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Effects of Altered Plasminogen Activator Inhibitor-1 Expression on Cardiovascular Disease

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Abstract

Plasminogen Activator Inhibitor-1 (PAI-1) is a multifunctional protein with the ability to not only regulate fibrinolysis through inhibition of plasminogen activation, but also cell signaling events which have direct downstream effects on cell function. Elevated plasma levels of this protein have been shown to have profound effects on the development and progression of cardiovascular diseases. However, results from a number of studies, especially those using PAI-1 deficient mouse models, have demonstrated that its function is ambiguous, with evidence of both preventing and enhancing various disease states. A number of lifestyle changes and pharmacological reagents have been identified that can regulate PAI-1 levels or function. Those reagents that target function are focused on its ability to regulate plasmin formation, and have been studied in vivo models of thrombosis. Further investigations involving regulation of cell function could potentially resolve paradoxical issues associated with the function of this protein in regulating cardiovascular disease.

Keywords

plasminogen activator inhibitor-1; gene knock out mice; cardiovascular disease

Plasminogen Activator Inhibitor-1 (PAI-1)

The plasminogen system is the major physiological surveillance mechanism for regulating fibrin clearance. The major components of the plasminogen system consist of plasminogen, the precursor of the serine protease, plasmin; plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA); an inhibitor of activation, PAI-1, and inhibitors of plasmin activity, α_2 -antiplasmin, and α_2 -macroglobulin. Activation of human plasminogen consists of the cleavage of a peptide bond (Arg560-Val561) generating the two-chain disulfide bond linked plasmin. PAI-1 is a 52 kDa protein and is a member of the SERPIN superfamily. PAI-1 regulates this plasminogen activation by forming stable serpin/protease complexes with uPA and tPA [1]. PAI-1 is also complexed with the plasma protein vitronectin (VTN) through the somatomedin B domain of VTN [2]. This interaction stabilizes PAI-1 in an active form [3]. VTN is also present in the ECM of many tissues and may serve to selectively localize PAI-1 function [3]. Studies have demonstrated that PAI-1 in complex with VTN can regulate thrombin activity in the endothelial cell matrix potentially regulating coagulant activity of thrombin and/or its activity as a cell signaling mediator through protease activated receptor (PAR)-1 activation. The receptor for uPA, uPAR, also binds to VTN and the N-terminal region of this matrix protein contains the binding domain for PAI-1 and uPA [4]. Occupancy of the receptor by

uPA results in an increased affinity for VTN [5] as well as activation of intracellular signaling and phosphorylation of proteins that regulate focal adhesion turnover, i.e., focal adhesion kinase (FAK) [6]. Colocalization of this complex with integrins is the result of RGD-containing binding sites for α_v integrins in VTN, located adjacent to the N-terminal domain of VTN [7]. This complex can lead to local dissolution of ECM protein through activation of a proteolytic cascade. Regulation of this process occurs as a result of active PAI-1 sterically interfering with binding of either uPAR or integrin to VTN [4,8,9]. Additionally, the interaction of PAI-1 with the surface-associated uPA/uPAR complex induces internalization of the complex via interaction with low-density-lipoprotein receptor-related protein (LRP) [10], thus altering cytoplasmic signaling by uPA/uPAR. Since cell proliferation relies on a cycle of cell attachment, spreading and flattening (to initiate DNA synthesis), and then rounding, the result of disengagement of cell contacts through dissolution of the matrix, it would appear that alterations in PAI-1 expression could have a profound effect on these cellular events. Indeed studies have found that a deficiency of PAI-1 in aortic EC has a profound effect on cell proliferation and regulation of this cellular event appears to involve the LRP interactive site of PAI-1 [11,12]. Examples of pathologies associated with dysregulated PAI-1 expression is illustrated in Figure 1.

PAI-1 Deficiency

The first reported clinical case of a PAI-1 deficiency occurred in 1989, a time shortly after the discovery of the first plasminogen activator inhibitor in 1984 [13]. In this case, the patient presented with PAI-1 antigen levels but diminished PAI-1 activity. A quantitative abnormality (low antigen and low activity) was identified 2 years later and a homozygous total deficiency in 1992 [14,15]. PAI-1 deficiencies are rare and result in mild to moderate bleeding episodes.

