic transition from tubular epithelium through EMT (4, 66); and 3) recruitment of bone marrow-derived progenitors (58, 67). EMT is a highly orchestrated, step-wise process characterized by loss of epithelial markers such as E-cadherin and ZO-1, *de novo* expression of mesenchymal markers including αSMA and fibroblast-specific protein-1 (Fsp-1), disruption of tubular basement membrane that normally confines the tubular epithelial cells, and enhanced migration of the transformed cells into the interstitium (4, 35, 67).

We previously showed that endogenous tPA facilitates tubular EMT in obstructive nephropathy by promoting the destruction of TBM integrity through induction of MMP-9 expression in interstitial fibroblasts (13). In tPA null mice, TBM integrity is largely preserved and tubular EMT is markedly reduced after obstructive injury. The expression of tPA is significantly upregulated in the obstructed kidney of mice and such induction mainly takes place in the interstitial compartment, concurrently accompanied by increased MMP-9 and LRP-1 expression (14). *In vitro*, addition of recombinant tPA directly induces MMP-9 gene expression and protein secretion in normal rat kidney interstitial fibroblasts (NRK-49F). Further investigation reveals that tPA induces MMP-9 expression by a mechanism independent of its protease activity. Instead, tPA elicits its action through binding to its plasma membrane receptor LRP-1, and activating downstream Mek-1/ERk-1/2 signaling (14). Similarly, tPA also induces MMP-9 gene expression in human

cerebral microvascular endothelial cells by a LRP-1-dependent pathway (68).

The notion that tPA-mediated MMP-9 induction, via destruction of TBM, facilitates tubular EMT suggests that TBM integrity is a critical determinant for EMT *in vivo*. TBM consists predominantly of collagen IV, laminin and entactin, and it provides a structural foundation for tubular epithelial cells to lie upon for proper function. TBM is also a physical barrier that separates tubular epithelial and interstitial compartments in the kidney; therefore, destruction of its integrity would facilitate the ready access of tubular epithelial cells to the interstitial matrix microenvironment. In addition, alterations in TBM composition also regulate tubular EMT *in vitro* (69), implying its importance in regulating the epithelial cell phenotype. All together, it appears that increased tPA promotes tubular EMT by impairing TBM integrity using a mechanism that depends on MMP-9 induction.

6.2. tPA potentiates myofibroblast activation by recruiting integrin signaling

Although myofibroblasts may derive from different sources as described above, interstitial fibroblast activation arguably remains the major pathway leading to the generation of matrix-producing myofibroblasts. Studies from our laboratory recently indicated that tPA promotes TGF-β1-mediated renal interstitial myofibroblast activation and ECM production by a mechanism independent of its protease activity (20). We have uncovered a novel signaling pathway that tPA elicits its fibrogenic action through its membrane receptor LRP-1 (20). In renal interstitial fibroblasts, tPA triggers tyrosine phosphorylation of the LRP-1 β subunit that, in turn, facilitates LRP-1/β1 integrin complex formation and subsequently activates ILK signaling (Figure 4).

Under basal conditions, LRP-1 and β1 integrin appear to constitutively interact with each other in renal interstitial fibroblasts. However, this complex formation between LRP-1 and β1 integrin is markedly enhanced after tPA stimulation, primarily by LRP-1-mediated recruitment of pre-existing β1 integrin in the plasma membrane (20). Such recruitment of β1 integrin is dependent upon the tPA-triggered tyrosine phosphorylation of the LRP-1 β subunit. Blockade of tPA binding to LRP-1 by the receptor-associated protein (RAP) or inhibition of LRP-1 tyrosine phosphorylation abrogates LRP-1/β1 intergrin complex formation and myofibroblast activation. Furthermore, tPA-mediated LRP-1/β1 integrin interaction leads to the activation of ILK, a major downstream effector kinase that plays an essential role in regulating cell growth, differentiation, ECM production and assembly (70-72). It appears that the β1 integrin/ILK pathway is indispensable for mediating the fibrogenic actions of tPA, since multiple manipulations, such as blockade of β1 integrin signaling, inhibition of ILK activity, or disruption β1 integrin/ILK engagement, completely abrogate tPA-mediated myofibroblast activation and matrix production. Consistently, ectopic expression of ILK in interstitial fibroblasts mimics the action of tPA and synergistically promotes TGF-β1-mediated myofibroblast activation. Therefore, tPA directly promotes interstitial myofibroblast activation by LRP-1-mediated recruitment of β1 integrin/ILK signaling (20). It is also worthwhile to point out that tPA also synergistically promotes TGF-β1-mediated myofibroblastic activation from human lung fibroblasts (MRC-5) and primary rat hepatic stellate cells (20), suggesting that these findings appear to have broader implications and may be of general importance in organ fibrogenesis.

