

Donor PAI-1 Expression Inhibits the Intimal Response of Early Allograft Vascular Disease

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Background: The development of allograft vascular disease (AVD) may be related to altered expression of the fibrinolytic system. We determined the extent to which plasminogen activator inhibitor type 1 (PAI-1) expression in donor tissue influences intimal proliferation (IP) in a mouse model of AVD.

Methods: We utilized an end-to-end abdominal aortic transplant model in mice to investigate the development of IP in 3 groups of 6 recipients. Group A (negative control) utilized C57BL/6J strain mice as both donors and recipients. In Groups B (positive control) and C, C57BL/6J mice were vessel donors and CBA/J mice were recipients. Both groups received intraperitoneal anti-CD4 and anti-CD8 monoclonal antibodies (250 $\mu\text{g}/\text{week}$ for 5 weeks). Group C recipients, however, were transplanted with vessels from C57BL/6J PAI-1 knockout mice. Animals were killed at 50 days. Transplanted aortas were removed and intimal areas calculated using morphometric analysis.

Results: Group A (mean intimal area $6,421 \pm 8,507 \mu\text{m}^2$) demonstrated very little IP in comparison to the other groups. IP was significantly higher in Group B (mean intimal area $56,357 \pm 35,629 \mu\text{m}^2$) than Group A ($p = 0.008$). Group C (mean intimal area $281,995 \pm 123,279 \mu\text{m}^2$) demonstrated significantly more intimal proliferation than either Groups A or B (vs B, $p = 0.003$; vs A, $p < 0.001$). The significance of these results is maintained if intimal thickness is measured as a stand-alone reference for the intimal response.

Conclusions: Lack of PAI-1 expression in donor tissue greatly exaggerates the extent of IP after allogeneic transplantation and suggests that PAI-1 is important in limiting the early phase of AVD. *J Heart Lung Transplant* 2003;22:515–518.

Impaired fibrinolytic activity clearly influences the development of allograft vascular disease (AVD).^{1–5} Despite this, the regulation of this system after transplantation and the relative contribution of individual fibrinolytic proteins to AVD are poorly characterized. As in native coronary artery disease (CAD), fibrin and

thrombus deposition in the microcirculation and the proliferative and migratory phase of the neo-intimal development are directly influenced by the relative state of the fibrinolytic system.^{6–11} Cellular components of the vascular wall, in particular endothelial cells (ECs) and smooth muscle cells (SMCs), are the

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major sites of synthesis of fibrinolytic proteins, tissue plasminogen activator (tPA), urokinase (uPA) and PAI-1. Our current level of knowledge of this system's relationship to AVD stems from in vivo determinations of plasma samples and by in situ analysis of tissue samples taken from diseased allografts.¹⁻⁵ Only a few animal studies exist that have isolated the fibrinolytic system to determine its role in the development of AVD. In the present study, we demonstrate the relative importance of PAI-1 in the early development of AVD by utilizing specific fibrinolytic protein knockout mice as donors in a well-established animal model of AVD.

METHODS

Animals

Male mice were bred and maintained in the animal resources facility at the University of Alabama at Birmingham at 25°C with 12-hour light/dark cycles. The American Association for Accreditation of Laboratory Animal Care accredits our facility. All procedures were performed by the investigators in accordance with *The Guide for the Care and Use of Laboratory Animals*, published by the National Institute of Laboratory Animal Resources and the National Institute of Health (NIH Publication No. 85-23, revised 1985).

Transplantation Procedure

We utilized C57BL/6J mice as donors and CBA/J mice as recipients. This strain combination reproducibly produces vascular disease consistent with chronic rejection in other mouse models of transplantation.^{12,13} All mice were 12 to 16 weeks of age. We performed the aortic transplantation technique, as previously described.¹³⁻¹⁵ Briefly, donor and recipient mice were anesthetized with an intraperitoneal injection of 0.12 to 0.16 ml per 20 g of ketamine/xylazine (ketamine [100 mg/ml] 1.74 ml and xylazine [100 mg/ml] 0.26 ml, in 8.52 ml of phosphate-buffered saline). A section of donor thoracic aorta was isolated and dissected, flushed and placed in chilled phosphate buffered saline (PBS). The infra-renal recipient aorta was transected between 2 vascular clamps (B-1 Clamp, S&T Co, Switzerland), followed by end-to-end anastomosis of the donor aorta to the recipient's abdominal aorta using a 10-0 nylon suture (Accurate Corp, Westbury, NY) via an interrupted technique. A single surgeon using an operating microscope with 20× magnification (Nikon Stereo SMZ800) performed the operations.

Experimental Groups

Three groups of mice were analyzed at 50 days post-transplant. Each group contained 6 mice.

