Pivotal Role of PAI-1 in a Murine Model of Hepatic Vein Thrombosis


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Keywords : PAI-1, venoocclusive disease, NO, hepatic thrombosis

Abbreviations: Plasminogen activator inhibitor-1 (PAI-1), venous occlusive disease (VOD), Nitric oxide (NO), N(omega)-nitro- L -arginine methyl ester (L –NAME).

Two of the authors (H.E. and D.L.C.) are employed by a company whose product was studied in the present work.
Abstract

Hepatic veno-occlusive disease (VOD) is a common complication of high-dose chemotherapy associated with bone marrow transplantation. While the pathogenesis of VOD is uncertain, plasminogen activator inhibitor-1 (PAI-1) has emerged as a diagnostic marker and predictor of VOD in humans. In this study, we investigated the role of PAI-1 in a murine model of VOD produced by long-term nitric oxide synthase inhibition using L-NAME. After 6 weeks, wild-type (WT) mice developed extensive fibrinoid hepatic venous thrombi and biochemical evidence of hepatic injury and dysfunction. In contrast, PAI-1 deficient mice were largely protected from the development of hepatic vein thrombosis. Furthermore, WT mice that received tiplaxtinin, an antagonist of PAI-1, were effectively protected from L-NAME-induced thrombosis. Taken together, these data indicate that NO and PAI-1 play pivotal and antagonistic roles in hepatic vein thrombosis, and that PAI-1 is a potential target in the prevention and treatment of VOD in humans.
Introduction

Hepatic veno-occlusive disease (VOD) is a common complication of high-dose chemotherapy associated with bone marrow transplantation. It is characterized clinically by hyperbilirubinemia, hepatomegaly and fluid retention. Histological features of VOD include fibrous occlusion of terminal hepatic venous lumen, dilatation and ultimately fibrosis of hepatic sinusoids and necrosis of zone 3 hepatocytes. VOD develops in 10-60% of patients undergoing allogenic transplantation, and severe VOD is associated with a mortality rate that approaches 100%. VOD has been treated using thrombolytics, such as tissue-type plasminogen activator, and with antithrombotic agents such as the polydeoxyribonucleotide defibrotide, with some success. However, the optimal treatment of VOD would theoretically employ agents that address the cause as well as the consequences of the disorder.

Several studies have provided evidence that injury to hepatic sinusoidal endothelial cells by chemotherapeutic agents is the initiating event in the pathogenesis of VOD. In cell culture, isolated sinusoidal endothelial cells were more susceptible to injury than hepatocytes when incubated with dacarbazine, an agent associated with the development of VOD. Recently, it was reported that decreased nitric oxide (NO) production contributed to the development of VOD. NO is the enzymatic end product of nitric oxide synthase (NOS), and plays a diverse role in regulating many physiological systems. In the liver, NO maintains the hepatic microcirculation and endothelial integrity.

Aside from the well-defined roles that endothelial NO plays in regulating vascular tone and structure, NO suppresses plasminogen activator inhibitor-1 (PAI-1)
production\textsuperscript{12}. PAI-1 serves as the primary physiological inhibitor of plasminogen activation and plays a critical role in regulating endogenous fibrinolytic activity\textsuperscript{13} and resistance to thrombolysis\textsuperscript{14}. In tissue, PAI-1 influences the response to injury by impairing cellular migration\textsuperscript{15} and matrix degradation\textsuperscript{16}. There is substantial evidence that PAI-1 may contribute to the development of thrombosis and fibrosis after chemical\textsuperscript{17} or ionizing injury\textsuperscript{18}. We reported that PAI-1 deficiency effectively prevents the development of arteriosclerosis and hypertension in mice treated with the nitric oxide synthase inhibitor L-NAME\textsuperscript{19}. Conversely, we have shown the transgenic mice that express a stable form of human PAI-1 develop spontaneous coronary arterial thrombosis\textsuperscript{20}. While 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\textsuperscript{21}. Taken together, these data suggest that increased PAI-1 may contribute to the pathophysiology of VOD. To test this hypothesis, we developed a murine model of hepatic vein thrombosis that involved administration of L-NAME to mice for six weeks. We investigated the role of PAI-1 in this model by characterizing the effects of L-NAME in wild-type (WT) and PAI-1\textsuperscript{-/--} mice, and by administering a novel, orally active PAI-1 antagonist, tiplaxtinin (PAI-039)\textsuperscript{22}, plus L-NAME to WT mice.
Materials and Methods