6.3. tPA promotes interstitial fibroblast/myofibroblast survival

In theory, the expanded population of fibroblasts/myofibroblasts in the fibrotic kidney could result from either an increase of proliferation or/and a decrease in cell death. Although studies indicate that fibroblasts from diseased kidney display an increased proliferative activity, the regulation of interstitial fibroblast/myofibroblast survival in pathologic conditions remains poorly understood. We recently found that tPA is a potent survival factor that protects renal interstitial fibroblast/myofibroblasts against apoptosis induced by a variety of death cues (38). *In vitro*, tPA is able to protect interstitial fibroblasts from apoptosis induced by staurosporine or oxidative stress, as evidenced by inhibition of caspase-3 activation, suppression of cytochrome C release from mitochondria into the cytosol, and prevention of cellular DNA fragmentation. The cytoprotective effect of tPA is also observed in TGF-β1-activated myofibroblasts. In agreement with its cytokine activity, the anti-apoptotic function of tPA is independent of its protease activity, but requires LRP-1. Deletion or knockdown of LRP-1 abolishes tPA-mediated cell survival, whereas re-introduction of an LRP-1 minigene into LRP-1-deficient fibroblasts restores the cytoprotective abilities of tPA (38). It is shown that tPA triggers a cascade of survival signaling that involves Erk-1/2, p90RSK and Bad phosphorylation (Figure 3). Phosphorylation of Bad leads to its inactivation and subsequent degradation (73, 74), thereby preventing it from entering the mitochondria to cause cytochrome C release. *In vivo*, increased apoptosis of interstitial myofibroblasts is found in tPA-/- mice after obstructive injury, which leads to a complete depletion of myofibroblasts at 4 weeks after relief of the obstruction. Hence, in addition to promoting EMT and myofibroblast activation, tPA expands the lifespan of renal interstitial fibroblasts and myofibroblasts by inhibiting their apoptosis. These combined effects should have a profound impact on the size and fate of this matrix-producing cell population, thereby having a critical role in the pathogenesis of renal interstitial fibrosis.

6.4. tPA modulates renal inflammatory responses

In response to renal injury, kidney cells often produce and release various cytokines or chemokines, which attract circulating inflammatory cells to migrate into the injured sites. Infiltrating inflammatory cells, including monocytes/macrophages, lymphocytes and neutrophils, in turn contribute to the initiation and progression of renal fibrosis in several ways. First, inflammatory cells produce and release their own profile of cytokines, growth factors,

and reactive oxygen species, thereby leading to the formation of a vicious self-accumulation cycle. Furthermore, production and secretion of profibrotic cytokines by inflammatory cells create a fibrogenic microenvironment, leading to generation of the matrix-producing effector cells through fibroblast activation and tubular EMT. Numerous studies demonstrate that the decline of renal function in CKD patients often correlates closely to the extent of inflammation (75). Logically, inhibition of renal inflammation by different maneuvers is therapeutically effective, resulting in an amelioration of renal fibrotic lesions in various experimental animal models (76, 77). Interestingly, tPA is also able to modulate the inflammatory response to tissue injury (17, 78). In an acute kidney injury induced by ischemia/reperfusion, de novo expression of tPA is found in proximal tubules, and it induces neutrophil influx into ischemic renal tissue, which is independent of plasmin generation, complement C3 activation, proinflammatory cytokines expression, and MMP activity in this model (17). tPA, in concert with its receptor LRP-1, also facilitates the migration of activated macrophages within an inflammatory environment by a novel Mac-1 ($\alpha_M\beta_2$ integrin)-dependent mechanism (78). It is shown that tPA augments the association of LRP and Mac-1 and eventually causes an increased migration of macrophages into inflammatory sites.