The first generation of a deficiency of a component of the plasminogen activation system in mice was PAI-1. These mice develop normally and are fertile, but demonstrate an enhanced basal level of fibrinolytic activity [16,17] and, in a number of mice, develop cardiac fibrosis at a late age [18]. While these spontaneous phenotypes were mild at best, studies utilizing challenge models in PAI-1^{-/-} mice, have demonstrated profound effects on a number of pathologies, *viz.*, skin wound healing [19], vascular injury/repair [20], and angiogenesis [21–23].

PAI-1 and Myocardial Infarction (MI)

In the U.S. alone, there are approximately 1.3 million cases of nonfatal MI/year, which indicates that there are approximately 600 cases/100,000 people. In most cases, plaque rupture results in exposure of procoagulant surfaces resulting in occlusive thrombus formation. This results in reduced blood/oxygen supply to parts of the myocardium eventually leading to myocardial necrosis. Other causes of MI have been documented and consist of vasculitis, emboli from infected heart valves, angina, and the use of narcotics such as cocaine and amphetamines. Risk factors for MI, following rupture of an atherosclerotic plaque, are excessive weight [24–26], diabetes mellitus [27–29], arterial hypertension [30–32], and smoking [33–35]. Additionally, a parental history of coronary heart disease (CHD) has been associated with risk to other family members and, therefore, genetic factors may play a role in the regulation of other vascular risk factors or predicting the incidence of MI [36].

Large epidemiological studies have indicated that PAI-1 is an important risk factor for the initial development and recurrence of cardiovascular disease (CVD). Elevated levels of plasma PAI-1 have been shown to precede MI in patients [37]. As a result, these patients have impaired fibrinolysis [38] and for those that survive their initial MI and still maintain

elevated levels of this inhibitor there is a high probability of early recurrence of another cardiac event [39]. Related to this, the renin-angiotensin system is also activated after acute MI and studies have shown that angiotensin II can upregulate the expression of PAI-1 [40]. A PAI-1 mutant consisting of a single base pair guanine deletion/insertion (4G/5G) polymorphism in the promoter region of the PAI-1 gene has been shown to increase plasma PAI-1 levels and is therefore a risk factor for coronary disease [41]. In a study consisting of 1179 healthy subjects, age and PAI-1 4G/5G were independent contributors to a family history of CHD [42]. Other single nucleotide polymorphism (SNP)s in the PAI-1 gene locus have also been implicated as risk factors [43–45]. Additionally, studies have suggested that Metabolic Syndrome is a prerequisite for high PAI-1 levels in patients with a predisposition to developing atherothrombosis [46,47].

Challenge studies in which myocardial infarction (MI) was induced by coronary occlusion in PAI-1^{-/-} and WT mice demonstrated that in PAI-1^{-/-} mice, there was a thickening of diastolic left ventricular anterior walls of the heart and increased infarction which was associated with hemorrhage and inflammation but not fibrosis [48]. Further, overexpression of PAI-1 resulted in increased left ventricular fibrosis after MI demonstrating that PAI-1 expression is profibrotic in hearts subject to infarction [49].

Atherosclerosis and PAI-1

Atherosclerosis is a complex, age-related disease involving a diverse array of cellular, protein, and lipid components and functions. It remains a major health issue in Western cultures where it is responsible for significant morbidity and mortality [50].

PAI-1 plasma levels have been shown to positively correlate with known risk factors for developing atherosclerosis, i.e., obesity, hyperinsulinemia, diabetes, and hypertriglyceridemia [37,51–53]. In atherosclerotic plaque tissue, mRNA for PAI-1 is elevated and expression has been identified in a number of cell types associated with the plaque, primarily localized around the base of the plaque [54,55]. PAI-1 could potentially contribute to the developing plaque by stabilizing the fibrin matrix, acting as a scaffolding protein for migrating cells [56,57] or potentially stimulating smooth muscle cell proliferation [58] and/or low density lipoprotein (LDL) uptake into the lesion [59]. Other studies have indicated that PAI-1 prevents atherosclerosis development and thus involvement of this protein in this disease remains a “paradox”. This may be due to the complex functions of PAI-1 and its preventive or enhancing involvement in atherosclerosis may be dependent on the vascular environment, composition of the plaque, or the different proteins that are interacting with PAI-1 [60].

