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PAI-1 in Tissue Fibrosis

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1. Summary

Fibrosis is defined as a fibroproliferative or abnormal fibroblast activation–related disease., Deregulation of wound healing leads to hyperactivation of fibroblasts and excessive accumulation of extracellular matrix (ECM) proteins in the wound area, the pathological manifestation of fibrosis. The accumulation of excessive levels of collagen in the extracellular matrix depends on two factors: an increased rate of collagen synthesis and or decreased rate of collagen degradation by cellular proteolytic activities. The urokinase-type/tissue-type plasminogen activator (uPA/tPA) and plasmin play significant roles in the cellular proteolytic degradation of ECM proteins and the maintenance of tissue homeostasis. The activities of uPA/tPA/plasmin and plasmin-dependent MMPs rely mostly on the activity of a potent inhibitor of uPA/tPA, plasminogen activator inhibitor-1 (PAI-1). Under normal physiologic conditions, PAI-1 controls the activities of uPA/tPA/plasmin/MMP proteolytic activities and thus maintains the tissue homeostasis. During wound healing, elevated levels of PAI-1 inhibit uPA/tPA/plasmin and plasmin-dependent MMP activities and thus help expedite wound healing. In contrast to this scenario, under pathologic conditions, excessive PAI-1 contributes to excessive accumulation of collagen and other ECM protein in the wound area and thus preserves scarring. While the level of PAI-1 is significantly elevated in fibrotic tissues, lack of PAI-1 protects different organs from fibrosis in response to injury-related profibrotic signals. Thus PAI-1 is implicated in the pathology of fibrosis in different organs including the heart, lung, kidney, liver and skin. Paradoxically, PAI-1 deficiency promotes spontaneous cardiac-selective fibrosis. In this review we discuss the significance of PAI-1 in the pathogenesis of fibrosis in multiple organs.

Keywords

PAI-1; fibrosis; fibroblasts; EMT/EndMT; TGF-β; Type I collagen

2. Introduction

Fibrosis is a very common end-stage pathologic manifestation of several diseases including systemic sclerosis, pulmonary hypertension, renal hypertension, cardiac hypertension, myocardial infarction (MI), alcoholic liver disease and nonalcoholic steatohepatitis (NASH) (Berk et al., 2007; Brunt, 2004; Ellmers, 2010; Ghosh, 2010; Jansen et al., 2004; Leask, 2010; Mastuzaki, 2010; Pohlers et al., 2009; Varga and Abraham, 2007; Vaughan et al., 2007; Wynn, 2008; Yang et al., 2010; Yue et al., 2010). Although the etiology of fibrosis in different organs is not properly defined, numerous studies suggest that at the molecular level, fibrogenic processes in different organs may share common mechanisms (Ghosh, 2010; Wynn, 2007, 2008). For example, numerous *in vitro* and *in vivo* studies suggest that accidental or surgery-related wounds, hypertension-induced stress, viral infection, or

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enhanced cellular proteolytic activity–induced vascular injury lead to inflammation (Wynn, 2007, 2008). It is also known that inflammation is an essential step for tissue repair during wound healing. However, prolonged or persistent inflammation may trigger responses such as profibrotic signaling via overproduction/activation of cytokines such as transforming growth factor-beta (TGF-β), the differentiation of resident fibroblasts or transformed vascular endothelial cells, epithelial cells, or hepatic stellate cells to myofibroblasts, which in turn synthesize elevated levels of ECM proteins (Krenning et al., 2010; Lee and Kalluri, 2010; Zeisberg and Kalluri, 2010). Non-physiologic levels of accumulated collagens and other matrix proteins disrupt tissue homeostasis. This is associated with stiffness, increased thickness, and loss of tissue elasticity, the pathologic manifestation of tissue fibrosis (Wynn, 2007, 2008). Therefore, a constellation of cellular events occurs during the onset and progression of fibrogenesis in different organs. These unique as well as common features cause increased morbidity and mortality in millions of patients in the United States and around the world. It has been estimated that approximately 45% of human deaths are related to fibroproliferative disorders including fibrosis (Bitterman and Henke, 1991).

It is well documented that tissue homeostasis in a particular organ is maintained by the balance of ECM synthetic machinery, contributing to an increased rate of collagen synthesis, and the cellular proteolytic activities of the uPA/tissue type plasminogen activator (tPA)/ plasmin/ MMP system, contributing to degradation of collagen (Nagase and Woessner, 1999; Visse and Nagase, 2003). Furthermore, the activities of the fibrinolytic system depend on the activity of potent inhibition of uPA/tPA by plasminogen activator inhibitor-1 (PAI-1). In fibrotic tissues, the level of PAI-1 is elevated, which prevents tissue proteolytic activities and thus contributes to a decreased rate of collagen degradation and tissue fibrogenesis. In this review article, we focus specifically on PAI-1 biology and its molecular involvement in the process of fibrogenesis in different organs.

3. Biology of PAI-1

PAI-1 is a member of the serine protease inhibitor (serpin) gene family and the major physiologic inhibitor of the serine proteases, urokinase-type plasminogen activator and tissue-type plasminogen activator. Inhibition of uPA/tPA results in the inhibition of plasminogen-to-plasmin conversion as well as plasmin-dependent MMP activation. PAI-1 is the most well studied member of the serpin superfamily (350–400 amino acids in length) and comprises over 100 members with conserved domains. Phylogenetic analysis of the serpin superfamily revealed the existence of serpin in virus (pox virus), higher plants, acoelomata (schistomes), coelomata (insects, horseshoe crab), pseudocoelomata (*Caenorhabditis elegans*), and chordata (lamprey and vertebrates) (Irving et al., 2000; Potempa et al., 1994; Whisstock et al. 1999). Two closely related family members of PAI-1 are PAI-2 and PAI-3. PAI-2 levels are generally higher in placental extracts and possibly play an important role during pregnancy in placental tissue homeostasis and fetal growth regulation (Kruithof et al., 1995; Kwano et al., 1970). However, PAI-2 is also expressed in other cells including macrophages (Astedt et al., 1986; Risse et al. 2000). Generally, PAI-3 is synthesized in liver and is present in plasma and urine. It inhibits uPA, tPA and activated protein C and thus is also known as protein C inhibitor (PCI). In seminal plasma, the levels of PAI-3 antigen and its complex with tPA/uPA are higher than that of PAI-1 and its complex with tPA suggesting PAI-3 may be a physiologic regulator of uPA/tPA in male reproductive tissue (Braumwald et al., 1995; Espana et al., 1993; Gilabert-Estelles et al., 2005; Maemura et al., 2000). While physiologic levels of PAI-1 in mouse and human plasma ranges from 10 to 20 ng/mL (Devy et al. 2002; Heckman et al., 1985), under pathologic conditions the plasma concentration of PAI-1 rises several fold depending on disease severity.

3.1 Molecular Structure and Function of PAI-1

The serpin family protein PAI-1 gene is located at chromosome 7q21.3-q22 in humans and on chromosome 5G2, 5 in mice, codes for 48–50 kDa polypeptide. PAI-1 is synthesized by a variety of cells including vascular endothelial cells, adipocytes, macrophages, cardiomyocytes and fibroblasts; half life of activated and latent PAI-1 in circulation is 32 and 7 minutes respectively (Reilly et al. 1991). The detailed structure of serpin family proteins has been reviewed by Irving et al. (2000). In brief, serpin family proteins contain three β-sheets and nine α-helices. Two well characterized functional domains of PAI-1 are highly conserved in different mammals including humans, mice and rats. One of these domains, encoded by exons 3 and 4, is involved in high-affinity binding to vitronectin, which anchors PAI-1 to ECM. The other functional domain of PAI-1, coded by exon 8, is the reactive center loop (RCL) that undergoes conformational changes, and is required for the inhibition of tPA/uPA activity (Gorlatova et al., 2007). Like other serpin family proteins which undergo different conformational changes, such as native, cleaved, latent, delta and polymeric changes, PAI-1 protein also undergoes transition from native/active form to a latent/non-inhibitory state (Levin and Santell, 1987). While uPA/tPA converts plasminogen to active serine protease plasmin, the latent form of TGF-β to its active form, and pro-MMPs to MMPs, PAI-1 interaction with uPA/tPA blocks uPA/tPA activation, plasmin formation, and plasmin-dependent MMP activation. Thus, PAI-1 protects ECM proteins from proteolytic degradation and helps expedite wound healing (Hertig et al., 2003; Lackie, 2008). However, sustained activities of PAI-1 may contribute to excessive collagen accumulation in a variety of tissues, which leads to tissue fibrosis. Recent finding indicates that other than controlling the serine protease activity of uPA/tPA/plasmin system, PAI-1 directly interacts with α-3 subunit of proteasome in vascular endothelial cells and prevents degradation of cellular p53 and IκBa Boncela et al. 2011. As elevated PAI-1 in endothelial cells is associated with increased cell apoptosis (Balsara and Ploplis, 2008), authors implicated PAI-1-mediated modulation of proteasome activity as a new mechanism of elevated PAI-1-induced apoptosis (Boncela et al. 2011). Additionally, PAI-1 forms stable complex with proprotein convertase furin and inhibits its activity. PAI-1 prevents furin-dependent matuaration and activation of insulin receptors and ADAM17 which are involved in the onset of metabolic syndrome including diabetes (Bernot et al. 2011). These results indicate that contribution of elevated PAI-1 in the development of metabolic syndrome may be via interaction and inactivation of furin and related other proprotein-convertases.

3.2 Transcriptional Regulation of PAI-1 Gene Expression

The biologic role of PAI-1 largely depends on its levels of expression as well as its activity. PAI-1 is expressed in a wide variety of cells, regulated at the levels of transcription and post-transcriptional as well as in post-translational modifications. PAI-1 gene expression is tightly regulated by a wide variety of cytokines and growth factors including TGF-β, interleukin-1-β IL-1β, epidermal growth factor (EGF), insulin, lipopolysaccharide (LPS [endotoxin]), and lipoproteins. Numerous transcription factors are involved in controlling the expression of PAI-1 in a cell type–dependent manner and in the context of physiologic and pathologic conditions (Liu et al., 2010; Liu and Pravia, 2010; Nagamine, 2008; Samarakoon and Higgins, 2008). PAI-1 gene expression and its activity are known to be regulated by circadian oscillation with peak activity in the early morning. Studies indicate that circadian oscillation of PAI-1 expression is transcriptionally regulated and transcription factors such as CLOCK, BMAL1, BMAL2, PERIOD2, PPAR-α and CLIF play a major role in this process. The CLOCK system cross-talks with the rennin angiotensin aldosterone system (RAAS) in the circadian regulation of PAI-1 gene expression. Additionally, the plasma PAI-1 concentration is higher in 4G4G homozygous subjects compared with 4G5G and 5G5G in the morning but not in the afternoon suggesting the 4G4G allele is associated with circadian oscillation of PAI-1 synthesis (Hayashida et al., 2010; Maemura et al., 2000;

Masuda et al., 2009; Oishi, 2009; Schoenhard et al., 2003; van der Bom et al., 2003). As this review article focuses on the involvement of PAI-1 in tissue fibrosis and TGF-β is essential for fibrogenesis (Ghosh, 2010; Mauviel, 2005; Schultz et al. 2002), in this section, the focal point of our discussion is on the Smad-dependent and Smad-independent TGF-β regulation of PAI-1 in different context- and cell type–dependent manners.