Animals  PAI-1<sup>−/−</sup> mice<sup>23</sup>, and WT mice on the same genetic background (C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, ME). Six male animals were studied in each of three experimental groups. L-NAME (Sigma, St. Louis MO) is a nonselective reversible inhibitor of nitric oxide synthase<sup>24</sup>, and was administered as described<sup>19</sup>. All control and untreated animals were fed a regular unmodified chow diet. Tiplaxtinin (PAI-039) was administered by mixing it into regular chow (1.0 mg/g), and administered in addition to L-NAME ad libitum. This dose has been previously shown to produce steady-state plasma levels of tiplaxtinin nearly equivalent to the in vitro IC<sub>50</sub> against PAI-1<sup>25</sup>. Systolic blood pressure was serially determined as described<sup>19</sup>.

Histopathology

Six weeks after the initiation of L-NAME treatment, animals were euthanized for gross and microscopic hepatic analyses. After extensive saline perfusion, livers were harvested, formalin fixed and embedded in paraffin blocks. Hepatic sections were stained with Masson’s trichrome, and hematoxylin and eosin stains, and photographed under 20x-80x magnification using an Olympus BX40 microscope with an Optronics Magnafire digital camera. Digital image analysis of each photomicrograph was performed with ImagePro Plus (MediaCybernetics, Silver Spring MD). The extent of hepatic venous thrombosis was determined by calculating the vascular luminal area obstructed by thrombi divided by the total vascular area in any given 20x field. For each liver, the obstructed and total vascular areas were calculated from five random 20x fields. In total, 240 individual veins were analyzed in each of the treatment groups. Sections were examined and characterized by a single blinded investigator.
**Clinical Chemistry**

Blood samples were taken by retro-orbital bleeding at week 0 and upon euthanasia. Samples were anticoagulated using acidified 3.8% sodium citrate. AST and bilirubin tests were performed at the Vanderbilt Clinical Diagnostics Laboratory (Nashville, TN) per clinical protocols. Plasma PAI-1 activity was measured using a functional ELISA assay\(^{26}\), that identifies only the active protein (Molecular Innovations, Southfield, MI).

**Statistical Analysis**

Data was analyzed by ANOVA and was performed by using SPSS 11.0 (SPSS Inc. Chicago, IL). When ANOVA indicated a statistically significant difference between treatment groups, Scheffe’s multiple comparison procedure was then used to determine which pairs of treatment groups were significantly different. Data are reported as the mean±standard error of the mean (SEM).

**Results and Discussion:**

*NOS inhibition by L-NAME induces hepatic venous thrombosis in WT mice.*

At baseline, there were no significant differences in systolic blood pressure between groups. After 6 weeks, systolic blood pressure was significantly higher in L-NAME–treated WT mice, compared to L-NAME–treated PAI-1\(^{-/-}\) mice (140.7±5.0 in WT vs. 121.4±7.3 in PAI-1\(^{-/-}\) mmHg; p<0.001). This observation is consistent with our previous studies and is attributable to the formation of perivascular fibrosis in WT mice receiving L-NAME. At the time of euthanasia, livers from WT+L-NAME mice exhibited a significant number of hepatic and portal veins occluded by fibrin thrombi compared to the PAI-1\(^{-/-}\)+ L-NAME mice (66.43±8.7% occluded area in WT vs. 18.36±5.6% occluded
area in PAI-1\(^{-/-}\); p<0.001). WT mice receiving L-NAME also exhibited other histologic changes associated with VOD including hepatocyte necrosis and fibrosis (data not shown). In contrast, these changes were not apparent in PAI-1\(^{-/-}\) mice receiving L-NAME (Figure 1g-i).

