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The role of fibrinolytic genes and proteins in the development of allograft vascular disease

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BACKGROUND: We have previously shown that lack of plasminogen activator inhibitor-1 (PAI-1) expression in donor tissue greatly increases intimal proliferation (IP) after allogeneic transplantation. We sought to determine the relative role of PAI-1 and other fibrinolytic proteins in the development of IP.

METHODS: We used an abdominal aortic transplant model in mice to investigate IP in 3 groups of 6 recipients. In the isograft group, CBA/J strain mice were donors and recipients, donors for allograft group were C57BL/6J mice, and for the allograft/knockout group, C57BL/6J PAI-1 knockout mice. All groups received weekly injections of anti-CD8/CD4 monoclonal antibodies. IP was calculated at 50 days, and sections were analyzed for fibrinolytic proteins, messenger RNA (mRNA) and PAI-1 activity using immunohistochemistry (IHC), in situ hybridization (ISH), reverse transcription-polymerase chain reaction (RT-PCR), and Western blot analysis.

RESULTS: Significantly more IP developed in the allograft/knockout group vs the isograft ($p < 0.001$) and the allograft groups ($p = 0.003$). There was marked intimal expression of tissue plasminogen activator (tPA), urokinase PA (uPA), and uPA receptor (uPAR) proteins and mRNA in the allograft and allograft/knockout groups vs the isograft group. Allografts also showed significant intimal staining for PAI-1 protein and mRNA. RT-PCR demonstrated a stepwise increase in profibrinolytic protein mRNA from isograft to allograft to allograft/knockout groups, particularly uPA ($p = 0.02$) and uPAR ($p = 0.016$). Western blot data showed complementary findings. PAI-1 activity was persistently present in isograft and allograft animals, only. Intimas in allograft and allograft/knockout groups were primarily smooth muscle cells.

CONCLUSIONS: PAI-1 reduces IP by limiting smooth muscle cell activity, with little change in matrix composition likely by modulating profibrinolytic protein expression.

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The development of an accelerated form of transplant coronary artery disease (TxCAD) significantly limits the long-term success of cardiac transplantation (CTx). TxCAD involves a complicated interplay between immune and nonimmune factors, and a distinct phase of marked intimal proliferation (IP) is noted early in the disease that is pathognomic for TxCAD. Among the nonimmune factors underlying IP, alterations in fibrinolytic activity appear to be important.[1–10] Changes in fibrinolytic activity, either *impaired* (reduced) or *enhanced,* are well-established risk factors for native CAD and restenosis, respectively,[11–16] and it is thus reasonable to explore fibrinolytic pathways to determine their contribution to IP after CTx.

Enhanced fibrinolytic activity resulting from an excess of plasminogen activators (PA), including tissue PA (tPA) or urokinase PA (uPA), may be particularly important in the development of the early intimal response due to promotion

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of cellular infiltration, vascular cell migration, and matrix turnover.[11,12,14,15,17–22] The major source of these fibrinolytic proteins are more than likely the donor vascular cells, including endothelial and smooth muscle cells (SMCs), with a lesser contribution from infiltrating inflammatory cells.[20,23]

Alternatively or in concert with PA involvement in TxCAD, enhanced fibrinolytic activity may also result from a reduction of PA inhibitor (PAI-1). If so, PAI-1 would present as a novel therapeutic target for TxCAD. The primary goal of the described studies was therefore to determine in a murine model of transplantation, the degree of association between the alterations in the expression of donor PAI-1 and the extent of IP. These studies were based on the hypothesis that the development of pathologic IP, which constitutes the early phase of TxCAD, can be promoted by enhanced fibrinolysis and exacerbated by reduced expression of PAI-1 in the donor vasculature.

Materials and methods

Animals

Male mice were bred and maintained at 25°C with 12-hour light/dark cycles in the Animal Resources Facility at the University of Alabama at Birmingham, which is accredited by the American Association for Accreditation of Laboratory Animal Care. CBA/J, C57BL/6J, and C57BL/6J PAI-1 knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME) All procedures were performed 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 Institutes of Health (NIH Publication No. 85-23, revised 1985).