In response to different environmental insults, cells generally generate ROS, and increased oxidative stress is known to play a significant role in the initiation of fibrogenesis (Liu, 2008; Liu et al., 2010; Liu and Pravia, 2010). Elevated ROS promotes fibrogenesis in different organs via activation of TGF-β which stimulates collagen as well as PAI-1 gene expression. Activated TGF-β also stimulates ROS production via activation of cell-membrane associated nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase 4 (Nox4) which release an elevated amount of H_2O_2. Recent studies indicate that activation of PAI-1 plays a significant role in ROS-induced fibrogenesis (Liu and Pravia, 2010). Another study demonstrated that increased ROS generation leads to inactivation of mitogen-activated protein kinase (MAPK) phosphatase1 and sustained activation of c-Jun N-terminal kinases (JNK) and p38 MAPK which contribute to TGF-β-induced PAI-1 transactivation. Furthermore, glutathione, a cellular antioxidant, blocks TGF-β-induced PAI-1 expression via suppression of JNK and p38 MAPK activation and via inhibition of the binding of Sp1 and Smad to the PAI-1 promoter (Liu et al., 2010; Vayalil et al., 2007). In bovine aortic endothelial cells, TGF-β induces PAI-1 promoter activity, mRNA expression and protein levels, and stimulation is p38 MAPK–dependent (Chen et al., 2001). These findings suggest that JNK and p38 MAPK play a significant role in ROS-induced PAI-1 expression and profibrotic signaling.

Several cis-acting regulatory elements and transcription factors (activators, coactivators and repressors) controlling PAI-1 expressions in different cell types have been well characterized and are summarized as follows. The PAI-1 promoter contains important regulatory elements which upon direct or indirect interaction with transcription factors and coactivators, including CCAAT box-binding transcription factor (CTF)/nuclear factor 1 (NF1), upstream stimulatory factors 1 and 2 (USF1/2), Smad2 and Smad3, CREB-binding protein (CBP) and specificity protein 1 (Sp1), control the expression of PAI-1 in a cell type–dependent manner (Nagamine, 2008). The TGF-β response elements, located between −800 bp and −500 bp in the set of PAI-1 gene upstream regulatory elements, contain binding sites for activator protein-1 (AP1) (Yingling et al., 1997), CTF/NF-1 (Riccio et al.,1992) and Smads (Dennler et al., 1998). Although TGF-β-induced PAI-1 gene expression is enhanced by the tumor suppressor p53, the transcription of uPA/tPA and MMP2 are negatively regulated by p53. This suggests that p53 differentially modulates TGF-β regulation of plasminogen activator and its inhibitor, PAI-1, of the fibrinolytic system (Ghosh et al., 2004a; Kunz et al., 1995).

In bone marrow-derived cultured mast cells, TGF-β stimulates PAI-1 gene transcription, and microphthalmia-associated transcription factor (MITF), a member of the basic-helix-loop-helix-leucine zipper (b-HLH-LZ) family of transcription factors expressed in mast cells, is required for this stimulation. The failure of TGF-β to transactivate PAI-1 in mast cells derived from mice expressing the dominant negative (DN) form of MITF suggests the significance of MITF in regulating PAI-1 expression in this cell type (Murakami et al., 2006). In human hepatoma (Hep) 3B cells, glucocorticoid receptor interacts with Smad3 and abrogates TGF-β-induced PAI-1 expression, further indicating the significance of Smad-dependent TGF-β signaling in PAI-1 gene expression (Li et al., 2006a). In NIH3T3 fibroblasts, expression of Caveolin-1, a unique membrane protein and component of Caveolie, significantly suppresses the TGF-β-induced expression of PAI-1 by suppressing TGF-β Receptor II (Tβ RII) expression (Lee et al., 2007). Recently, Zhang et al. (2011)

demonstrated that Caveolin-1 is significantly lower in keloid-derived fibroblasts compared to healthy controls and Caveolin-1 peptide blocks TGF-β -induced collagen and PAI-1 expression via suppression TβRI expression and Smad2/3 phosphorylation. These findings suggest that Caveolin may be a target for controlling PAI-1 expression under pathologic conditions and for controlling disease progression.

Increased PAI-1 expression in adipose tissues plays a significant role in cardiovascular diseases and obesity. Like insulin, TGF-β also stimulates PAI-1 in adipocytes, in a ERK1/2 MAPK– and protein kinase C (PKC)–dependent manner (Pandey et al., 2005). TGF-β induces PAI-1 expression in preadipocytes and fenofibrate, a peroxisome proliferator-activated receptor-α (PPAR-α) agonist, abrogates TGF-β-induced PAI-1 expression. These findings indicate that incorporating fenofibrate to downregulate PAI-1 levels may be a potential therapeutic approach to reduce cardiovascular disease (Zirik et al., 2009). Recently, Kishore et al. (2010) reported that adipocyte-derived factors potentiate free fatty acid–induced production of a key inflammatory adipokine (PAI-1) by adipose macrophages. Other studies (Samarakoon et al., 2008; Samarakoon and Higgins, 2008) showed that in primary cultures and established vascular smooth muscle cells (VSMC), TGF-β induces PAI-1 gene expression. In these cases, stimulation is dependent on activation of epidermal growth factor receptor (EGFR) and c-src/pp60 kinase, which phosphorylates EGFR. The inhibition of EGFR and src-kinase in VSMC significantly decreased TGF-β-stimulated ERK1/2 MAPK (but not Smad2) activation and suppressed TGF-β-induced PAI-1 expression. Inhibition of Rho and ROCK protein kinases also abrogated TGF-β-mediated activation of Smad2 and induced PAI-1 expression in VSMC (Zirik et al. 2008). However, only TGF-β activation of Smad2 was not adequate to activate PAI-1 in the absence of intact EGFR signaling, suggesting the TGF-β-induced signaling network may be an ideal target to control PAI-1 expression in VSMC and PAI-1-related vascular diseases.

In a study by Guo et al. (2005) with rat mesangial cells, TGF-β stimulated PAI-1 gene expression via activation of the ERK1/2 MAPK and JNK MAPK pathways, independent of Smad activation. Treatment of cells with ERK-MAPK inhibitors blocks MAPK activation and AP1 binding to PAI-1 promoter and abrogates TGF-β-induced PAI-1 synthesis. This study further demonstrated that IFN-γ antagonizes TGF-β-induced PAI-1 expression via suppression of the ERK/AP1 activation pathway. On the other hand, Song et al. (2005) reported that oxidized low-density lipoprotein (LDL), known to be involved in glomerulosclerosis, stimulates PAI-1 expression in human mesangial cells via activation of Smad-dependent TGF-β signaling, suggesting that elevation of PAI-1 in glomerulosclerosis is due to oxidized LDL-induced Smad-dependent TGF-β responses (Song et al., 2005). Furthermore, Smad3 binding sequence CAGA elements that are present in PAI-1 promoter are required for oxidized LDL-activated PAI-1 synthesis (Kim et al., 2007). Das and colleagues (2008) reported that in renal mesangial cells, TGF-β-induced Smad2/Smad3 signaling cross-talks with phosphatidylinositol 3-kinase (PI3K)/Akt signaling and stimulates PAI-1 gene expression. Inhibition of the PI3K/Akt pathway abrogates TGF-β-induced PAI-1 expression by disrupting the TGF-β-induced interaction of transcriptional coactivator and acetyltransferase CBP with activated Smad3. This indicates that the TGF-β-induced PI3K/Akt pathway plays a pivotal role in CBP-Smad3 interaction and elevated PAI-1 expression (Das et al., 2008). In renal tubular epithelial cell, Angiotensin II stimulates PAI-1 via ATI receptor activation (Fintha et al. 2007). A recent study by He and colleagues (2010) demonstrated that in renal tubular epithelial cells (HKC-8), TGF-β induces PAI-1 expression. However, in contrast to previous reports, He et al. (2010) demonstrate that overexpressed Smad2/Smad3 blocks TGF-β-induced PAI-1 expression, and knockdown of Smad2/3 potentiates TGF-β-induced PAI-1 expression, suggesting Smad2/3 antagonizes TGF-β-induced PAI-1 expression in renal tubular epithelial cells. This study further demonstrated that while TGF-β-activated p38MAPK and JNK pathways are partially

responsible for TGF-β-induced PAI-1 expression, TGF-β-inducedβ-catenin stimulates PAI-1 expression. Inhibition of the β-catenin pathway completely abrogated TGF-β-induced PAI-1 gene expression, indicating TGF-β-induced β-catenin signaling in renal tubular epithelial cells plays a pivotal role in renal fibrogenesis.

Collectively, the results from these *in vitro* studies clearly indicate that molecular regulations of PAI-1 gene expression under physiologic as well as pathologic conditions in response to TGF-β are cell-type–, tissue-type– and stimuli-dependent. It is expected, therefore, that the physiologic and pathologic roles of PAI-1 *in vivo* will be cell/tissue selective as has been hinted upon by Eren et al. (2003). Concrete knowledge about the molecular regulation of PAI-1 expression in tissue- and stimuli-dependent mechanisms will be helpful to design tissue-specific therapeutic approaches to control PAI-1 abnormality-associated diseases including tissue fibrosis.

3.3 Posttranslational Modification of PAI-1

In addition to its involvement in transcriptional regulation, posttranslational modification of PAI-1 by glycosylation also plays an important role in controlling PAI-1 activity and physiologic mechanisms. Adipocytes from abdominal fat in the white adipose tissue express only the glycosylated form of PAI-1 (55 kDa), which shows stronger inhibitory activity on uPA/tPA serine proteases than that of non-glycosylated form of PAI-1 (~48 kDa), which is synthesized by other tissues including liver (Samad and Loskutoff, 1996; Serrano et al., 2009). Therefore, it will be beneficial to identify the non-glycosylated and glycosylated forms of PAI-1 in different abnormal fibrinolysis-related disorders in humans, because use of a pharmacologic inhibitor specifically targeting the glycosylated form of PAI-1 will be a more efficient therapeutic approach to control PAI-1 abnormality–related diseases. At present the role of glycosylated and non-glycosylated PAI-1 in tissue fibrosis is unknown.

3.4 Disease Association of PAI-1

Increased levels of PAI-1 are associated with age-related subpathologic and pathologic conditions such as obesity, insulin resistance, diabetes and cardiovascular disease (Cesari et al., 2010; Oishi, 2009). Recent study also indicates that higher PAI-1 levels are associated with a sedentary lifestyle as compared with the lifestyle of trained athletes. There is a positive correlation between PAI-1 and total cholesterol or LDL or triglyceride, suggesting that an increased risk of cardiovascular disease associated with a sedentary lifestyle is linked with elevated PAI-1 levels (Lira et al., 2010). The activities of tPA/uPA/plasmin, the major serine protease enzymes in the fibrinolytic system, are controlled by PAI-1 and thus play a pivotal role in hemostasis, which efficiently stops hemorrhage after injury (Meltzer et al., 2009). Mutation in PAI-1 and other serpin family genes are associated with disorders/diseases such as abnormal bleeding, impairment of wound healing, emphysema, and cirrhosis (Diéval et al., 1991; Fay et al., 1997; Gilabert-Estelles et al., 2005, 2006; Lee et al., 1993; Schleef et al., 1989). Several studies demonstrated that the 4G/4G polymorphism in the PAI-1 gene locus at −675 bp upstream of the transcription start site is a risk factor for coronary artery disease as compared with a 4G/5G or 5G/5G polymorphism (Böttiger et al., 2003; Margaglione et al., 1998). Recently, however, a meta-analysis of 32 independent clinical studies demonstrated that 1) the 4G/4G polymorphism of PAI-1 gene is not associated with MI and coronary artery disease; and 2) that it has only a marginal association with atherosclerosis (Koch et al., 2010). Further studies are needed to better understand the influence of the 4G/4G polymorphism in the PAI-1 upstream regulatory element and its impact on different disease development.

