PAI-1 ANTAGONISTS: THE PROMISE AND THE PERIL

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ABSTRACT

The plasminogen activator (i.e., fibrinolytic) system is one of the key endogenous defense mechanisms against intravascular thrombosis. Thrombolytic agents represent the only direct way of augmenting fibrinolytic activity in humans, and have proven to be of value in the treatment of acute myocardial infarction and stroke. Although these agents are efficacious in the acute setting, they are not a viable option for long-term use. Net fibrinolytic activity is plasma is largely determined by the balance between tissue-type plasminogen activator (t-PA) and its natural, fast-acting inhibitor, plasminogen activator inhibitor-1 (PAI-1). The recent development of specific PAI-1 antagonists promises to expand the limits of understanding of the role of the fibrinolytic system in human disease, and to break through the current confines of therapeutic options that can effectively restore and augment the activity of the fibrinolytic system.

The fibrinolytic system is one of the key endogenous defense mechanisms against intravascular thrombosis (1). At present, thrombolytic agents represent the only direct way of augmenting fibrinolytic activity in humans. Although these agents are proven to be efficacious in the treatment of acute thrombotic events, they are not a viable option for long-term use. Net fibrinolytic activity in plasma is largely determined by the balance between tissue-type plasminogen activator (t-PA) and its natural, fast-acting inhibitor, plasminogen activator inhibitor-1 (PAI-1). There are numerous drugs available that indirectly increase fibrinolytic activity by reducing plasma levels of PAI-1, including angiotensin converting enzyme (ACE) inhibitors, insulin-sensitizing agents, and the hormones used in hormone-replacement therapy in women. Plasma PAI-1 is derived from several sources, including the

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vascular endothelium, adipose tissue, and the liver. Plasma PAI-1 levels reflect a complex calculus of genetic factors; hormonal, metabolic, and inflammatory stimuli; and body mass. There is also a well-recognized circadian variation in the plasma activity of PAI-1, and this fluctuation in PAI-1 activity is responsible for the diurnal variation in net fibrinolytic activity (2).

Probably the most common clinical condition associated with increased PAI-1 production is obesity. Although plasma PAI-1 activity is consistently elevated in patients with obesity and insulin resistance, the precise source of PAI-1 is debated, and may include the vascular endothelium, liver and/or adipose tissue, including both adipocytes and stromal cells (3) located within visceral fat. In encoding a protein involved in fibrinolysis, the PAI-1 gene is remarkable for its responsiveness to a variety of metabolic and hormonal factors that are associated with obesity. Analytical studies of the upstream regulatory region of the PAI-1 gene has allowed the identification of relevant transcriptional response sites, including a glucocorticoid response element (GRE) that also localizes aldosterone responsiveness (4), a very-low-density lipoprotein (VLDL) response site (5), and two Sp1 sites that appear to mediate glucose/glucosamine responsiveness (6). In the aggregate, probably the most important determinants of plasma PAI-1 in a given individual include the molecular clock, body mass index (BMI), genetic determinants, and hormonal, inflammatory, and metabolic factors. At the molecular level, the hierarchy of factors that regulate transactivation of the PAI-1 promoter are the molecular clock, inflammatory cytokines, and hormonal and metabolic factors, while the suppressors of PAI-1 expression are less well defined, but include nitric oxide and cyclic nucleotides (7).

PAI-1 AND THROMBOSIS

The most predictable application of a PAI-1 antagonist would involve the prevention or treatment of thrombotic disease (Table 1). There is substantial experimental and epidemiologic evidence that PAI-1 may in fact contribute to the development of ischemic cardiovascular disease (8, 9). An excess of PAI-1 has been identified in youthful survivors of acute myocardial infarction (MI) (10) and predicts recurrent MI (11). There is also experimental evidence that these epidemiologic links to PAI-1 are more than casual associations. Transgenic mice that overexpress a stable form of human PAI-1 driven by the murine preproendothelin promoter develop age-dependent spontaneous macrovascular coronary thrombosis and subendocardial MI in the absence of

TABLE 1
Potential Therapeutic Applications of PAI-1 Antagonists.