Group A consisted of negative intimal proliferation controls and utilized the C57BL/6J strain as both recipients and donors; Group B consisted of positive intimal proliferation controls and utilized the CBA strain as recipients and the C57BL/6 strain as donors; Group C was the experimental group and utilized the CBA strain as recipients and C57BL/6 PAI-1 knockouts as donors. Animals that died perioperatively were eliminated from the study and replaced. Immunosuppression consisted of weekly anti-CD4 and anti-CD8 monoclonal antibodies (250 µg) administered intraperitoneally for 5 weeks.¹³

Morphometric Analysis

Vessels from control and experimental groups were perfusion-fixed in 10% buffered formalin. Vessels were then embedded in paraffin and serially sectioned for morphometric analysis. Tissue was stained with hematoxylin and eosin (H&E) and elastic van Gieson. Morphometric analysis of each arterial ring segment was performed with a computer-based Bioquant II morphometric system. Intimal area (IA) was calculated by subtracting lumen area from the area encircled by the internal elastic lamina (IEL). The areas were calculated from the internal diameter (ID) and the lumen diameter (LD) using the formula: $A = \pi r^2$. Three sections of each vessel were examined and measurements averaged for statistical analysis.

Statistical Analysis

Numeric data are expressed as mean ± SD. Statistical analysis was performed using the STATGRAPHICS statistical program (Statistical Graphics Corp, Rockville, MD) and *t*-tests. The differences were considered statistically significant at $p < 0.05$. Data on IA and intimal thickness (IT) are presented.

RESULTS

Representative sections from each of the 3 groups of mice (defined in Methods) are shown in Figure 1. Group A (mean IA $6,421 \pm 8,507 \mu\text{m}^2$, mean IT $4.3 \pm 5.8 \mu\text{m}$) demonstrated very little intimal proliferation in comparison to the other groups. Intimal proliferation was significantly higher in Group B (mean IA $56,357 \pm 35,629 \mu\text{m}^2$, mean IT $72 \pm 69 \mu\text{m}$) than Group A (IA $p = 0.008$, IT $p < 0.01$). Group C (mean IA $281,995 \pm 123,279 \mu\text{m}^2$, mean IT $151 \pm 52 \mu\text{m}$) demonstrated significantly more intimal proliferation than either Groups A or B (IA: vs B, $p = 0.003$; vs A, $p < 0.001$; IT: vs B or A, $p < 0.01$). The differences in IA are noted in Figure 2. Therefore, the lack of PAI-1 expression in donor tissue greatly exaggerates the extent of intimal proliferation after allogeneic trans-

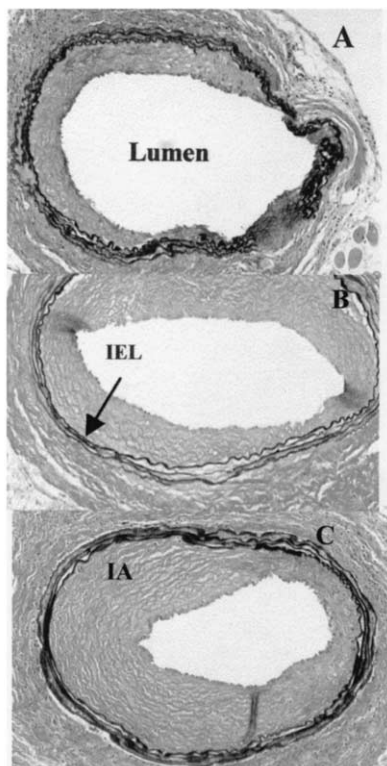


FIGURE 1 Representative sections from each group of mice. (A) Group A. (B) Group B. (C) Group C.

plantation and suggests that PAI-1 is important in limiting this early form of AVD.

DISCUSSION

The intima remains the most affected portion of the vessel wall in AVD. Intimal thickening confined to proximal/mid-regions of the epicardial coronaries is the most common morphologic feature seen at <1 year after transplantation and is due to an increase in extracellular matrix as well as the proliferation and migration of SMCs.^{16–20} Impaired fibrinolytic activity

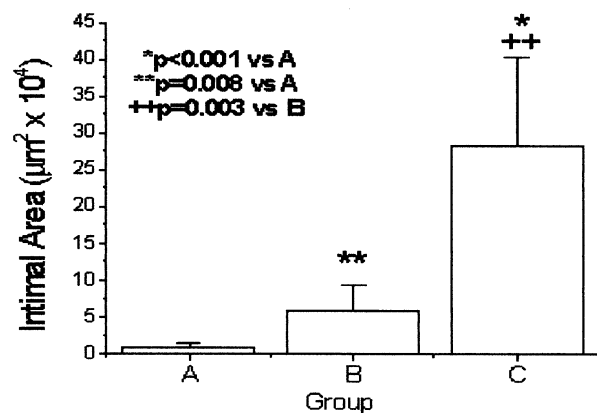


FIGURE 2 Differences in intimal area between groups.

may be integral to the development of this pathogenic intima.