Non-physiologically increased PAI-1 activity is associated with the development of diseases such as thrombosis, atherosclerosis, endometriosis, cancer and multiple organ fibrosis (De

Taeye et al., 2005; Gilabert-Estellas et al., 2005, 2006; Zorio et al., 2008). Paradoxically, complete deficiency of PAI-1 is associated with cardiac-selective fibrosis in aged mice (Ghosh et al., 2010; Moriwaki et al., 2004; Xu et al., 2010). Several studies indicate that PAI-1 levels in plasma may be used as a promising marker for different fibrinolytic and age-related disease progression (Cesari et al., 2010; De Taeye et al., 2005; Dieval et al., 1991; Fay et al., 1997; Gilabert-Estelles et al., 2005, 2006; Lee et al., 1993; Schleef et al., 1989; Vaughan et al., 2007). Increased PAI-1 activity prevents tissue fibrinolysis and promotes coronary artery diseases such as atherothrombosis (Meltzer et al., 2009). In rodents, PAI-1 deficiency is associated with faster vascular fibrinolysis and increased bleeding (Carmeliet et al., 1993a,b). Additionally, several studies strongly suggest that C-reactive protein (CRP) is directly involved in the artherosclerotic process and in endothelium dysfunction (Teoh et al., 2008; Verma 2004; Verma et al., 2004). Interestingly, CRP stimulates PAI-1 synthesis and its activity in human coronary artery endothelial cells suggesting CRP promotes coronary artery disease via induction of PAI-1, the major inhibitor of fibrinolysis (Devaraj et al., 2003). PAI-1 is also implicated in endometriosis where the concentration of PAI-1 is higher compared with healthy endometrium. The elevated PAI-1 may inhibit uPA activity and thus contributes to excess accumulation of collagen and other ECM in endometrium (reviewed in Gilabert-Estelles et al., 2005). Therefore, findings of numerous case studies establish the strong association of abnormal (deficient or elevated) PAI-1 level/activity with a variety of human diseases.

3.5 PAI-1 Deficiency in Humans

Generally most of the PAI-1 related human diseases are due to elevated antigenic concentrations and their inhibitory activity. However, absence of cellular PAI-1 activity also causes several diseases. A few rare clinical reports strongly suggest that PAI-1 deficiency is associated with prolonged surgical- or menstrual-related bleeding due to unstable hemostatic plug formation and impaired hemostasis, where increased fibrinolytic activity (tPA/uPA/plasmin) plays a significant role. For example, hyperfibrinolytic bleeding may be due to any of the following conditions: 1) high tPA activity, undetectable free plasma PAI-1 antigen, and minimal tPA/PAI-1 complex levels (Dieval et al., 1991); 2) low PAI-1 activity and normal PAI-1 antigen (Schleef et al., 1989); 3) low concentration of plasma PAI-1 and high tPA activity with normal tPA antigen (Lee et al., 1993); 4) elevated levels of tPA activity and low PAI-1 activity (Stankiewicz et al.,1991); or 5) deficiency of active PAI-1 in plasma (as was shown in an Amish family and due to a dinucleotide insertion in exon 4 of the PAI-1 gene which resulted in premature termination of translation). Compared with homozygous PAI-1 deficiency, heterozygous PAI-1 was not associated with abnormal or delayed bleeding (Fay et al., 1997).

4. Pathobiology of Tissue Fibrosis

In response to physical, chemical, immunologic or microbial injury, the body's reactions in promoting wound healing are characterized by inflammation, elevated levels of cytokine/chemokine release, cellular migration to the wound area, and conglomeration and activation of circulating mononuclear cells/fibroblasts. Activated resident fibroblasts or epithelial-to-mesenchymal transition (EMT)/endothelial-to-mesenchymal transition (EndMT)-derived fibroblasts differentiate to myofibroblasts and synthesize extracellular matrix proteins such as collagen and fibronectin to heal the wound. However, abnormal tissue repair may lead to scarring or fibrosis that impairs organ function. At present there is no effective drug for the treatment of fibrosis. In recent years, enormous progress has been made in our understanding of the molecular bases of tissue fibrosis. Along with a series of *in vitro* experiments using cultured cells from different organs, various animal models (induced/injured and spontaneous/uninjured) of tissue fibrosis have been used to explore the molecular basis of fibrosis *in vivo* (Ellmers, 2010; Leask, 2010; Mastuzaki, 2010; Yang et

al., 2010; Yue et al., 2010). The widely used animal models of tissue fibrosis include the following: For skin and lung fibrosis, the bleomycin-induced mouse model (Murray et al., 2008; Wu et al., 2004a,2009; Yamamoto et al., 1999) and the graft-versus-host disease (GVHD) mouse model (McCormick et al., 1999); for cardiac fibrosis, the transverse aortic banding (TAB) model (Sun et al., 2007), angiotensin II (AngII)–induced model (Lijnen et al., 2000), phenylephrine-induced model (Farivar et al., 1995), isoproterenol-induced model (Rohrer et al., 1996), and aldosterone-induced model (Lijnen and Petrov, 2000); for kidney fibrosis, the unilateral ureteral obstruction (UUO)–induced model and aldosterone-induced renal injury model (Ma et al., 2006; Ma and Fogo, 2009; Oda et al., 2001); for liver fibrosis, the carbon tetrachloride (CCl_4)–induced mouse model and bile duct ligation model (Wang et al., 2007); and for gastrointestinal tract fibrosis, the chronic 2,4,6-trinitrobenzene sulfonic acid–induced colitis model (Wu and Chakravarti, 2007). Besides the use of induced models of tissue fibrosis, spontaneously developed tissue fibrosis has also been reported in genetically modified animals. For example, mice lacking Fli-1 or TGF-β RII develop spontaneous skin fibrosis (Asano et al., 2009; Denton et al., 2005). PAI-1 deficiency is associated with spontaneous development of age-dependent cardiac-selective fibrosis (Ghosh et al., 2010; Moriwaki et al., 2004, Xu et al., 2010) (Figure 1). These animal models of organ fibrosis are extremely valuable tools to understand the molecular bases of fibrogenesis initiation and progression, and for testing the efficacy of various new drugs for the treatment of this devastating fibroblast abnormality-related disease in humans.

Extracellular matrix maintains the tissue integrity and homeostasis which is made up of fibrillar type I, III, IV, V and VI collagens, fibronectin, elastin, desmin, laminin, fibrillin, proteoglycans, etc. (Kitamura et al., 2001; Weber et al., 1988). In fibrotic tissue, almost 70–80% of accumulated ECM proteins are Type I collagen, a trimer of gycine- and proline-rich two collagen Iα1 and one collagen Iα2 polypeptide chains, products of two genes synthesized by mesenchymal cells. Therefore, investigating the regulation of these genes will facilitate our understanding of the molecular basis of fibrotic diseases. After synthesis, the polypeptide chains undergo hydroxylation in endoplasmic reticulum, cross-linked by hydrogen bonds, and form triple helix procollagen. Upon secretion into extracellular space, the N- and C-terminal peptides are cleaved off by proteolytic activities and the mature triple helical collagen molecules aggregate to form collagen fibrils. Upon context-dependent interaction with different transcription factors (activators or repressors) and coactivators, the regulatory regions control the basal as well as cytokine-modulated expression of both genes (procollagen 1α1 and procollagen 1α2) (Ghosh 2002; Ghosh and Varga, 2007; Ruiz-Ortega et al., 2007). TGF-β stimulates Type I collagen synthesis in mesangial cells via modulation of the levels or activity of many transcription factors and coactivators including Sp1, CBF, Smads, Fli-1, PPAR-γ, and p300, and contribute in the pathogenesis of fibrosis (Asano et al., 2009; Ghosh 2002; Ghosh and Varga, 2007; Ghosh et al., 2009; Ihn et al. 2006; Wei et al., 2010). Along with transcriptional regulators, elevated levels of TGF-β and TGF-β receptors (TβRI/II) in fibrotic tissues play a pivotal role in fibrogenesis (Mauviel, 2005; Pannu and Trojanowska, 2004). TGF-β is known to transduce its signal from receptor to nucleus via activation of TGF-β receptor kinase and its substrates, Smad2/3. Ligand activated receptors phosphorylate at the SSXS motif of Smad2/3, and then heterodimerize with co-Smad4, translocate to nucleus, interact with Smad binding elements and activator Sp1, and acetyltransferase p300/CBP, thus activating the expression of Type I collagen. Increased expression and synthesis of Type I collagen may also be due to suppression of repressors of TGF-β signaling including Smad7 (Dong et al., 2002), PPAR-γ (Ghosh et al., 2004b, 2008, 2009; Wei et al., 2010; Wu et al. 2009), p53 (Ghosh, 2004a), and Fli-1 (Asano et al., 2009). Additionally, antifibrotic cytokines TNF-α and IFN-γ antagonize TGF-β-induced collagen synthesis via activation of suppressors such as NF-κB (Bitzer et al., 2000), C/EBP-β (Ghosh et al., 2006; Iraburu et al., 2000), STAT-1 α (Ghosh et al., 2001), AP1 (Verrecchia et al., 2000), and YB1 (Dooley et al., 2006; Higashi et al., 2003). Collectively,

these studies suggest that an elevated level of collagen accumulation under pathologic conditions is at least partly due to increased collagen gene expression in response to elevated TGF-β-induced profibrotic signaling.

While the physiologic levels of collagen synthesis and its accumulation in the matrix is important in tissue remodeling, the activities of the cellular uPA/tPA/plasmin/MMPs proteolytic system (uPA/tPA-activated serine protease, plasmin and plasmin activated MMPs) are involved in matrix protein turnover and also play a pivotal role in the maintenance of the physiologic levels of ECM proteins and in tissue homeostasis (Li et al. 2000; Takeshita et al., 2004; Visse and Nagase, 2003; Zaman et al., 2009). The serine protease inhibitor PAI-1 inhibits uPA/tPA activities and thus subsequently controls the activities of plasmin and plasmin-dependent MMPs (Gramley et al., 2007; Lackie, 2008) suggesting that uPA/tPA inhibitor PAI-1 plays a pivotal role in matrix degradation. Elevated levels of PAI-1 are implicated in several diseases, including tissue fibrosis, largely due to decreased uPA/tPA/plasmin and MMP-mediated ECM degradation or altered cellular migration and proliferation (Kortlever et al., 2008; Pedroja et al., 2009; Petrov et al., 2002; Ploplis et al., 2004). Here, the involvement and significance of PAI-1 in fibrogenesis in multiple organs are discussed.