Thrombotic Disorders	Fibrotic Disorders	Metabolic/Hormonal Disorders	Other
Acute coronary syndromes	Arteriosclerosis	Obesity	Amyloidosis
Veno-occlusive Disease	Nephrosclerosis	Type 2 Diabetes	Alzheimer's disease
Atrial thrombosis	Pulmonary fibrosis	Polycystic Ovarian Disease	Alopecia
Venous thrombosis	Myelofibrosis		Aging

hyperlipidemia or hypertension (12). Preclinical data have been reported indicating that administration of an orally active small-molecule antagonist of PAI-1 can delay the time to occlusion and enhance the rate of spontaneous reperfusion in a canine model of electrical coronary injury (13).

Specific, uncommon thrombotic disorders may be particularly well-suited for treatment with a PAI-1 antagonist. Hepatic veno-occlusive disease (VOD) is an uncommon but devastating complication of high-dose chemotherapy associated with bone-marrow transplantation (14). It is characterized clinically by hyperbilirubinemia, hepatomegaly, and fluid retention. Histologic features of VOD include fibrous occlusion of the terminal hepatic venous lumen, dilatation and ultimately fibrosis of hepatic sinusoids, and necrosis of zone-3 hepatocytes. Currently, experimental treatment of VOD includes the administration of thrombolytics agents, such as tissue-type plasminogen activator (t-PA) (15), and antithrombotic agents, such as the polydeoxyribonucleotide defibrotide (16). However, the optimal treatment of VOD would theoretically employ agents that address the cause as well as the consequences of this disorder. Although the pathogenesis of VOD is largely unknown, PAI-1 has emerged as both an independent diagnostic marker of VOD and a predictor of the severity of the disease (17, 18).

We developed a murine model of hepatic-vein thrombosis that involved long-term inhibition of nitric oxide (NO) synthase by administering N(ω)-nitro-L-arginine methyl ester (L-NAME) to mice for 6 weeks. In this study, the mechanistic role of PAI-1 in VOD was explored by investigating the effects of genetic PAI-1 deficiency and by administering tixplatinin—a first-generation, orally active small molecule PAI-1 antagonist (19)—to wild-type (WT) mice. After 6 weeks of treatment, livers from WT mice exhibited extensive fibrinoid hepatic venous thrombi as well as increased levels of total bilirubin and aspartate amino transferase (AST). In contrast, PAI-1-deficient mice

were largely protected from the development of hepatic-vein thrombosis. Furthermore, WT mice that received tiplaxtinin were also effectively protected from L-NAME-induced thrombosis. Taken together, these data indicate that NO and PAI-1 play pivotal and antagonistic roles in the pathology of hepatic-vein thrombosis, and that PAI-1 is a potential target in the prevention and treatment of VOD in humans.

Other relatively common thrombotic disorders might also be considered targets for treatment with a direct PAI-1 antagonist. Venous thromboembolic disease is generally managed by administering anticoagulants (heparin or warfarin) to prevent further thrombosis. The actual clots in the legs or pulmonary circulation in venous thromboembolic disease are often left to be dismantled by the activity of the endogenous fibrinolytic system, except in patients with massive pulmonary embolism (PE) or extensive deep-venous thrombosis (DVT), which necessitate acute thrombolysis or other invasive measures (thrombectomy). Although endogenous fibrinolysis is often eventually effective in clearing intravascular clots, it is viewed as limited in capacity and velocity in removing extensive intravascular thrombi. The availability of an agent that could be administered for days or even weeks to augment endogenous fibrinolysis would theoretically provide an improved strategy for the debulking or disposal of intravascular clots, and potentially reduce the risk of stroke from left-atrial thrombi or the development of post-phlebetic syndrome in patients with extensive venous thrombi in the lower extremities.