Cardiac Fibrosis

Heart disease is the leading cause of death in the Western world (> 2,500 Americans die from heart disease/day). Most patients who suffer from heart disease have scarring of cardiac tissue (cardiac fibrosis), the result of pathological wound healing processes following cardiac tissue injury. The underlying cause of this disease is unknown and there is currently no effective therapy to treat it. The hallmark feature of this disease is the activation and migration of alpha smooth muscle cell actin-expressing myofibroblasts that synthesize and deposit extracellular matrix protein, i.e., collagen, in an unregulated manner [61]. The origin of these myofibroblasts is unknown. However, it is believed that they originate from resident fibroblasts that are differentiated to myofibroblasts by growth factors, i.e., transforming growth factor-beta (TGF- β), platelet-derived growth factor, connective tissue growth factor (CTGF) or vasoconstrictors, i.e., angiotensin II and endothelin-1 [62]. Alternatively, recent studies have demonstrated that endothelial cells can undergo endothelial-mesenchymal transition (EMT), when stimulated with TGF- β , generating

collagen-producing fibroblasts [63]. In that study administration of bone morphogenic protein 7 (BMP-7) inhibited EMT and the development of cardiac fibrosis in a mouse model. The physiological outcome of this dysregulated wound healing process is stiffening of the cardiac tissue with resultant inability of the heart to function properly.

TGF- β expression is upregulated in response to injury. This growth factor is known to control a number of cellular responses, i.e., proliferation and differentiation [64]. TGF- β is initially expressed in a latent form as a large precursor protein consisting of an N-terminal signaling peptide, a pro-region (latent associated peptide (LAP)), a C-terminus containing the mature TGF- β protein sequence which is generated by proteolytic processing of the pro-region [65,66]. The mature protein dimerizes to form the active TGF- β [67]. Active TGF- β signals through the Smad pathway by initially binding to a type II receptor. This interaction results in the recruitment and activation (phosphorylation) of a type I receptor which recruits and activates (phosphorylation) a receptor regulated Smad (R-Smad). R-Smad then binds to the common Smad (coSmad) forming a heterodimer complex that translocates to the nucleus where it acts as a transcription factor for a number of genes [68].

Another profibrotic protein, angiotensin II, indirectly induces collagen production through upregulating expression of its downstream target, TGF- β [69,70]. Through activation of Smads, E26 transformation-specific-1 (Ets-1), protein kinase C or RAt sarcoma/mitogen-activated protein kinase kinase/extracellular (ras/MEK/ERK) pathway, TGF- β has also been shown to regulate expression of another profibrotic growth factor, CTGF [71–73]. Activation of the ras/MEK/ERK pathway also regulates ECM contraction and actin stress fiber formation [74,75]. CTGF and TGF- β have been shown to act in a synergistic manner in regulating fibrotic responses in the heart. Another profibrotic regulator, endothelin-1, which is a potent vasoconstrictor, has been shown to induce ECM synthesis and contraction [76–78]. TGF- β has been shown to induce the expression of this protein in fibroblasts through a Jun N-terminal protein kinase (JNK)-dependent but Smad-independent manner. Endothelin-1 also acts in synergy with TGF- β in regulating fibrosis. These observations indicate that TGF- β plays a central and critical role in fibrosis.

It has been shown that cell-associated uPA activates cell bound plasminogen to plasmin [79] which releases both active and latent TGF- β from extracellular matrices [80]. In support of these observations, antibodies to uPA or blocking its interaction with its cell surface receptor, uPAR, block activation of TGF- β [81]. Further, addition of the serine protease inhibitors aprotinin or 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) also inhibited TGF- β activation. Additionally, since thrombospondin-1 (TSP-1) has been implicated as an activator of TGF- β [66], and TSP-1 binds plasminogen [82,83], TSP-1 could potentially act as a scaffolding protein for plasmin in mediating TGF- β activation on cells. Therefore, dysregulated plasmin formation (altered plasminogen activator or plasminogen activator inhibitor expression) could be a driving force for cardiac fibrosis.