4.1 PAI-1 in Skin Fibrosis

Skin, the largest organ in the body, performs numerous physiologic roles including body temperature control, protection of the body from environmental insults, and excretion. Excessive synthesis and accumulation of collagen and other ECM proteins in the dermal region by activated dermal fibroblasts or myofibroblasts leads to thickening and hardening of skin, the pathologic manifestation of skin fibrosis in scleroderma (Ghosh, 2002; Mauviel, 2005; Pannu and Trojanowska, 2004; Varga and Abraham, 2007). In response to injury, a series of events occur during epidermal wound closure and repair of internal wounded areas. These events include initiation of inflammation due to infiltration of mononuclear cells, secretion of cytokines and chemokines, cellular migration and differentiation to myofibroblasts, and the synthesis and accumulation of collagen and other ECM proteins in the matrix for the purpose of repair. The magnitude of ECM protein accumulation is tightly controlled by the synthesis of ECM and its degradation by cellular proteolytic activities. As is true in other tissues, imbalance of these two events during wound healing leads to dermal fibrosis and loss of normal skin structure and function.

Another fibrotic skin condition is benign tumor such as the outgrowth called keloids. This condition results from injury and the increased injury-related fibroblast proliferation, synthesis and accumulation of excessive collagen in the dermal region. While elevated levels of Type I collagen contribute to skin fibrosis, an impaired uPA/tPA/plasmin/MMP proteolytic system, resulting from elevated PAI-1, is also important in the process of dermal fibrogenesis (Toriseva and Kahari, 2009). In this section of the review, we discuss the role of PAI-1 in both types of skin scars: keloids and the skin fibrosis in scleroderma. Fibroblasts derived from keloids, skin scarring due to fibroproliferation, synthesized elevated levels of collagen, and PAI-1 (Dong et al., 2002; Higgins et al., 1999). In these studies, keloid fibroblasts exhibited an intrinsically high level of PAI-1 and a low level of uPA. This altered ratio of uPA to PAI-1 causes less degradation of fibrin and other ECM proteins in keloid fibroblasts and thus contributes to dermal fibrogenesis (Tuan et al., 1996). Furthermore, while adenoviral-mediated overexpression of PAI-1 in normal and keloid skin fibroblasts causes significantly elevated levels of collagen accumulation, siRNA mediated depletion of cellular PAI-1 causes a reduction in the levels of collagen, suggesting PAI-1 controls the levels of collagen, an important profibrotic marker and contributor to hypertrophic scarring (Tuan et al., 2008).

Hypoxia is known to play a significant role in fibrogenesis (Ueno et al., 2011). Hypoxia induces multiple signaling pathways including PI3K/Akt and ERK1/2 MAPK which are involved in hypoxia-mediated stimulation of PAI-1 expression in keloid fibroblasts via activation of hypoxia-inducible factor-1 alpha (HIF-1α) (Zhang et al., 2003, 2004). Wu et al. (2004b) reported that the levels of vascular endothelial growth factor (VEGF) are significantly higher in keloid tissues compared with normal skin. VEGF stimulates the expression of PAI-1 via activation of ERK1/2 MAPK signaling in keloid fibroblasts, suggesting VEGF plays a significant role in increased PAI-1 expression, impaired degradation of ECM, and keloid formation. Interestingly, a recent study showed that vitamin D ameliorates elevated collagen and PAI-1 synthesis in keloid skin fibroblasts (Zhang et al., 2010). The vitamin D–mediated suppression of profibrotic activity may be due to activation of anti-fibrotic hepatocyte growth factor (HGF). Treatment of keloid fibroblasts with vitamin D stimulates the levels of anti-fibrotic HGF and reverses the profibrotic cytokine TGF-β-induced suppression of HGF, suggesting vitamin D exerts its antifibrotic effects via activation of HGF in this fibroproliferative disease.

The levels of PAI-1 are elevated in skin fibroblasts derived from scleroderma patients compared with normal healthy controls (Dong et al., 2002). The elevation of PAI-1 in scleroderma skin is due to increased Smad2/3 activation and decreased levels of inhibitory Smad, Smad7. Furthermore, overexpression of exogenous Smad7 in scleroderma skin fibroblasts normalize the level of PAI-1, suggesting that increased Smad-dependent TGF-β signaling is responsible for an elevated PAI-1 level and skin fibrosis. Moreover, a recent study showed that the levels of PAI-1 mRNA are significantly elevated in skin biopsies derived from patients with skin fibrosis compared with those of healthy controls, further indicating the possible role of PAI-1 in the pathogenesis of skin fibrosis in scleroderma (Wei et al., 2010).

Study of wound healing in fetal tissues provides valuable information on the molecular basis of scarring. It is known that while the healing of fetal mouse skin is scarless, healing in adult mice skin is commonly associated with scar formation. Huang et al. (2002) demonstrated that in normal skin while uPA level was higher in E14.5 than E18 mice, PAI-1 was lower in E14.5 than in E18 mice. Upon injury in the skin, E14.5 wounds show a moderate increase of uPA and a minimal increase in PAI-1. In contrast, E18 wounds show a transient increase of uPA but a significant and sustained increase in PAI-1. The authors concluded that differential expression of uPA and PAI-1 in fetal and adult skin may be responsible for the extent of hypertrophic skin scarring (Huang et al., 2002). It has been postulated that elevated levels of TGF-β3 in fetal mouse skin may be associated with scarless healing. Interestingly, TGF-β3 is known to downregulate PAI-1 expression (Huang et al., 2002) and the levels and activity of PAI-1 are increased in skin from TGF-β3 null embryos at E14.5 in an ex-vivo limb-wounding model (Li et al., 2006b). Together these results suggest that TGF-β3-mediated downregulation of PAI-1 may be responsible for scarless healing in fetal skin. Therefore, activation of the TGF-β3 axis and lowering of PAI-1 levels may be an ideal approach to control dermal fibrosis.

In response to injury, keratinocytes in the epidermis express increased levels of PAI-1. Although the cellular events of wound healing processes are comparable between wildtype and PAI-1 knockout mice, the rate of wound closure is significantly faster in PAI-1 knockout mice (Chan et al., 2001). This may be because PAI-1 inhibits cell migration by blocking the interaction of vitronectin with its receptor αvβ3, and in the absence of PAI-1, vitronectin interaction withαvβ3 accelerates cell migration and wound healing, as has been hinted upon by Stefansson et al. (1996). This expedited wound healing may also be due to elevated levels of uPA/tPA/plasmin fibrinolytic activity and MMP activities in PAI-1 deficient mice because both systems are responsible for faster skin closure (Chan et al.,

2001). PAI-1 plays a significant role in adult skin fibrosis; evidence for this has been further demonstrated by the significantly slower accumulation of fibrotic tissue within subcutaneously implanted polyvinyl alcohol sponges in PAI-1 deficient mice compared with wildtype controls. This study also showed that a fibrin-rich matrix that accumulated within the sponges was rapidly removed in the PAI-1 deficient mice compared with wildtype controls, further suggesting that PAI-1 deficiency is associated with reduced dermal fibrosis (Chuang-Tsai et al., 2003). Additionally, treatment of skin fibroblasts with profibrotic cytokine TGF-β1/β2 significantly stimulates PAI-1 mRNA expression and protein levels, suggesting that the profibrotic effect of TGF-β is not only due to elevated collagen synthesis but also due to elevated levels of PAI-1, a major serine protease inhibitor that blocks collagen degradation.

Several *in vivo* studies revealed that the levels of PAI-1 mRNA and proteins were significantly elevated in bleomycin-treated fibrotic mice skin compared to controls. Although PAI-1 plays an important role in ECM remodeling through inhibition of proteolytic degradation of matrix proteins, the degree of bleomycin-induced skin fibrosis in PAI-1 deficient mice is comparable with that in wildtype controls suggesting that 1) PAI-1 is not essential for the development of bleomycin-induced skin fibrosis, and 2) PAI-1 deficiency is not protective against the development of bleomycin-induced skin fibrosis (Matsushita et al., 2005). Paradoxically, fibroblast-specific overexpression of a kinase-deficient Type II TGF-β receptor leads to constitutive activation of TGF-β signaling and development of spontaneous skin fibrosis. Interestingly, the basal levels of PAI-1 mRNA and proteins in TβRII kinase deficient transgenic skin fibroblasts are significantly higher compared with controls, which further indicates the possible role of PAI-1 in the development of skin fibrosis (Denton et al., 2005). However, the exact molecular basis of PAI-1-promoted skin fibrosis is not clear and this is an important area for future skin fibrosis research.

4.2 PAI-1 in Lung Fibrosis

Lung fibrosis is a fatal disease with a median survival of 3 to 4 years. Idiopathic pulmonary fibrosis (IPF) is the most common pulmonary disease and is characterized by increased fibroblast proliferation and matrix remodeling. In response to alveolar epithelium injury due to infection or exposure to chemical agents, a series of events occurs in the repair of damaged tissues. Epithelial injury leads to release of growth factor and to the fibrinolytic activities of uPA/tPA/MMPs, which promote fibroblast proliferation, migration, transformation of epithelial cells to mesenchymal cells (EMT), myofibroblast differentiation, and extracellular matrix deposition (Deterding et al., 1997; Lazar et al., 2004). While increased epithelial cell migration and proliferation causes reduction in fibrosis in an animal model, TGF-β-induced epithelial-to-mesenchymal transitions are involved in the pathogenesis of lung fibrosis (Arciniegas et al., 2007). Rapid activation of coagulation factors and deposition of fibrin are important steps during wound healing in which fibrin serves as a chemotactic agent, promoting cell migration to the wound site. However, presence of prolonged periods of fibrin accumulation may contribute to deregulation of wound healing and to accumulation of excessive matrix protein deposition, the pathologic manifestation of fibrosis. As fibrin is a substrate of plasmin, the major serine-protease fibrinolytic enzyme, the level of fibrosis is also controlled by the plasminogen fibrinolytic system which also controls the migration of alveolar epithelial cells during wound healing. Lazar and colleagues (2004) further demonstrated that the wound healing process in an *in vitro* model was adversely affected in the absence of uPA or in the presence of excess PAI-1 (Figure 2)

Several studies showed that PAI-1 deficiency protects lungs from excess fibrin accumulation and bleomycin-induced fibrosis (Bauman et al., 2010; Chung-Tsai et al., 2003; Eitzman et

al., 1996; Hattori et al., 2000; Senoo et al., 2010). This protective mechanism is at least partly due to enhanced fibrinolytic activity but it is not due to altered cell migration because it has been demonstrated that a similar number of leukocytes are present in both bleomycin-treated wildtype and PAI-1 deficient lungs (Hattori et al., 2000). This has been supported by two observations: first, that PAI-1deficient mice lungs accumulate less fibrin than those of wildtype mice in response to bleomycin, and second, that inhibition of plasmin activity by tranexamic acid reverses fibrin clearance and the protective effect of PAI-1 deficiency against bleomycin-induced lung fibrosis (Hattori et al., 2000). This study also shows that fibrin removal is not the sole cause of reduction of fibrosis in PAI-1 deficient mice because fibrinogen deficient mice developed bleomycin-induced pulmonary fibrosis to the same magnitude as that in fibrinogen heterozygous controls (Hattori et al., 2000). This finding has been supported by the observation of Wilberding and colleagues (2001) who demonstrated that the levels of bleomycin-induced collagen accumulation are comparable between fibrinogen deficient and heterozygous control mice. The implication of enhanced fibrinolytic activity in the protection of lungs from bleomycin-induced fibrosis development has been further supported by the observations that while uPA infused into lungs protects mice from bleomycin-induced lung fibrosis (Sisson et al., 1999), plasminogen deficient mice develop significantly increased levels of lung fibrosis in response to bleomycin (Swaisgood et al., 2000). Collectively, therefore, these results imply the pivotal role of PAI-1 in lung fibrogenesis via suppression of uPA/tPA/plasmin proteolytic activities (Eitzman et al., 1996; Hattori et al., 2000; Sisson et al., 1999; Swaisgood et al., 2000).