PAI-1 AND FIBROSIS

Aside from the role of PAI-1 in thrombosis, the fibrinolytic system also plays an important role in vascular and tissue remodeling. Metabolic derangements alone are sufficient to increase the arterial vascular content of PAI-1, even in the absence of atherosclerosis, as reported to be the case in the internal mammary artery of diabetics patients undergoing bypass grafting (20). In tissues in which PAI-1 is overproduced, local plasminogen activation is impaired, and this in turn has profound effects on vascular housekeeping and remodeling capacity (21). Indeed, we have shown that PAI-1 deficiency effectively prevents the development of arteriosclerosis and hypertension in mice treated with the NO synthase inhibitor L-NAME for periods of 8–16 weeks (22, 23). Although local impairment of the plasmin/plasminogen activator system appears to play an important role in the progression of atherosclerotic cardiovascular disease in general, we have hypothesized that increased vascular PAI-1 production and accumulation

plays a major role in the arterial remodeling that contributes to the development of hypertension in obesity and the metabolic syndrome. More recently, the effects of tiplaxtinin on angiotensin II(Ang II) induced cardiovascular injury in uninephrectomized mice during a high-salt intake for 8 weeks were investigated (24). The effect of pharmacologic PAI-1 inhibition on Ang II/salt-induced aortic remodeling and cardiac fibrosis was compared with the effect of genetic PAI-1 deficiency. Ang II caused significant medial, adventitial and aortic-wall thickening as compared with vehicle alone. Tiplaxtinin attenuated Ang II-induced aortic remodeling without altering the pressor response to Ang II. Interestingly, tiplaxtinin did not attenuate the effect of Ang II on cardiac hypertrophy and fibrosis. The effect of tiplaxtinin on Ang II/salt-induced aortic remodeling and cardiac fibrosis was comparable to the effect of genetic PAI-1 deficiency (24).

Progressive accumulation of extracellular matrix (ECM) in glomeruli and interstitium is almost universally seen in chronic renal disease, regardless of the underlying disease. There is considerable evidence that decreased ECM degradation contributes to matrix accumulation, and that ECM degradation is largely controlled by the plasminogen activator/plasmin system. Plasmin contributes to ECM degradation directly, by degrading matrix proteins, including fibronectin, laminin, proteoglycan, and type IV collagen, as well as fibrin, and indirectly, by converting inactive matrix metalloproteinases to active forms that degrade collagenous proteins. Among the most widely studied diseases that appears to be influenced by PAI-1 are glomerulosclerosis and tubulointerstitial fibrosis (25). Numerous studies have identified increased expression of PAI-1 in rodent models of renal injury provoked by a variety of insults, including ionizing radiation, Ang II, aldosterone, and ureteral ligation. Furthermore, the extent of fibrosis corresponds with the pattern and extent of PAI-1 expression (26). Although direct PAI-1 antagonists have not been studied in any of these rodent models of renal injury, a mutant, noninhibitory PAI-1 (PAI-1R) has been shown to increase glomerular plasmin generation and reduce disease in anti-thy-1 nephritis (27). PAI-1R reduces the pathologic accumulation of ECM, in large part by effectively competing with native PAI-1, thereby restoring plasmin generation and increasing plasmin-dependent degradation of matrix components (28).

Impaired fibrinolytic activity the lung is a common manifestation of acute and chronic inflammatory lung diseases. Because the fibrinolytic system is active during repair processes that restore injured tissues to normal, reduced fibrinolytic activity has been proposed to contribute to the development of pulmonary fibrosis. The relationship between the

fibrinolytic system and pulmonary fibrosis was examined by administering bleomycin to mice that either overexpressed or were completely deficient in murine PAI-1 (29). The lungs of transgenic mice overexpressing PAI-1 contained significantly more hydroxyproline than did those of littermate controls. In contrast, the lung hydroxyproline content of bleomycin-treated PAI-1−/− mice was not significantly different from that of control animals receiving saline. These data have been confirmed and extended, and consistently demonstrate a direct relationship between PAI-1 expression and the extent of pulmonary fibrosis that follows inflammatory lung injury (30). (Recent studies with a second-generation, orally-active PAI-1 antagonist demonstrated its efficacy in preventing bleomycin-induced pulmonary fibrosis in mice [31]) Thus, it seems reasonable to suggest that human diseases that generate fibrotic responses in pulmonary tissue, including idiopathic pulmonary fibrosis, should be investigated for their response to and the effect of PAI-1 antagonists.