PAI-1 and Spontaneous Cardiac Fibrosis

Studies have demonstrated that cardiac PAI-1 levels increase significantly as a function of age [84,85]. Additionally, studies have demonstrated that altered PAI-1 expression results in altered cardiac function associated with aging.

A spontaneous increase in macrophage accumulation followed by increased fibrosis has been observed in cardiac but not other tissues in PAI-1^{-/-} mice [18]. These studies would indicate that PAI-1 may have opposite effects in uninjured versus injured tissue (MI induced by coronary occlusion), which may be dictated by the different functional domains of PAI-1. Studies in our laboratory and that of others have demonstrated that TGF- β is enhanced in uninjured fibrotic cardiac tissue from aging PAI-1^{-/-} mice [86,87]. In one of these studies

[87], phosphorylated Smad 2 and 3, a common signaling pathway involved in tissue scarring, as well as phosphorylated ERK1/2 MAPK were enhanced in aging PAI-1^{-/-} myocardial tissue. Additionally, endothelial cells from PAI-1^{-/-} mouse cardiac tissue demonstrated enhanced EMT when stimulated with TGF- β , supporting an endothelial origin to the profibrotic fibroblasts associated with cardiac fibrosis. Since plasmin has been shown to activate TGF- β allowing its interaction with a receptor this ultimately enhances the generation of PAI-1 which would have a negative feedback on Pm-mediated TGF- β activation thus diminishing fibrosis. A summary of these observations pertinent to cardiac fibrosis is seen in Table 1.

Activation of other signaling pathways has been associated with cardiac fibrosis and ultimately cardiac failure. Clinically, enhanced activated Akt has been observed in heart failure patients [88]. Additionally, Akt has been shown to promote endocardial-mesenchyme transition [89]. Pertinent to this observation, studies in our laboratory have demonstrated that a PAI-1 deficiency leads to enhanced activation of Akt in EC [12]. Therefore, multiple pathways involving different functional properties of PAI-1 may play a role in regulating cardiac fibrosis.

Potential Therapies

Some of the therapeutic approaches toward diminishing plasma PAI-1 levels are implementing life style changes. Physical exercise has been shown to lower plasma PAI-1 and other hemostasis and inflammatory biomarkers, i.e., C-reactive protein (CRP), fibrinogen (Fg), tPA [90–93]. In addition to physical activity, weight loss has been shown to diminish PAI-1 levels [94–96].

Clinically approved drugs for hypertension and those for limiting cholesterol synthesis have also been shown to impact PAI-1 levels. Angiotensin II stimulates PAI-1 expression in adipocytes and ramipril, an angiotensin-converting enzyme (ACE) inhibitor has been shown to lower PAI-1 in patients with acute MI [97]. Simvastatin, which is a HMG-CoA reductase inhibitor (statin) which lowers cholesterol levels has been shown to lower plasma PAI-1 protein and mRNA in rabbits fed an atherogenic diet [98]. Similarly, atorvastatin and rosuvastatin also decreased PAI-1 levels as well as levels of the inflammatory biomarker, CRP [99]. The antidiabetic drugs of the class thiazolidinedione, troglitazone and pioglitazone, which were designed to activate peroxisome proliferator-activated receptor- γ (PPAR- γ) also have been shown to diminish PAI-1 levels as well as components of the metabolic syndrome [100–102]. However, due to enhanced liver toxicity troglitazone is no longer available.

Catalytic DNA enzymes (DNAzyme) that target cleavage of PAI-1 mRNA have also been developed. Infusion of these reagents at the site of balloon-induced arterial injury in obese diabetic rats demonstrated that at early times after injury (48 hr) endothelial expression of PAI-1 is diminished. By 2 weeks, fibrin deposition is diminished, there is a 5 fold lower level of proliferating smooth muscle cells, and a 2 fold lower level of neointima/media ratio relative to that seen with a scrambled DNA enzyme [103].