Recently, Bauman et al. (2010) elaborately described the molecular basis by which PAI-1 deficiency protects lungs from bleomycin-induced lung fibrosis. This study shows that in response to bleomycin, PAI-1 deficient mice lungs produce elevated levels of the anti-fibrotic lipid mediator prostaglandin E2 (PGE2), and this increase in PGE2 may be associated with increased plasminogen activation. Plasminogen activation is associated with upregulation of PGE2 in alveolar epithelial cells and in normal fetal and adult lung fibroblasts. However, the degree of elevation of PGE2 was significantly higher in cells derived from PAI-1 deficient mice. This study further demonstrated that enhanced PGE2 formation required plasmin-mediated proteolytic activation and release of HGF and induction of COX-2. This is consistent with the previous observation that the levels of HGF proteins are elevated in bleomycin-treated PAI-1 deficient mice lungs compared to wildtype (Hattori et al. 2004). As a selective inhibitor of the HGF receptor cMet, PHA-665752 reduced the levels of COX-2 and PGE2, and increased the levels of lung fibrosis in PAI-1 deficient mice compared with wild-type mice, the authors concluded that the plasmin/HGF/COX-2/PGE2 axis mediates *in vivo* protection from bleomycin-induced-fibrosis in PAI-1 deficient mice. This conclusion was further supported by the observation that fibroblasts derived from patients with idiopathic pulmonary fibrosis failed to induce COX-2 and were unable to stimulate PGE2 synthesis in response to plasmin or HGF (Bauman et al., 2010). Additional evidence for the pivotal role of PAI-1 in the development of lung fibrosis is provided by a recent observation by Senoo et al. (2010) that suppression of PAI-1 by intranasal instillation of PAI-1 siRNA ameliorates bleomycin-induced lung fibrosis. As EMT plays a significant role in lung fibrosis and TGF-β fails to induce EMT in PAI-1 depleted mouse-lung epithelial cells, the authors concluded that inhibition of EMT may be partly responsible for attenuation of bleomycin-induced lung fibrosis in the absence of PAI-1. Another recent report suggests that increased HIF-1α mediates Smad-dependent TGF-β-induced elevated PAI-1 expression in bleomycin-induced lung fibrosis (Ueno et al., 2011).

Environmental pollution is one of the major causes of lung injury and injury related lung diseases including idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD). The lungs are the major target of environmental pollutants because during

respiration they are directly exposed to the environmental atmosphere. The molecular mechanisms of lung fibrosis in response to environmental pollution have also been reported. For example, inhalation of nickel dust is associated with an increased incidence of pulmonary fibrosis. Nickel subsulfide particulate is known to inhibit cellular fibrinolysis by inducing the expression of PAI-1. Nickel activates PAI-1 transcription in human airway epithelial cells via ERK1/2 MAPK-dependent activation of AP1 and HIF-1α and independent of ROS, suggesting HIF-1α-dependent activation of PAI-1 plays an important role in nickel-induced pulmonary fibrosis. (Andrew et al., 2001a,b). PAI-1 is also known to play a significant role in silica-induced pulmonary fibrosis. Treatment of human lung epithelial cells with silica leads to increased synthesis of PAI-1 in an AP-1 activation-dependent manner. Like nickel, silica also activates ERK1/2 MAPK which in turn activates AP-1 and its binding to PAI-1 promoter. An inhibitor of ERK1/2MAP abrogates silica-induced AP-1 activation and induced PAI-1 synthesis. Together, these results suggest that silica- and nickel-induced ERK1/2 MAPK play a significant role in activation of PAI-1 and the development of lung fibrosis (Hu et al., 2008). The pivotal role of PAI-1 and fibrinolytic components in the development of pulmonary fibrosis has been further documented by the observations that the extracellular matrix component hyaluronan, a profibrotic agent, induces the levels of mRNA, protein, and the activity of PAI-1, and suppresses the expression of uPA and its activity in the alveolar macrophage cell line as well as in inflammatory alveolar macrophages derived from bleomycin treated rats (Horton et al., 2000). Therefore, it is reasonable to conclude that lowering the level/activity of PAI-1 using pharmacologic inhibitors of PAI-1 or RNAi-mediated PAI-1 gene silencing may be an ideal approach to control lung fibrosis.

4.3 PAI-1 in Kidney Fibrosis

Kidney fibrosis, including glomerulosclerosis and tubulointerstitial fibrosis, is a very common end-stage manifestation of hypertension- or diabetes-related renal injury. Numerous studies established a strong link between elevated levels of PAI-1 with glomerulosclerosis and kidney fibrosis (Eddy, 2000; Ma and Fogo, 2009). The association of elevated levels of PAI-1 with kidney fibrosis has been clearly evidenced by the observations that in a unilateral ureteral obstruction (UUO) mouse model, the levels of PAI-1 are 8–16 fold higher compared with controls. PAI-1 deficiency is associated with significantly decreased levels of TGF-β, collagen accumulation, and renal interstitial fibrosis in the UUO mouse model. Although, the levels of plasmin activities are not altered in PAI-1 deficient UUO mice, the significantly lower number of interstitial macrophages and myofibroblasts, compared with the wildtype UUO model, may be responsible for protection against UUO-induced renal fibrosis in PAI-1 deficient mice (Oda et al., 2001). The significance of PAI-1 in kidney fibrosis has been further supported by the observation that while overexpressed TGF-β1 stimulates PAI-1 expression and interstitial collagen deposition in TGF-β1 transgenic mice, PAI-1 deficiency blocks TGF-β1-induced collagen deposition and kidney fibrosis (Krag et al., 2005). In contrast, in response to aldosterone, PAI-1 deficient mice are not protected against macrophage infiltration, activation of TGF-β, and development of renal interstitial fibrosis (Ma et al. 2006). Recently, Han et al. (2010) demonstrated that in response to UUO, within two weeks mice shows severe renal tubulointerstitial fibrosis which is associated with increased levels of PAI-1, TGF-β1 and TGF-β-receptor kinase phosphorylated Smad2. This finding indicates elevated Smad-dependent TGF-β signaling and increased PAI-1 activity contribute to the development of renal fibrosis in the UUO murine model.

The significance of TGF-β in renal fibrosis has also been demonstrated in phosphoenolpyruvate carboxykinase (PEPCK)-TGF-β1 transgenic mice, in which renal expression of TGF-β1 is associated with tubulointerstitial fibrosis due to excessive synthesis

of collagen and accumulation of PAI-1. (Clouthier et al.,1997). A recent study (Cheng et al. 2010) demonstrated that the expression levels of PAI-1 and MMP9 mRNA and protein are significantly elevated in hypertensive renal interstitial fibrosis in the kidney derived from spontaneously hypertensive rats. Interestingly, the levels of anti-aging gene Klotho are significantly decreased in this model of hypertensive rats. Treatment of hypertensive rats with Fosinopril and valsartan causes an increase in Klotho mRNA and protein, and decreased the levels of PAI-1 and MMP9 expression suggesting anti-aging Klotho may act as an antifibrotic agent in kidney via suppression of PAI-1 (Cheng et al., 2010). Furthermore, Klotho blocks TGF-β-induced profibrotic signaling and renal fibrosis via direct interaction with TGF-β receptor II and the blocking of TGF-β binding to its receptor (Doi et al. 2011). In contrast, PAI-1 deficiency is associated with an early and severe glomerulanephritis compared with wildtype mice in response to anti-GBM serum administration. Importantly, the glomerulonephritis in this murine model is associated with increased uPA/tPA activities and overactivation of TGF-β, possibly due to tPA/uPA-induced conversion of latent TGF-β into its active form. Antibody neutralization of TGF-β ameliorates the severity of glomerulonephritis suggesting severe manifestation of glomerulonephritis in PAI-1 deficient mice is associated with elevated TGF-β signaling (Hertig et al., 2003). PAI-1 is also involved in oxidative stress-induced pathogenesis of glomerulosclerosis in humans, where oxidized LDL stimulates PAI-1 via activation of Smad-dependent TGF-β signaling in mesangial cells (Song et al., 2005).

Recent studies indicate that PPAR-γ agonists 15d-PGJ_2 and troglitazone abrogate renal fibrosis in both diabetic nephropathy and nondiabetic chronic renal diseases via suppression of TGF-β-induced PAI-1 expression and its activities (Han et al. 2010; Li et al. 2006c). Furthermore, PPAR-γ ligands ameliorate renal fibrosis via activation of antifibrotic HGF, its receptor c-Met and Smad repressor TGIF, and thus abrogates Smad-dependent profibrotic signaling. This finding suggests that HGF plays a pivotal role in PPAR-γ mediated amelioration of renal fibrosis (Li et al., 2006c). Similarly, another study demonstrates that treatment of UUO mice with the PPAR-γ agonist/antidiabetic drug pioglitazone causes a decrease in the levels of TGF-β1, pSmad2 and PAI-1 and is associated with reduced interstitial fibrosis, suggesting suppression of PAI-1 by PPAR-γ agonists may be useful as a potential therapeutic approach for amelioration of kidney fibrosis (Han et al., 2010).

4.4 PAI-1 in Liver Fibrosis

The liver, the largest gland in the body, is responsible for a wide variety of body functions including food metabolism, glycogen storage, bile secretion for lipid emulsification, and detoxification. Most of the chronic liver diseases are characterized by excessive accumulation of collagen and other ECM proteins, a pathologic manifestation of liver fibrosis or cirrhosis that leads to liver failure. Cirrhosis is associated with very high rates of morbidity and mortality. Liver fibrosis and cirrhosis, due to chronic insults such as excessive alcohol consumption, fat accumulation, nonalcoholic steatohepatitis (NASH), and viral infection, are characterized by inflammation, hepatocyte to mesenchymal transition, hepatic stellate cell activation, elevated profibrotic signaling, increased collagen accumulation, and disruption of physiologic liver function. Activated hepatic stellate cells are the major source of collagen in fibrotic liver (Friedman et al., 1985). Increased expression of the major profibrotic agents TGF-β and PDGF and their receptors are associated with increased proliferation and activation of hepatic stellate cells (HSC), differentiation of HSC to myofibroblasts, and elevated synthesis of collagen and other ECM proteins which leads to liver fibrosis (Bataller and Brenner, 2005; Brunt, 2004; Gong, 1998; Gressner, 1996).