Impaired fibrinolysis has an important role in the pathogenesis of native and transplant atherosclerosis.^{1–5,21–23} Transplant patients with AVD have significantly higher mean circulating tPA and PAI-1 antigen levels than patients without AVD.^{4,5} Similarly, severity of AVD correlates positively with increased levels of PAI-1 and tPA antigen.^{4,5} Labarrere et al. demonstrated in biopsies from cardiac transplant recipients that depleted arteriolar SMC tPA correlated with graft failure and AVD.³ In addition, certain genetic variants of the PAI-1 gene carried in the donor tissue may influence the early development of this disease.²⁴ Therefore, perturbations in the balance of this system that occurs after transplantation may promote AVD.

As alluded to earlier, abnormal fibrinolytic activity may lead to the development of an abnormally thick neo-intima after transplantation. In this regard, vascular injury, secondary to the transplant procedure, results in the activation of this system and increased plasmin activity, a process similar to that which occurs after percutaneous transluminal coronary angioplasty (PTCA).^{16,25} This heightened activity is localized to both the lumen and wall of the injured vessels and involves a complicated interplay between tPA, uPA and PAI-1. Each of these components may then influence the extent of vascular remodeling after this initial injury via their actions on metalloproteinase or plasminogen activation (plasmin generation).

Plasmin plays a pivotal role in normal and pathogenic cellular functions related to CAD, including matrix remodeling and cellular migration of SMCs,^{22,26} early fibrin deposition and initiation of atherogenesis.^{8,22} Plasminogen knockout mice²⁷ exhibit decreased migration of SMCs after injury to vessels that may protect against vasculopathy. PAI-1 is a key regulator of plasmin generation induced by either tPA or uPA. Increased PAI-1 is associated with CAD risk factors^{28,29} as well as atherosclerotic plaque regions and vessels.^{23,30} Paradoxically, PAI-1 can also inhibit the mitogenic effect of tPA on SMCs⁹ and deficiencies of this protein can accelerate the rate of SMC migration and neo-intima formation after injury.⁶ Thus, the degree of neo-intima formation appears regulated by changes in the rate of SMC migration, which may be influenced by PAI-1's action on plasmin proteolysis. This mechanism is further supported by observations that the neo-intimal response to arterial injury in PAI-1-deficient mice is mitigated primarily through an effect on SMC migration.^{8,13} Thus, these observations strongly support the following 2 notions. First, PAI-1 plays an early inhibitory role in arterial

neo-intima formation after arterial injury. Second, this inhibition is likely related to PAI-1's affect on altering the rate of SMC migration. Our work and the work of others support the hypothesis that factors that affect the dynamic balance of the fibrinolytic system, particularly PAI-1, may contribute significantly to the intimal proliferative response after vascular injury. The present study, however, suggests that PAI-1 may also have a regulatory role in limiting the intimal response early after transplantation. Future studies will expand on these initial observations and define the time course of change of PAI-1 and other fibrinolytic components, both mRNA and protein, in this and other knockout systems. We will make use of various immunohistologic and molecular techniques to define these relationships and determine how each component influences the other and intimal development after transplantation.