PAI-1 IN OBESITY, DIABETES, AND POLYCYSTIC OVARIAN DISEASE

Obesity is an increasingly important risk factor for cardiovascular disease in men and women, and is associated with the premature development of atherosclerosis, increased risk of stroke, and the development of congestive heart failure (32). A classical perspective of cardiovascular risk does not adequately explain all of the cardiovascular events associated with obesity (33). Elevations in plasma levels of PAI-1 are one of the biochemical hallmarks of obesity and are likely to contribute to the increased risk of atherothrombotic events in patients with obesity and metabolic syndrome (34). In fact, it has been suggested that fibrinolytic dysfunction mediates the increased risk of cardiovascular disease in patients with metabolic syndrome (35). Recent studies in our laboratory have shown that inhibition of ACE can significantly reduce plasma levels of PAI-1 in obese human subjects (36), and that PAI-1 strongly correlates with serum aldosterone levels. Based on the numerous interactions listed above, it is not at all surprising that a strong correlation exists between BMI and plasma PAI-1 concentration (37).

With the epidemic of obesity, there has been a remarkable global increase in the incidence of type 2 diabetes mellitus (T2DM). It was recognized very early that individuals with T2DM have increased plasma levels of PAI-1 (38). Treatment of patients with T2DM with insulin-sensitizing agents, including metformin or thiazoladinediones,

can effectively reduce plasma levels of PAI-1. Although the literature has largely focused on elevated levels of PAI-1 as a consequence of insulin resistance, and as a mechanistic contributor to cardiovascular disease in patients with T2DM, the tissue has more recently shifted to a potential role of PAI-1 in the development of T2DM. The plasma PAI-1 level predicts the development of T2DM (39), and is viewed as a robust, non-metabolic predictor of T2DM. Numerous theoretical mechanisms about how PAI-1 might contribute to the development of diabetes deserve investigation.

Although not as common as obesity or T2DM, polycystic ovarian syndrome (PCOS), a diagnosis with life-long implications for cardiovascular and reproductive health affects from 5 to 10% of reproductive-age women (40). PAI-1 is consistently elevated in women with PCOS, regardless of their metabolic status. Interestingly, the plasminogen system plays an important role in proteolytic processes within the dynamic ovary. A non-physiologic elevation in PAI-1 may thus contribute systemically to increased cardiovascular risk, and may contribute locally to abnormal ovarian phenotype and function. Recently, we systematically characterized the phenotypic alterations in ovaries and the plasma testosterone levels of transgenic mice that constitutively express a stable form of human PAI-1 as compared with those of WT littermates (41). More than half of the ovaries from transgenic mice were found to contain large cystic structures, whereas similar cysts are seen in less than 6% of ovaries from WT controls. Plasma testosterone was nearly twofold greater in transgenic female mice than in WT females. Augmented ovarian PAI-1 expression appears to predispose female mice to the development of this abnormal ovarian architecture, which in turn is associated with an increase in plasma testosterone. Interestingly, ovarian tissue from patients with PCOS exhibits intense staining for PAI-1 around the borders of cysts, whereas little PAI-1 is detected in normal ovarian tissue. On the basis of these findings, we propose that dysregulated and augmented gonadal PAI-1 expression contributes to the development of polycystic ovaries. These findings may help to inform and motivate efforts to develop and test PAI-1 antagonists in the treatment of this increasingly common syndrome.