6.5. tPA and renal blood flow dynamics

Another important factor that is implicated in the pathogenesis of renal fibrosis is the alteration of systemic renal blood flow dynamics. Increased vessel tone, caused by contraction of vascular smooth muscle cells, can result in both systemic and microvascular hypertension. This eventually causes glomerular hypertension, hyperfiltration, and hyperperfusion, setting the kidney in motion towards a vicious cycle (79). It has been reported that tPA, independent of its catalytic activity, promotes smooth muscle cell activation and increases vessel tone (8, 80). Intriguingly, tPA-mediated vasoconstriction and calcium mobilization from intracellular stores also requires the formation of a complex between LRP and $\alpha_v\beta_3$ integrin in vascular smooth muscle cells (80).

7. CONCLUSION AND PERSPECTIVES

Studies in the last several years have uncovered that tPA can act as a cytokine that binds to plasma membrane receptors, activates a diverse array of intracellular signal pathways, and controls gene expression (14, 20, 68). These novel actions of tPA are clearly divergent from its classic role as a serine protease, and represent a paradigm shift in our understanding of tPA biology. As summarized in Figure 5, both protease activity and cytokine functions of tPA play crucial, and sometimes opposite, roles in the evolution of chronic kidney diseases. As a protease, tPA may ameliorate or exacerbate the onset and development of tissue fibrosis in an organ-, disease- and perhaps stage-specific manner. As a cytokine, tPA triggers the interaction of its receptor LRP-1 and various integrins, leading to an increased myofibroblast activation, altered blood flow dynamics and enhanced macrophage migration. In addition, tPA also promotes tubular EMT by inducing MMP-9 expression, and expands the lifespan and size of the interstitial fibroblast and myofibroblast population by inhibiting apoptosis (Figure 5). Therefore, tPA can not be viewed merely as a serine protease any more. Similar to other kringle-containing proteins such as HGF and MSP, the unique structural features of tPA also endow it as a cytokine, able to activate intracellular signaling and dictate gene expression, and thereby elicit a wide variety of cellular activities.

Not surprisingly, many basic questions regarding tPA cytokine function as well as the biochemical pathways along which its signals are transduced remain unanswered. The cytokine activities and intracellular signaling pathways of tPA are primarily mediated by the LRP-1 receptor. However, LRP-1 also functions as a scavenger receptor that plays an essential role in the clearance and endocytosis of tPA and tPA/PAI-1 complexes (53). How cells determine when LRP-1 mediates its scavenging versus signaling functions after tPA binding remains mysterious. Furthermore, our current knowledge on the immediate downstream signaling subsequent to tPA binding is limited. While tPA triggers a rapid and robust tyrosine phosphorylation of LRP-1 β subunit and such phosphorylation is vital for its cellular functions, exactly which tyrosine kinase is responsible for LRP-1 phosphorylation after tPA stimulation is yet to be determined.

The novel cytokine functions of tPA unraveled recently have posed a great challenge for us to translate this new-found knowledge into clinical applications in the setting of chronic kidney diseases. While tPA is clinically beneficial in the management of ischemic stroke patients (7), and may be advantageous for a subset of glomerular disease (30), the fibrogenic cytokine functions of both exogenously administrated and endogenously induced tPA have to be taken into consideration. It is conceivable that the cytokine functions of tPA may be separable from its proteolytic activity, and can be targeted independently, as exemplified by recent studies demonstrating that selective tPA antagonists reduces its neurotoxic effects without compromising its fibrinolytic activity (42). Clearly, more studies are needed in this area in order to develop specific therapeutic agents that discretely target the protease activity or cytokine function of tPA in clinical settings.