REFERENCES

- Labarrere CA, Pitts D, Halbrook H, Faulk WP. Natural anti-coagulant pathways in normal and transplanted human hearts. *J Heart Lung Transplant* 1992;11:342-7.
- Labarrere CA, Nelson DR, Faulk WP. Myocardial fibrin deposits in the first month after transplantation predict subsequent coronary artery disease and graft failure in cardiac allograft recipients. *Am J Med* 1998;105:207-13.
- Labarrere CA, Pitts D, Nelson DR, Faulk WP. Vascular tissue plasminogen activator and the development of coronary artery disease in heart-transplantation recipients. *N Engl J Med* 1995;333:1111-6.
- Hunt BJ, Segal H, Yacoub M. Hemostatic changes in heart transplant recipients and their relationship to accelerated coronary sclerosis. *Transplantation* 1993;55:309-15.
- Warshofsky MK, Wasserman HS, Wang W, et al. Plasma levels of tissue plasminogen activator and plasminogen activator inhibitor-1 are correlated with the presence of transplant coronary artery disease in cardiac transplant recipients. *Am J Cardiol* 1997;80:145-9.
- Carmeliet P, Collen D. Role of plasminogen/plasmin system in thrombosis, hemostasis, restenosis and atherosclerosis: evaluation in transgenic animals. *Trends Cardiovasc Med* 1995;5:117-22.
- Reidy MA, Irvin C, Lindner V. Migration of arterial wall cells: expression of plasminogen activators and inhibitors in injured rat arteries. *Circ Res* 1996;78:405-14.
- Carmeliet P, Moons L, Ploplis VA, Plow E, Collen D. Impaired arterial neointima formation in mice with disruption of the plasminogen gene. *J Clin Invest* 1997;99:200-8.
- Xiaoli M, Wenying H, Mingpeng S. Effects of mechanism of tissue-type plasminogen activator and plasminogen activator inhibitor on vascular smooth muscle cell proliferation. *Int J Cardiol* 1998;66(suppl):S57-64.
- Carmeliet P, Moons L, Lijnen R, et al. Inhibitor role of plasminogen activator inhibitor-1 in arterial wound healing and neointima formation: a gene targeting and gene transfer study in mice. *Circulation* 1997;96:3180-91.
- Jackson CL, Reidy MA. The role of plasminogen activation in smooth muscle cell migration after arterial injury. *Ann NY Acad Sci* 1992;667:141-50.
- Chow LH, Huh S, Jiang J, Zhong R, Pickering JG. Intimal thickening develops without humoral immunity in a mouse aortic allograft model of chronic vascular rejection. *Circulation* 1996;94:3079-82.
- Koglin J, Glysing-Jensen T, Mudgett JS, Russell ME. Exacerbated transplant arteriosclerosis in inducible nitric oxide-deficient mice. *Circulation* 1998;97:2059-65.
- Koulack J, McAlister MB, Giacomantonio CA, Bitter-Suermann H, Lee TG. Development of a mouse aortic transplant model of chronic rejection. *Microsurgery* 1995;16:110-13.
- Mennander A, Tilsala S, Halttunen J, Yilmaz S, Paavonen T, Hayry P. Chronic rejection in rat aortic allografts: an experimental model for transplantation arteriosclerosis. *Arterioscler Thromb* 1991;11:671-80.
- Johnson DE, Gao S-Z, Schroeder JS, DeCampi WM, Billingham ME. The spectrum of coronary artery pathologic findings in human cardiac allografts. *J Heart Transplant* 1989;8:349-59.
- Bieber CP, Stinson EB, Shumway NE, Payne R, Kosek J. Cardiac transplantation in man. VII. Cardiac allograft pathology. *Circulation* 1970;61:753-72.
- Billingham ME. Cardiac transplant atherosclerosis. *Transplant Proc* 1987;19:19-25.
- Billingham ME. Pathology of graft vascular disease after heart and heart-lung transplantation and its relationship to obliterative bronchiolitis. *Transplant Proc* 1995;27:2013-6.
- Uys CJ, Rose AG. Pathologic findings in long-term cardiac transplants. *Arch Pathol Lab Med* 1984;108:112-6.
- Hamsten A, Blomback M, Wiman B, et al. Haemostatic function in myocardial infarction. *Br Heart J* 1986;55:58-66.
- Schwartz CJ, Valente AJ, Kelley JL, Sprague EA, Edwards EH. Thrombosis and the development of atherosclerosis: Rokitansky revisited (review). *Semin Thromb Hematol* 1988;14:189-95.
- Padro T, Emeis JJ, Steins M, Schmid KW, Kienast J. Quantification of plasminogen activators and their inhibitors in the aortic vessel wall in relation to the presence and severity of atherosclerotic disease. *Arterioscler Thromb Vasc Biol* 1995;15:893-902.
- Benza RL, Grenett HE, Bourge RC, et al. Gene polymorphisms for plasminogen activator inhibitor-1/tissue plasminogen activator and development of allograft coronary artery disease. *Circulation* 1998;98:2248-54.
- Sawa H, Lundgren CH, Sobel BE, Fujii S. Increased intramural expression of plasminogen activator inhibitor type 1 after balloon injury: a potential progenitor of restenosis. *J Am Coll Cardiol* 1994;24:1742-8.
- Smith EB, Thompson WD. Fibrin as a factor in atherogenesis. *Thromb Res* 1994;73:1-19.
- Juhan-Vague I, Alessi MC. Plasminogen activator inhibitor 1 and atherothrombosis. *Thromb Haemost* 1993;70:138-43.
- Meade TW, Chakrabarti R, Haines AP, North WF, Stirling Y. Characteristics affecting fibrinolytic activity and plasma fibrinogen concentrations. *BMJ* 1979;1:153-6.
- Andersen P, Arnesen H, Hjermmann I. Hyperlipoproteinaemia and reduced fibrinolytic activity in healthy coronary high-risk men. *Acta Med Scand* 1981;209:199-202.
- Lupu F, Bergonzelli GE, Heim D, et al. Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. *Arterioscler Thromb* 1993;13:1090-100.