There were no significant differences in AST or bilirubin between the treatment groups at baseline. After 6 weeks of L-NAME treatment both AST (159.4±13.5 U/L in WT vs. 93.8±20.5 in PAI-1\(^{-/-}\); p=0.018) and bilirubin (1.56±0.5 mg/dl in WT vs. 0.20±0.05 mg/dl in PAI-1\(^{-/-}\); p<0.001) were increased in WT mice compared to PAI-1\(^{-/-}\) mice (Figure 2a). Elevated levels of AST and bilirubin are associated with VOD in humans and reflect injury to hepatocytes and obstruction of the liver. In this model, WT mice exhibit similar increases in serum total bilirubin and AST, while PAI-1\(^{-/-}\) mice do not suggesting that PAI-1\(^{-/-}\) deficiency is sufficient to protect against hepatic injury despite decreased NO. Furthermore, this observation suggests that PAI-1 plays an early role in the pathogenesis of VOD and may provide insight into the sequence by which endothelial damage leads to hepatic thrombosis. It is likely that increases in serum total bilirubin reflect the formation of obstructive hepatic and portal venous thrombi that result from damage to endothelial cells, while changes in AST occur subsequently and reflect the resulting hepatocyte injury.

Effects of PAI-1 inhibition by tiplaxtinin (PAI-039) in L-NAME-treated mice.

We have previously demonstrated that inhibition of PAI-1 by tiplaxtinin protects against Ang II-induced aortic remodeling\(^{25}\). This compound inhibits PAI-1 by binding directly to the protein and inhibiting its activity\(^{22}\). As shown in figure 1, L-NAME induced numerous and extensive hepatic thrombi in WT mice. Consistent with the data observed
in PAI-1−/− mice, tiplaxtinin, a small molecule inhibitor of PAI-1, significantly attenuated the number and extent of L-NAME induced venous thrombi in WT mice (41.55±3.6 vs. 66.43±8.7% occluded area; p<0.05) (Figure 1j). As expected, plasma PAI-1 activity was decreased in WT mice receiving the tiplaxtinin+L-NAME compared to those mice that received L-NAME alone (17.68±1.6 vs. 36.05±6.34 ng/ml; p=0.011. Figure 2b), and had no effect on L-NAME induced increases in systolic blood pressure (136.6±11.7 vs. 140.8±11.74 mmHg, p>0.05). The consistency of the findings that both pharmacological inhibition and genetic deletion of PAI-1 reduce the extent and severity of hepatic venous thrombi confirms that PAI-1 is directly involved in the molecular pathogenesis the disease.

In summary, this study provides direct evidence that PAI-1 is more than a biochemical marker of VOD. Indeed, these results establish that PAI-1 is essential to the pathogenesis of hepatic venous occlusive disease. Since both genetic deficiency and pharmacological inhibition of PAI-1 provided protection against hepatic thrombosis, this study also provides proof of concept for the strategy of developing pharmacological antagonists of PAI-1 for the treatment of VOD. Importantly, while other chemical classes of PAI-1 inhibitors have been reported that include both direct-acting small-molecule inhibitors and antibodies16,22; none has shown the oral activity and efficacy of tiplaxtinin, or has been profiled in a model of this disease. The present findings also suggest that PAI-1 is a rational and druggable target for the prevention and treatment of VOD in humans.
References:

Figure legends:

Figure 1: Masson’s trichrome stains of representative livers from WT+L-NAME (A-C), WT + L-NAME + tiplaxtinin (PAI-039) (D-F) and PAI-1-/-+L-NAME (G-I) mice after 6 weeks of L-NAME treatment at the indicated magnifications. Arrows illustrate the extent of venous thrombi in WT mice. (J) Calculated percent occluded luminal area in all three treatment groups (p<0.001 for WT vs. PAI-1 KO, and p<0.001 for WT vs. WT+tiplaxtinin by ANOVA).

Figure 2: (A) Total serum bilirubin and AST levels are elevated in WT mice receiving L-NAME, but not in PAI-1-/- mice or in WT mice receiving tiplaxtinin (PAI-039). (B) Tiplaxtinin (PAI-039) reduced plasma PAI-1 activity in WT mice receiving L-NAME.
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A

- Total Bilirubin (mg/dl)
- AST (U/L)

PAX1 KO + L-NAME
WT + L-NAME + Tiplaxtinin
WT + L-NAME

B

- Active PAI-1 (ng/mL)

WT + L-NAME + Tiplaxtinin
WT + L-NAME
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