As is the case in other organs, the extent of fibrosis in the liver depends on the rates of collagen synthesis and its degradation by cellular proteolytic activities, uPA/tPA/plasmin, and MMPs. PAI-1, the potent inhibitor of fibrinolytic activity, is also implicated in liver

fibrosis. This is a result of two factors: first, the level of PAI-1 is significantly elevated in fibrotic liver (Clouthier et al.,1997); and second, PAI-1 deficiency is associated with increased activity of tPA and MMP9 and with reduced cholestatic liver fibrosis, in response to bile duct ligation, as compared with wildtype mice (Wang et al., 2007). Interestingly, there was no induction of hepatic uPA and plasmin activities in PAI-1 deficient mice compared with wildtype controls indicating that less cholestatic liver fibrosis may not be directly due to altered serine protease activity. As in other tissues, TGF-β also plays a pivotal role in hepatic fibrogenesis. For example, transgenic mice overexpressing constitutively active TGF-β1 in liver developed liver fibrosis (Clouthier et al.,1997), and the levels of latent and active TGF-β1 are significantly elevated in liver after bile duct ligation. However, the levels of profibrotic cytokine TGF-β and major ECM protein Type I collagen mRNA were comparable in PAI-1 deficient liver compared with wildtype controls after bile duct ligation suggesting collagen synthesis was unaltered in PAI-1 deficient liver (Wang et al., 2007). This study showed that the level of tPA substrate HGF, an antifibrotic factor, was elevated in PAI-1 deficient liver. Furthermore, after bile duct ligation, the levels of pro-MMP9 and active MMP9 but not pro-MMP2 or active MMP2 were significantly elevated in liver homogenates derived from PAI-1 deficient mice compared with wildtype controls. These results collectively suggest that reduced levels of liver fibrosis in PAI-1 deficient mice in response to bile duct obstruction is largely due to activation of tPA-induced HGF, a known antifibrotic agent, elevated MMP9, and increased collagen degradation (Wang et al., 2007). Treatment of a CCl4-treated or bile duct–ligated rat model of cirrhosis with pirfenidone, an antifibrotic drug, or IFN-α 2a resulted in reduction in collagen and PAI-1 synthesis and amelioration of cirrhosis. This finding further signifies the role of PAI-1 in induced liver fibrogenesis (García et al., 2002; Bueno et al., 2000). Administration of TGF-β in mice causes activation of PAI-1 and progression of steatohepatitis. Interestingly, treatment of this murine model of fibrosing steatohepatitis with fenofibrate, a PPAR-α agonist, activates AMPK signaling, increases nuclear receptor small heterodimer partner (SHP) and reverses fibrosis through suppression of PAI-1 gene expression (Chanda et al., 2009). These results, therefore, further support the significant roles of PAI-1 and fibrinolytic components in the progression of liver fibrogenesis, and PAI-1 may be an ideal therapeutic target for treatment of liver fibrosis.

4.5 PAI-1 in Cardiac Fibrosis

The fibrotic heart fails to pump blood because it lacks myocardial tissue elasticity. Cardiac fibrosis is the major cause of heart failure–related deaths. Cardiac fibrosis is often preceded by hypertension-associated cardiac hypertrophy and myocardial infarction (Ruiz-Ortega et al., 2007) and is characterized by a loss of contractility due to myofibroblast differentiation, and excessive synthesis and accumulation of matrix protein, collagen, and the cellular inhibitor of uPA/tPA and MMP proteolytic activities, PAI-1 (Takeshita et al., 2004). As is true for an elevated level of collagen, an elevated level of PAI-1 is also an important diagnostic profibrotic marker of cardiac fibrosis.

Although, PAI-1 deficiency protects most organs, including the heart, from induced fibrosis (Takeshita et al., 2004), the lack of PAI-1 and elevated levels of uPA/tPA in macrophages are associated with spontaneously developed age-dependent cardiac-selective fibrosis (Moriwaki et al., 2004). Studies from different laboratories indicate that vascular injury in aged PAI-1 deficient mice may be due to an increase in uPA/tPA/MMP activities, which leads to inflammation, infiltration of macrophages and macrophage-specific overexpression of uPA. This leads to fibrosis in the PAI-1-deficient heart (Ghosh et al., 2010; Heymans et al., 2005; Moriwaki et al., 2004; Weisberg et al., 2005; Xu et al., 2010). As macrophage infiltration is not only found in cardiac tissue but is also present in other organs, such as kidney and lungs, it is difficult to reconcile that macrophage infiltration is the sole cause of

cardiac selective fibrosis in PAI-1 deficient mice (Moriwaki et al., 2004). In a mouse model of myocardial infarction (MI), the levels of PAI-1 mRNA and protein are significantly elevated compared with controls and this is associated with progressive development of interstitial and perivascular fibrosis in post MI heart. Importantly, PAI-1 deficient mice develop less cardiac fibrosis after MI suggesting that under an injured condition, lack of PAI-1 is protective against the development of cardiac fibrosis(Takeshita et al., 2004). Furthermore, nitric oxide inhibitor L-NG-nitro arginine methyl ester (L-NAME) induces cardiac fibrosis in wildtype mice. However, PAI-1 deficient mice develop less vascular fibrosis in response to L-NAME suggesting that cellular PAI-1 contributes in hypertension-induced vascular fibrosis (Kaikita et al., 2001). However, although PAI-039, a small molecule inhibitor of PAI-1, protects against AngII-induced aortic remodeling, it did not inhibit the AngII-induced cardiac hypertrophy and cardiac fibrosis, suggesting that pharmacologic suppression of PAI-1 is tissue selective (Weisberg et al., 2005). Therefore, the nature of the molecular basis of spontaneous cardiac fibrosis development in PAI-1 null mice and how it differs from cardiac fibrosis developed from elevated levels of PAI-1 in injury-induced models of cardiac fibrosis was not clear.

Further studies have delineated the possible molecular basis of spontaneously developed cardiac fibrosis in aged PAI-1 deficient mice. These studies demonstrated in cardiac tissues derived from PAI-1 deficient mice that age-dependent cardiac fibrosis is associated with an increased number of Mac3 or CD45 positive cells (macrophages), indicating the presence of inflammation (Ghosh et al., 2010; Moriwaki et al., 2004; Xu et al., 2010). The proteolytic activity of MMP2 and MMP9 are known to play a significant role in the initiation and progression of cardiac fibrosis (Ducharme et al. 2000; Matsusaka et al. 2006). Interestingly, lack of PAI-1 is associated with increased levels of MMP2/MMP9, suggesting that in aged PAI-1 deficient mice, elevated MMP2/MMP9 might contribute in spontaneous cardiac fibrogenesis. Presence of an increased number of fibroblasts (major collagen producing cells) in PAI-1 deficient myocardial tissues indicates that an increased number of fibroblasts is responsible for elevated collagen deposition in PAI-1 deficient heart. Increased numbers of fibroblasts may be due to increased proliferation of resident fibroblasts (Kortlever et al., 2008) or due to increased proliferation of endothelial cells (Ploplis et al., 2004) and their transition to mesenchymal cells in the PAI-deficient myocardial tissue (Ghosh et al., 2010). Furthermore, the elevated levels of profibrotic cytokines and growth factors in PAI-1 deficient myocardial tissues indicate increased downstream profibrotic signaling which may play a significant role in spontaneously developed cardiac fibrosis (Ghosh et al., 2010; Xu et al., 2010). Indeed, PAI-1 deficient myocardial tissues showed increased levels of phosphorylated-Smad2/3 and ERK1/2 MAPK, suggesting elevated TGF-β signaling and activation of both Smad- and MAPK-dependent signaling may contribute to profibrotic responses in PAI-1 deficient mice (Ghosh et al., 2010).

As PAI-1 induces internalization of uPA and αvβ3 integrins from the cell surface, the increased TGF-β signaling in PAI-1 deficient tissues may also be associated with elevated levels of cell surface expression of α vβ 3 integrins, increased expression of TGFβRII, increased plasmin activation, and elevation of Smad-dependent TGF-β signaling, as has been reported by Pedroja et al. (2009). Therefore, excessive accumulation of collagen in fibrotic heart in PAI-1 deficient mice is associated with increased inflammation, increased number of fibroblasts, elevated αvβ3 levels, increased MMP2/9 activity, elevated TGF-β levels, and activation of profibrotic Smad- and ERK1/2 MAPK–dependent pathways (Ghosh et al., 2010; Moriwaki et al., 2004; Xu et al., 2010). Development of spontaneous cardiac fibrosis in aged PAI-1 deficient mice further suggests that while physiologic levels of PAI-1 are cardioprotective, elevated or complete absence of PAI-1 is profibrotic and cardiopathologic.

In summary, it is evident from all these *in vivo* studies using different animal models of tissue fibrosis that TGF-β signaling and the PAI-1 axis play pivotal roles in the pathogenesis of tissue fibrosis. Different downstream signaling molecules/transcription factors in this axis may be ideal targets for therapeutic approaches to treat this devastating fibroblast-related disease.

5. Epithelial-to-Mesenchymal transition (EMT) and Endothelial-to-Mesenchymal Transition (EndMT) in Tissue Fibrosis: Involvement of PAI-1

Although fibroblasts are the major source of ECM proteins during tissue repair as well as during development of fibrosis under pathologic conditions, the origins of participating fibroblasts in tissue fibrosis is not well understood. Originally it was thought that adult fibroblasts are only derived from embryonic mesenchymal cells and in response to myocardial infarction, intracardiac resident fibroblasts are the primary origin of myofibroblasts contributing in repair processes (Yano et al., 2005). However, numerous recent studies suggest that other than resident tissue fibroblasts, adult fibroblasts also originate from epithelial cells and endothelial cells by epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT) respectively. EMT and EndMT are common biologic processes during embryonic development of the heart and other organs such as lung, kidney and liver. However, abnormal activation of EMT and EndMT and transformation to collagen producing myofibroblasts, in adults play significant roles in the development and progression of fibrosis in organs such as skin, kidney, liver, lung and heart (Arciniegas et al., 2007; Chapman, 2011; Cufí et al., 2010; Goumans et al. 2008; Hertig et al., 2010; Kaimori et al., 2010; Krenning et al., 2010; Liebner et al. 2004; Nakamura and Tokura, 2011; Senoo et al., 2010; Wada et al., 2011; Yoshino et al., 2007; Zeisberg et al., 2007; Zhang et al., 2007). While EMT is characterized by gradual reduction of epithelial or hepatocyte markers like E-cadherin, and appearance of mesenchymal markers such as α-SMA, collagen, EndMT is characterized by endothelial cell disaggregation, shape change related to myofibroblast differentiation, gradual loss of endothelial markers such as CD31, VECadherin, and vWF, and the gradual appearance of fibroblastic markers such a s FSP1 and collagen (Zeisberg et al., 2007). It is now well documented that elevated TGF-β signaling plays a significant role in both EMT and EndMT processes.