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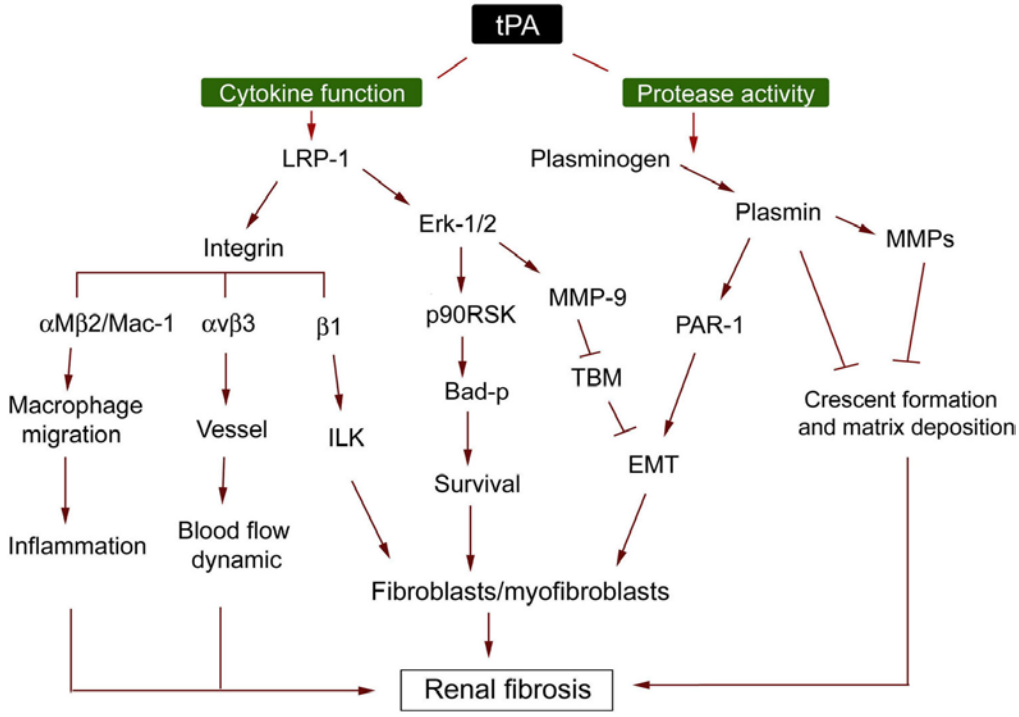

Figure 5. An overview of the potential roles of tPA in the pathogenesis of renal fibrosis. As a protease, tPA converts plasminogen to plasmin, which promotes fibrinolysis and matrix degradation, thereby providing beneficial effects in the pathogenesis of some glomerular diseases. However, plasmin may directly induce tubular EMT by a PAR-1-dependent mechanism (34). As a cytokine, tPA binds to its receptor LRP-1, triggers intracellular MAPK signaling, and induce MMP-9 expression, thereby facilitating EMT via destruction of TBM (13, 14). tPA-mediated MAPK signaling is also essential for fibroblast and myofibroblast survival, leading to an expansion of this matrix-producing cell population (38). Furthermore, tPA-activated LRP-1 interacts with distinct integrins in different cells. It recruits β1 integrin and its downstream effector ILK in renal interstitial fibroblasts, leading to promotion of myofibroblast activation (20). tPA/LRP-1 also works in concert with β2 integrin in the macrophages, promoting their migration (78). Finally, tPA promotes LRP/β3 integrin complex formation in vascular smooth muscle cells (80), modulating blood flow dynamics. Therefore, tPA as a cytokine plays a critical role in the pathogenesis of renal fibrosis by regulating tubular EMT, renal inflammation, blood flow dynamics, myofibroblast activation and survival.

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Abbreviations: CKD, chronic kidney disease; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; Erk-1/2, extracellular signal-regulated kinase-1 and -2; HGF, hepatocyte growth factor; ILK, integrin-linked kinase; LDL, low-density lipoprotein; LRP-1, LDL receptor-related protein 1; MMP-9, matrix metalloproteinase-9; PAR-1, protease-activated receptor-1; RAP, receptor-associated protein; α-SMA, α-smooth muscle actin; TBM, tubular basement membrane; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of matrix metalloproteinases; tPA, tissue-type plasminogen activator; UUO, unilateral ureteral obstruction.

Key Words: tPA, Renal Fibrosis, Myofibroblast, Integrin, Lrp-1, Protease, Review

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