Transplantation procedure

Aortic transplantation was performed at age 12 to 16 weeks, as previously described.[3] Briefly, donor and recipient mice were anesthetized with a 0.12 to 0.16 ml/20 g intraperitoneal injection of ketamine/xylazine (ketamine [100 mg/ml], 1.74 ml; and xylazine [100 mg/ml], 0.26 ml in phosphate-buffered saline [PBS, 8.52 ml]). A section of donor thoracic aorta was isolated and dissected, flushed, and placed in chilled PBS. The infrarenal recipient aorta was transected between 2 vascular B-1 clamps (S&T Co, Dübendorf, Switzerland), followed by end-to-end anastomosis of the donor aorta to the recipient's abdominal aorta using 10-0 nylon suture (Accurate Corp, Westbury, NY) with an interrupted technique.[24] A single surgeon using a Nikon Stereo SMZ800 operating microscope at ×20 magnification (Nikon Instruments, Melville, NY) performed the operations.

Experimental groups

Three groups of mice ($n = 6$ per group) were analyzed at 50 days after transplantation. Positive controls consisted of wild-type CBA/J recipients and C57BL/6J donors (allografts), negative controls consisted of CBA/J recipients and CBA/J donors (isografts), and the experimental group consisted of CBA/J recipients and C57BL/6J PAI-1 knockout donors (allograft/knockout). Animals that died within 2 to 3 days after transplantation were eliminated from the study and replaced.

Immunosuppression consisted of weekly anti-CD4 (clone GK1.5) and anti-CD8 (clone 116-13.1 for CBA/J recipients, clone 2.43 for C57BL/6 recipients) monoclonal antibodies (250 μg) administered intraperitoneally.[3,25] Animals were humanely killed at Day 50 with vessels removed, as described below.

Morphometric analysis

Vessels from control and experimental groups were perfusion-fixed in 10% buffered formalin. Transplant vessel segments were embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin (H&E) and elastin van Gieson. Morphometric analysis of each arterial ring segment was performed with a computer-based Bioquant II Morphometric System (Bioquant Image Analysis Corp, Nashville, TN). Intimal area (IA) was calculated by subtracting lumen area from the area encircled by the internal elastic lamina (IEL). The areas are 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.

Quantitative real-time reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from the harvested donor aortas using RNAeasy Mini Kit (Qiagen, Valencia, CA). The total RNA (400 ng) was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) using Oligo dT as primer, according to the manufacturer's instructions. The reverse transcription-polymerase chain reaction (RT-PCR) was done on Applied Biosytems *Taqman* using SYBR Green Master Mix (Applied Biosytems, Foster City, CA) and specific oligonucleotides for murine mRNA:

PAI-1: forward 5′-TGC ATC GCC TGC CAT TG-3′ and reverse 5′-CCT GAG ATA GGA CAG TGC TTT TTC C-3′;

tPA: forward 5′-GGC CTG GCA CGA CAC AAT-3′ and reverse 5′-TCA TCA CAT GGC AAC AAG GT-3′;

uPA: forward 5′-CGA TTC TGG AGG ACC GCT TA-3′ and reverse 5′-CCA GCT CAC AAT CCC ACT CA-3′;

uPAR: forward 5′-GCT TTG AGA CAG GAT CTC ACC AT-3′) and reverse 5′-ACT CCT GGA GCC CGT CAG TA-3′; and

GAPDH: forward 5′-TGT GTC CGT CGT GGA TCT GA-3′ and reverse 5′-CCT GCT TCA CCA CCT TCT TGA-3.

Oligonucleotides were designed with the help of the Applied Biosystems Primer Express 2.0. The thermal cycling parameters were 2 minutes at 50°C, 10 minutes at 94°C, followed by 40 cycles of 20 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. The data were analyzed with the ABI 7700 software, and the PCR products were electrophoresed on 2% agarose gel with ethidium bromide to rule out primer dimer signals read by the software.

Fluorescent in situ hybridization and riboprobe synthesis

A full-length murine PAI-1 complementary DNA (cDNA), subcloned into the vector pBS+ (Stratagene/Agilent, Santa Clara, CA) was provided by Dr M. Cole (Princeton University). The full-length cDNA for murine tPA and murine uPAR in vector pCMVSport6 (Invitrogen) and the partial-length murine uPA in