OTHER POTENTIAL APPLICATIONS OF PAI-1 ANTAGONISTS

In addition to spontaneous coronary thrombosis and polycystic ovaries, PAI-1-stab transgenic mice exhibit multiple phenotypic abnormalities, including alopecia, splenomegaly from extramedullary hema-

topoiesis, and hepatic enlargement from amyloid accumulation (42). A thickened epidermis, with impaired keratinization and pigment organization in hair, is present in PAI-1 transgenic mice. This suggests that the regulation of keratinocyte growth and differentiation is influenced by local expression of PAI-1-stab in the hair follicles of these mice. This confirms and extends the original observations of delayed hair growth and abnormal epidermal morphology in transgenic mice overexpressing WT human PAI-1 under the control of the metallothionein promoter (43). The mechanisms responsible for splenomegaly and extramedullary hematopoiesis in PAI-1-stab mice are more enigmatic. Plasminogen activator (PA) activity typically exceeds that of PAI-1 in the bone marrow, producing a robust fibri-nolytic environment, in contrast with the normal preponderance of PAI-1 activity in plasma (44). Proteases have recently been recognized as playing important roles in the recruitment of stem and progenitor cells (44). Interestingly, the proteolytic release of soluble kit ligand (sKitL) is impaired in mice deficient in matrix metalloproteinase-9 (MMP-9) a condition associated with the failure of hematopoietic stem cells (HSC) to translocate from their niche into the vascular zone of bone, where cells enter the circulation (45). Because plasmin is an activator of MMP-9, these findings anticipate a role for the PA system in the proliferation, maturation, and migration of HSC in the marrow (46). In fact, we detected abundant levels of PAI-1-stab expression in bone-marrow cells, which corresponded with a 64% decrease in the plasma levels of sKitL as compared with those of WT mice. These findings suggest that PAI-1-stab mice may have reduced MMP-9 activation, and therefore impaired migration of HSC to the vascular zone of bone. We therefore hypothesize that chronic overexpression of PAI-1 affects cell proliferation and migration, and promotes a functional isolation of the bone-marrow elements, from the circulation, a phenomenon we have termed “marrow exit block”, leading to a compensatory need for the establishment of sites of extramedullary hematopoiesis in the spleen. These findings certainly suggest that PAI-1 can influence the mobilization of hematopoietic precursors, and support the suggestion that specific PAI-1 antagonists deserve consideration for studies in the treatment of bone-marrow disorders associated with splenomegaly and marrow pathology, such as myelofibrosis.

The amyloid accumulation in multiple tissues of PAI-1-stab mice is perhaps more predictable. Amyloid beta (Aβ) peptides have been shown to induce elevated tissue plasminogen activator (t-PA) and urokinase-type plasminogen activatus (u-PA) levels in brain tissue *in vivo* and in astrocytes *in vitro* (47). Accumulation of the amyloid-beta

(Aβ) peptide depends on both its generation and its clearance. The time-dependent and consistent accumulation of amyloid aggregates observed in multiple tissues in PAI-1-stab mice is likely to reflect impairment of PA and/or another protease system by chronic overexpression of active PAI-1. Recently, it has been reported that PAI-1 antagonists can augment the activity of t-PA and plasmin in the hippocampus, can significantly reduce plasma and brain levels of Aβ, can restore long-term potentiation deficits in hippocampal slices from transgenic Aβ-producing mice, and can reverse cognitive deficits in these mice (48).

THERAPEUTIC OPTIONS: CURRENT LIMITATIONS AND FUTURE DEVELOPMENTS

At present, thrombolytic agents represent the only direct way of augmenting fibrinolytic activity in humans. Although these agents are proven to be efficacious in the treatment of acute thrombotic events, they are not a viable option for long-term administration. There are numerous agents available that indirectly but nevertheless effectively reduce plasma PAI-1 levels, including ACE inhibitors, insulin-sensitizing agents (including metformin and thiadolazinediones), and hormone-replacement therapy in women. ACE inhibitors are the only agents shown to augment endogenous t-PA release. Efforts are underway to develop and test synthetic, orally active PAI-1 antagonists. While the first such agent, tiplaxtinin, has been shown to prevent venous thrombosis, Ang II-induced arteriosclerosis, and obesity in experimental models, clinical studies of its use in humans have not yet been reported.