Recent reports indicate the possible role of PAI-1 in EMT and EndMT. TGF-β induces the levels of PAI-1 in both epithelial cells and endothelial cells and promotes EMT and EndMT (Ghosh et al., 2010; Senoo et al., 2010; Yoshino et al., 2007) and siRNA mediated depletion of PAI-1 in mouse lung epithelial cells prevents TGF-β-induced EMT (Senoo et al., 2010). However, in response to unilateral ureteral obstruction in plasminogen deficient mice, EMT is significantly reduced and is associated with lower levels of TGF-β signaling, reduced ERK1/2 activation, and renal interstitial fibrosis compared with UUO-wildtype mice *in vivo*. These findings suggest that plasmin may play an important role in EMT and profibrotic signaling (Zhang et al., 2007). This suggestion is consistent with observations that treatment of mouse cardiac endothelial cells with TGF-β induces EndMT in both wildtype and PAI-1 deficient cells. However, PAI-1 deficient endothelial cells are more susceptible to EndMT compared with wildtype cells. This susceptibility may be due to increased cellular uPA/tPA/plasmin activities in the PAI-1 deficient cells which activate endogenous latent TGF-β to active TGF-β and the resultant increased autocrine TGF-β signaling may contribute to increased EndMT in PAI-1 deficient mouse cardiac endothelial cells (Ghosh et al., 2010; Vaughan, 2006) (Figure 3). Increased susceptibility of PAI-1 deficient endothelial cells to EndMT is associated with increased levels of TGF-β signaling and activation of downstream Smad and ERK1/2 MAPK dependent signaling. These results indicate that in aged mice, increased EndMT-derived fibroblasts in the absence of PAI-1 may contribute to elevated collagen synthesis and spontaneous cardiac fibrosis (Ghosh et al., 2010). The origin of

cardiac fibroblasts contributing to spontaneously developing cardiac fibrosis in aged PAI-1 deficient mice needs further *in vivo* investigation.

6. Concluding Remarks and Future Direction

Physiologic levels of PAI-1, the most potent cellular inhibitor of the uPA/tPA/plasmin fibrinolytic system, plays a pivotal role in controlling blood clotting, wound healing, the levels of MMP activities, in the magnitude of TGF-β activation, cellular proliferation, and differentiation, and matrix remodeling. However, an abnormal level or activity of PAI-1 ignites numerous diseases in humans. While elevated levels of PAI-1 causes thrombosis, atherosclerosis and tissue fibrosis, deficient PAI-1 activity is associated with excessive or delayed bleeding, increased cellular proliferation, and increased profibrotic signal-induced cardiac fibrosis. Importantly, while stress or injury-induced elevated PAI-1 contributes to tissue fibrosis, PAI-1 deficiency protects different organs from stress-activated or injury-induced tissue fibrosis. Therefore, it is apparent that while physiologic levels of PAI-1 are beneficial from the standpoint of its role in different biologic process, either excessive PAI-1 or PAI-1 deficiency contributes to disease development. Further, PAI-1 deficiency protects tissues from injury-related fibrogenesis via activation of an antifibrotic agent, and elevated PAI-1 contributes to induced fibrogenesis by suppressing cellular proteolytic activities and increasing extracellular matrix stability. As the proteolytic activities are elevated in the absence of PAI-1, development of tissue fibrosis is attenuated in response to profibrotic induction. In contrast to these scenarios, development of age-dependent spontaneous cardiac-selective fibrosis in PAI-1 deficient mice is due to elevated TGF-β-induced profibrotic signaling, suggesting that a physiologic PAI-1 level is cardioprotective. Collectively, all these studies on PAI-1 biology and tissue fibrosis indicate that while the development of fibrosis is largely associated with the elevated ROS, activation of TGF-β-induced Smad and ERK1/2 MAPK pathways, EMT/EndMT, myofibroblast differentiation, decreased proteolytic activity and excessive collagen deposition (Figure 4), protection from induced fibrosis in most of the PAI-1 deficient organs is associated with activation of antifibrotic HGF-1α. Furthermore, these studies suggest that while some common molecular mechanism of fibrogenesis is operating in different organs, organ-specific and cell-type–specific regulation of PAI-1 and its activity also determine its unique role in injury-specific, age-specific and tissue-specific fibrogenesis (Figure 5). In conclusion, pharmacologic or RNAi-or miRNA-mediated normalization, but not complete depletion, of elevated PAI-1 or suppression of elevated PAI-1 via ligand-mediated induction of PPAR-γ and downstream HGF-1α will be ideal approaches to control tissue fibrosis in different organs.

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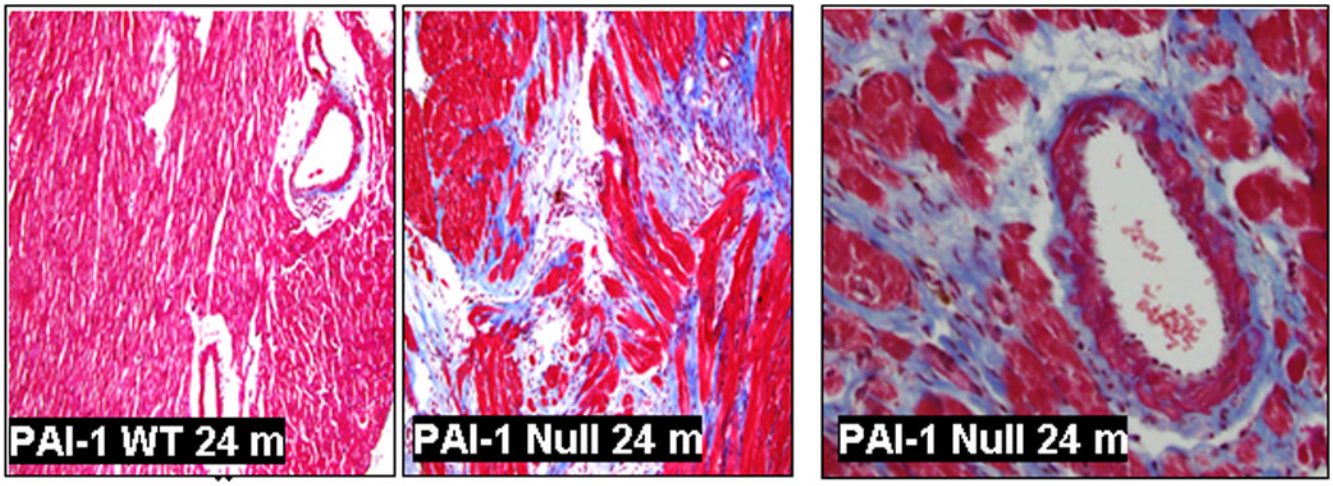

Figure 1. PAI-1 deficiency and spontaneous cardiac fibrosis

Images show the Masson's Trichrome staining of myocardial tissues derived from 24-m old wildtype mice and PAI-1 knockout mice. Note the excessive perivascular and interstitial collagen deposition (blue color) in aged PAI-1 knockout mice in heart (middle and right) and no such pathologic manifestation of fibrosis is present in aged wildtype heart (left).

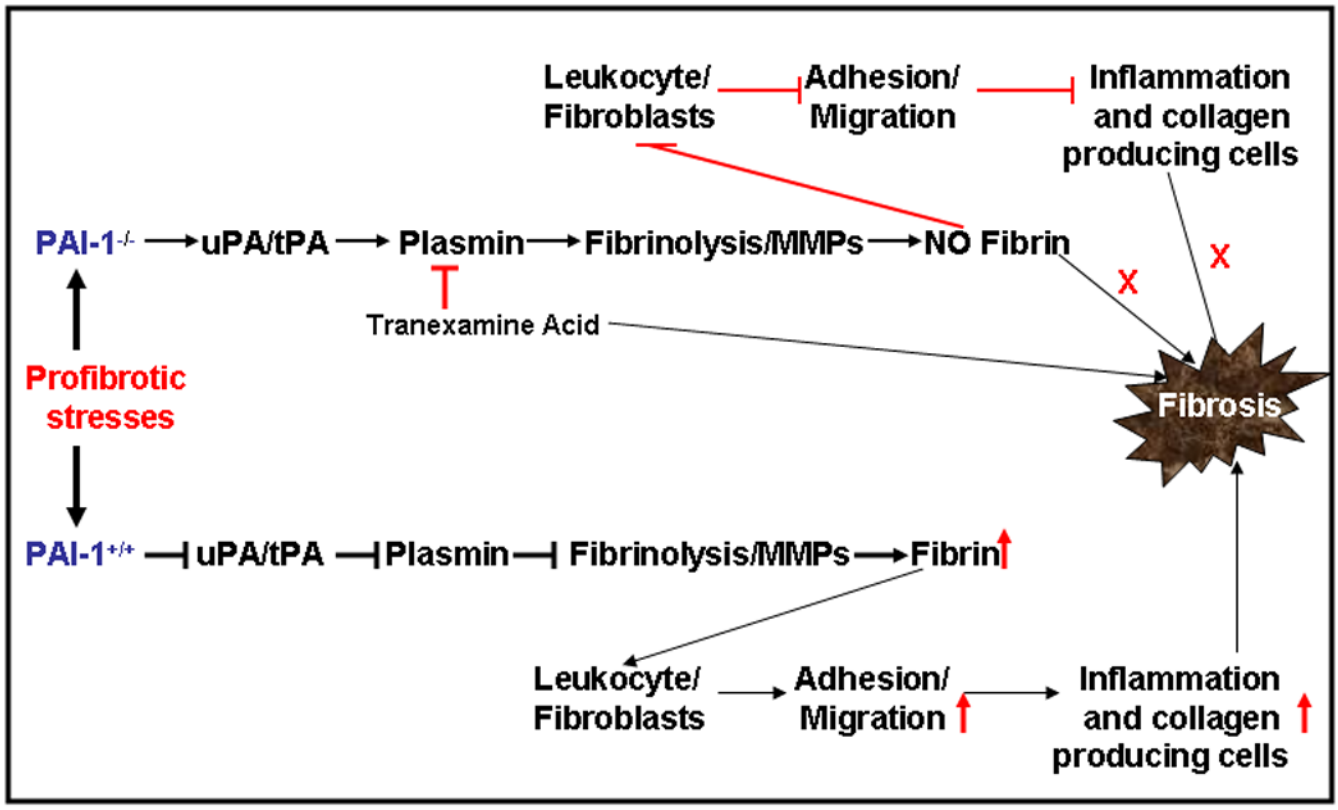

Figure 2. Possible role of PAI-1 in induced fibrosis in different organs: involvement of fibrin

PAI-1 controls uPA/tPA/plasmin/MMPs proteolytic activity, levels of fibrin and the migration of inflammatory- and collagen-producing cells. In PAI-$1^{-/-}$ mice, more fibrinolytic activity leads to a lack of fibrin, impaired cellular migration/adhesion, and less inflammation and collagen production. In the PAI-$1^{+/+}$ mice, the elevated level of fibrin causes increased adhesion and migration of inflammatory- and collagen-producing cells and tissue fibrosis.