Other small molecule pharmacological agents have been developed for targeting PAI-1. The indole oxoacetic acid derivative, tiplaxtinin, has been shown in both in vitro and in vivo assays to act as a select PAI-1 inhibitor ($IC_{50} = 2.7 \mu M$) [104]. This reagent was shown to have excellent bioavailability and metabolic stability. However, its affinity to PAI-1 is relatively low and it does not interact with PAI-1 in the presence of VTN [105]. TM5007, another orally active molecule was found to interact with the strand 4 position (s4A) of the A β -sheet of PAI-1 inhibiting PAI-1 activity and increasing fibrinolysis. In vivo, this compound inhibited coagulation in 2 animal models, a rat arteriovenous shunt model and a

mouse ferric chloride arterial injury model. [106]. Based on the structure of this molecule and *in silico* docking observations, the same researchers designed a new inhibitor of PAI-1, TM5275, which demonstrated antithrombotic efficacy *in vivo* in rats and nonhuman primate thrombosis models [107]. This compound was equivalent to ticlopidine and clopidogrel in antithrombotic activity but without adverse bleeding side effects. PAI-749 is another small molecule inhibitor of PAI-1 and neutralizes this protein utilizing dual mechanisms involving blocking the ability of the inhibitor to interact with plasminogen activators, uPA ($IC_{50} = 87$ nM) and tPA ($IC_{50} = 150$ nM), as well as rendering PAI-1 susceptible to plasmin mediated degradation [108]. More recently, novel bis-arylsulfonamide and arylsulfonamide derivatives have been developed that are 30X more potent than tiplaxtinin in regulating PAI-1 function as an antifibrinolytic protein [109]. Another class of active small molecules that target PAI-1 is polyphenolics. These molecules have demonstrated an IC_{50} for PAI-1 between 10–200 nM. Inhibition by these molecules is reversible and involves preventing the stabilization of the noncovalent Michaelis complex between PAI-1 and target proteases [110]. Additionally, these compounds inactivate PAI-1 in the presence of VTN.

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Abbreviations

Akt	a serine/threonine kinase
CHD	coronary heart disease
CRP	C-reactive protein
CTGF	connective tissue growth factor
CVD	cardiovascular disease
EC	endothelial cell
ECM	extracellular matrix
EMT	endothelial-mesenchymal transition
ERK	extracellular signal-regulated protein kinase
Ets-1	E26 transformation-specific-1
Fg	fibrinogen
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FAK	focal adhesion kinase
FSP-1	fibroblast-specific protein-1
JNK	Jun N-terminal protein kinase
LDL	low density lipoprotein
LRP	low density lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
MI	myocardial infarct
PAI-1	plasminogen activator inhibitor-1

Pm	plasmin
rasMEK/ERK	RAt sarcoma/mitogen-activated protein kinase kinase/extracellular
SERPIN	serine protease inhibitor
SNP	single nucleotide polymorphism
TGF-β	transforming growth factor-beta
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
uPAR	urokinase receptor
VTN	vitronectin

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Figure 1.
Pathologies associated with abnormal expression of PAI-1

Table 1Changes in Aging Murine PAI-1^{-/-} Cardiac Tissue

Observation *	Potential effects on cardiac tissue
↑ uPA	↑ formation of Pm (TGF β activator)
↑ Vascular leakage	↑ inflammation (early event of fibrosis)
↑ macrophage	↑ inflammation
↑ proinflammatory cytokines (iNOS, iCAM, KC)	↑ inflammation
↑ TGFβ-1, -2	↑ activation of Smad signaling, common profibrotic pathway
↑ phosphorylated Smad-2, -3	↑ extracellular matrix production
↑ phosphorylated ERK1/2	↑ activation of Smad signaling
↑ MMP-2, -9, TIMP-2	↑ tissue remodeling
↑ FSP-1	↑ marker of fibroblasts (enhanced during EMT)
↑ TGFβ-induced EMT	↑ EC differentiation into profibrotic fibroblasts
↑ FGF2 and FGFR2	↑ EMT and fibrillar collagen
↑ collagen	↑ tissue scarring
↓ cardiac function	↑ physiological manifestation of cardiac fibrosis

* From references 18, 86, 87