Although the potential applications of PAI-1 antagonists should be apparent, some cautionary notes deserve consideration. While the common clinical situation involves PAI-1 excess, humans with complete PAI-1 deficiency have been identified (49). Clinical manifestations of PAI-1 deficiency are generally restricted to abnormal bleeding, which is typically observed after trauma or surgery in individuals homozygous for this deficiency. A spectrum of bleeding patterns has been reported, including intracranial and joint bleeding after mild trauma, delayed surgical bleeding, severe menstrual bleeding, and frequent bruising. Therefore, the goal of treatment with PAI-1 antagonists should be constrained to reducing PAI-1 excess rather than to completely eradicating PAI-1 activity. This cautionary note extends to the potentially undesirable effects of complete PAI-1 deficiency in atherosclerosis. Although PAI-1 deficient mice have never been re-

ported to develop atherosclerotic lesions, there are reports that Apo E-deficient mice develop more extensive atherosclerotic lesions when crossed into a PAI-1 deficient background (50). The effects of lifelong PAI-1 deficiency on the development of atherosclerosis in humans have not been investigated. The availability of specific PAI-1 antagonists promises to expand the limits of understanding the role of the fibrinolytic system in human disease and to break through the current confines of therapeutic options that can effectively restore and augment the activity of the fibrinolytic system. However, at this time, PAI-1 remains a low-hanging fruit on the tree of therapeutic opportunity and has yet to be picked.

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DISCUSSION

August, New York: Thank you, Doug. That was a great talk. Two questions. One is in your transgenic animals with overexpression of PAI-1. What did their kidneys look like?

Vaughan, Chicago: The kidneys look relatively normal. There is some mild evidence of glomerular sclerosis, but it's really not very striking, and we've never made a big deal out of it. It's kind of surprising, but I think one of the phenotypic abnormalities that we see is related not just to the overexpression of PAI-1, but to where it gets expressed. With the promoter that we use, we're not expressing much in the kidney.

August, New York: I guess my second question has to do with serum levels in human beings. For example, how much variation is there? How do they range in different disease states? Do you have data on that?

Vaughan, Chicago: Plasma levels of PAI-1 are highly variable. There is a marked circadian variation. The PAI-1 gene is the prototypical diurnal-response gene. It's driven by the molecular clock, and in humans, PAI-1 levels are high in the morning and are low late in the afternoon, so that circadian variation in PAI-1 tends to synchronize with times of day that are associated with increased risk of myocardial infarction. We've measured PAI-1 levels in thousands of people over the past several years, and we've seen levels as low as zero and levels greater than 200 ng/mL. So the variability in the human population is enormous, and it confounds measuring in clinical groups, because you've got to do it at a specific time of day and be very consistent with that.

Rounds, Providence: Very interesting. I am wondering if PAI-1 has an effect on autophagy, seeing the amyloid deposition in the liver. Also, there is a congenital form of pulmonary fibrosis that's probably related to accumulation of a protein that's toxic to epithelial cells. So an effect of a PAI-1 antagonist on that would be very interesting as well.

Vaughan, Chicago: We've never specifically looked at the role of PAI-1 and autophagy. Most of its proteolytic inhibition is extracellular. There is a PAI-2 that is a cousin of PAI-1 and that's similar to it; very structurally similar, very functionally similar, but it's more intracellular. It could play a role in that. The congenital form of pulmonary fibrosis is quite interesting, and could be a reasonable target for a PAI-1 antagonist. As I mentioned, genetic PAI-1 deficiency and PAI-1 antagonists have been shown to reduce the development of fibrotic changes in the lungs of mice treated with bleomycin.

Moore, New York: In our patients getting chemotherapy, their most dreaded complication is hair loss. Have you treated any of your mice with cyclophosphamide or adriamycin?

Vaughan, Chicago: Clearly the plasminogen activator system plays a role in hair-follicle cycling and growth. I don't know if PAI-1 plays a direct role in hair loss associated with the administration of chemotherapy. As I showed a few moments ago, PAI-1 antagonists can promote the growth of hair in PAI-1-overexpressing mice. We will soon test this in other models of alopecia.