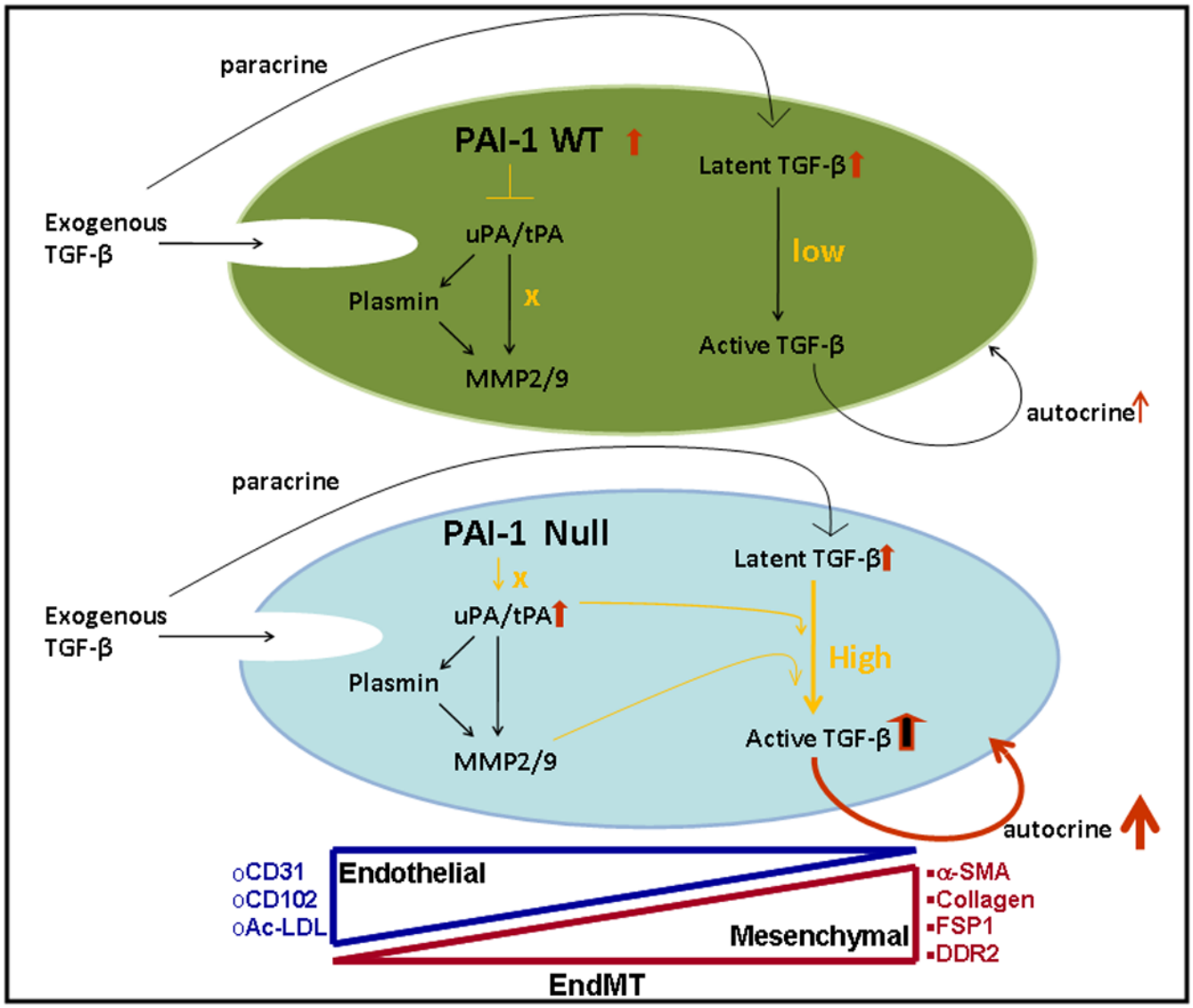

Figure 3. PAI-1 and EndMT

Model showing possible role of PAI-1 deficiency in EndMT. Exogenous TGF-β activates both wildtype and PAI-1 deficient endothelial cells. However, in the absence of PAI-1, autocrine TGF-β signaling is higher in PAI-1 null cells compared to wildtype cells. Increased autocrine TGF-β signaling may contribute to faster EndMT in PAI-1 deficient endothelial cells.

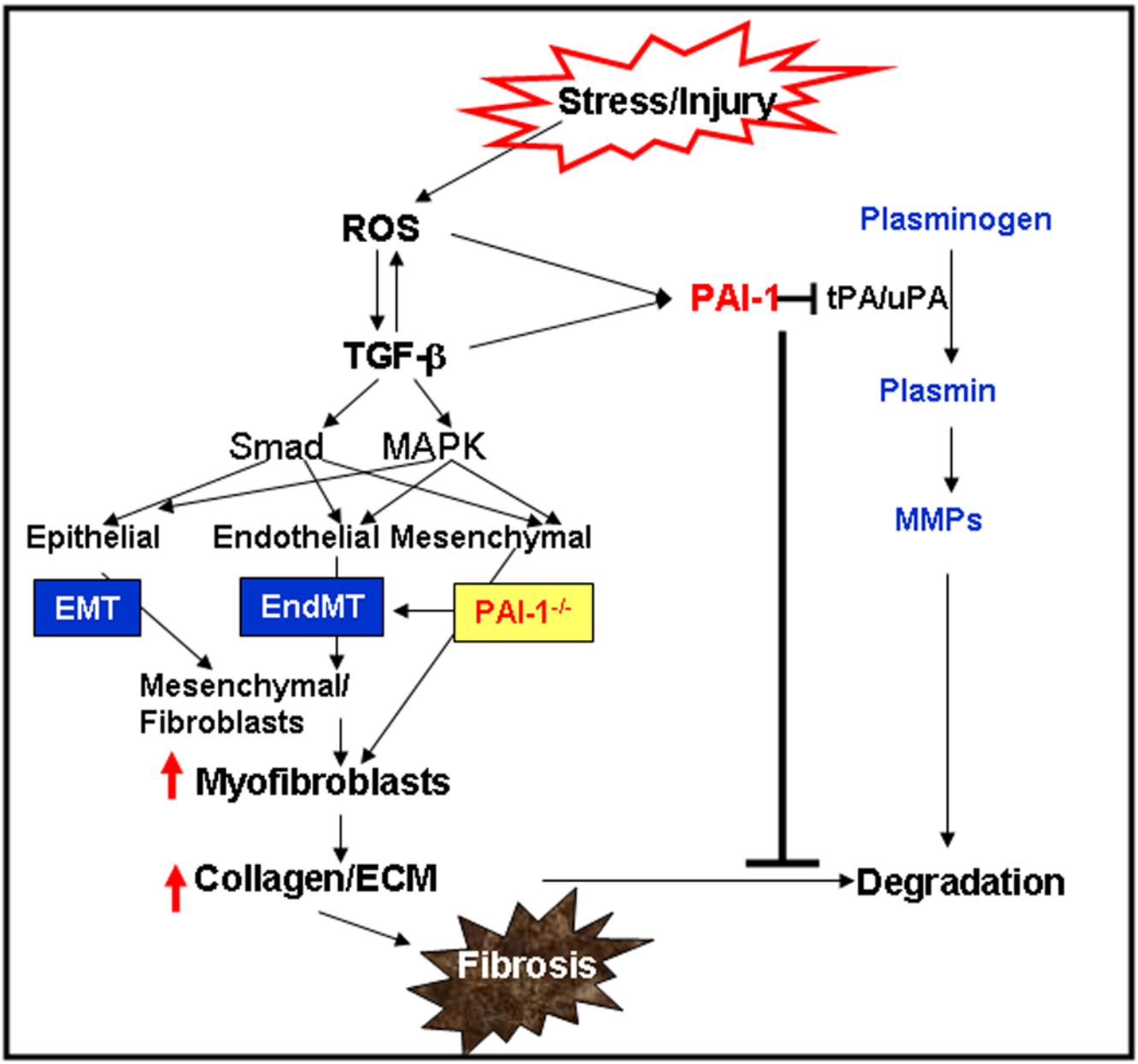

Figure 4. Model demonstrating possible molecular basis of induced tissue fibrosis and role of PAI-1

Different extracellular and intracellular stresses induce ROS production; inflammation and inflammatory mononuclear cells secrete profibrotic cytokines such as TGF-β which activates Smad and MAPK, which in turn activates resident fibroblasts as well as induces EMT/EndMT. Resident and EMT/EndMT-derived fibroblasts differentiate to myofibroblasts and produce collagen and other ECM proteins. ROS and TGF-β also induce PAI-1, which inhibits proteolytic activities and prevents collagen/ECM degradation. Additionally, PAI-1 contributes in tissue fibrosis by influencing EMT/EndMT and the migration of inflammatory/collagen producing cells.

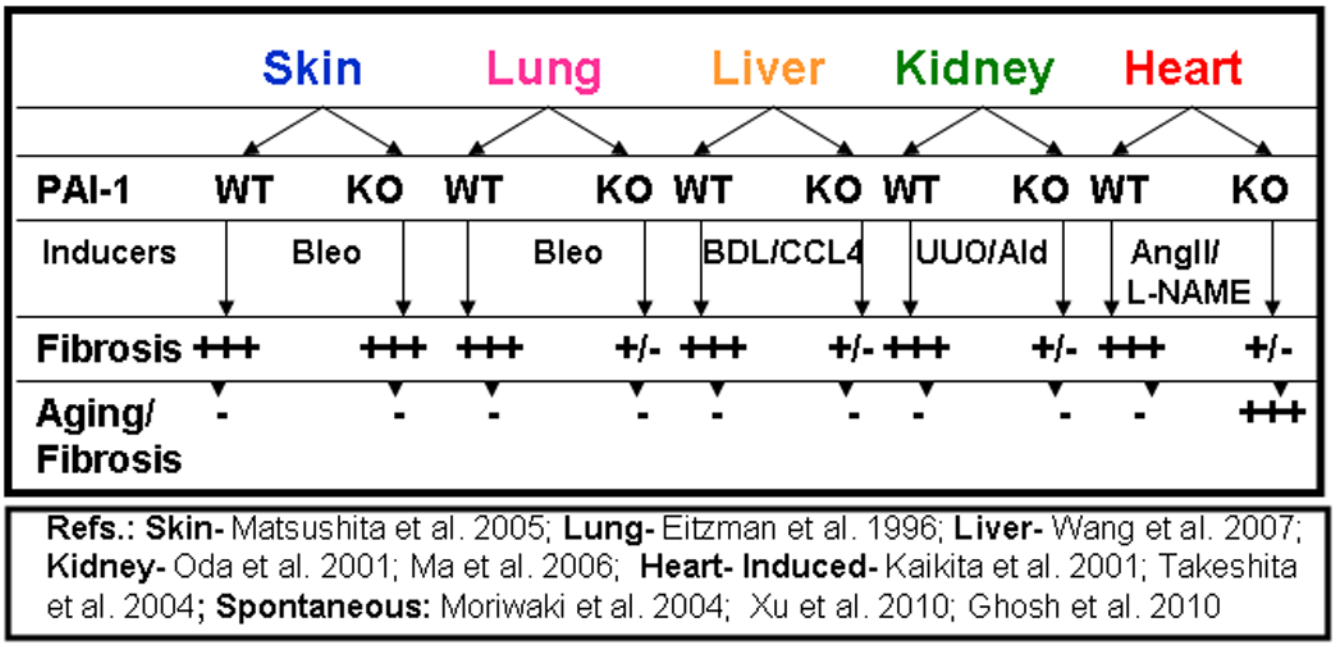

Figure 5. Significance of PAI-1 in injury or stress–induced tissue fibrosis and in age-dependent spontaneously developed tissue fibrosis

While PAI-1 deficiency protects organs from induced fibrosis except skin, PAI-1 deficiency develops spontaneous cardiac-selective fibrosis in aged mice. Role of PAI-1 is organ specific.