Inhibition of Transplant Vasculopathy in a Rat Aortic Allograft Model After Infusion of Anti-Inflammatory Viral Serpin

Leslie W. Miller, MD; Erbin Dai, MD; Piers Nash, PhD; Liying Liu, MD; Carolyn Icton, BSc; Dennis Klironomos, BSc; Lilly Fan, BSc; Patric N. Nation, DVM; Robert Zhong, MD; Grant McFadden, PhD; Alexandra Lucas, MD

Background—Transplant vasculopathy remains a difficult therapeutic problem, resulting in the majority of late cardiac graft losses. This chronic vascular disease is thought to be triggered by alloantigen-dependent and alloantigen-independent inflammatory factors. Despite improved 1-year survival, the incidence of transplant vasculopathy has not improved with current immunosuppressive protocols. Highly effective strategies have evolved in the large DNA viruses that shield infecting viruses from host inflammatory responses. Serp-1 is a secreted myxoma virus anti-inflammatory serine proteinase inhibitor. Serp-1 inhibits plasminogen activators in a manner similar to plasminogen activator inhibitor (PAI-1), a vascular protein that plays a pivotal regulatory role in vascular wound healing. In this study, we tested the ability of purified Serp-1 protein to ameliorate posttransplant vasculopathy after rat aortic allograft surgery.

Methods and Results—Serp-1 protein or controls were infused into 98 rats immediately after segmental aortic allograft transplantation. After either late (28 days, 64 rats) or early (12 to 48 hours, 24 rats) follow-up, transplanted aortic segments were harvested for morphological and immunohistochemical analysis. Significant reductions in intimal plaque growth (P<0.002) and mononuclear cell invasion (P<0.033) were detected after Serp-1 infusion at nanogram doses. Serp-1 reduced early macrophage (P<0.0016) and nonspecific lymphocyte (P<0.0179) invasion into medial and adventitial layers and inhibited associated depletion of medial smooth muscle cells (P<0.0006).

Conclusions—Infusion of a viral anti-inflammatory serpin, Serp-1, significantly reduces early inflammatory responses and later luminal occlusion in a rat aortic allograft model. (Circulation. 2000;101:1598-1605.)

Key Words: rejection • transplantation • viruses • serpin • inflammation

Immunosuppressive therapy for acute rejection has improved early survival after heart transplantation,\(^1\) such that it is now an accepted therapeutic option for the treatment of end-stage heart failure.\(^2\) Currently available immunosuppressive agents, however, fail to prevent either acute refractory rejection or chronic rejection; factors that limit long-term survival.\(^1,2,3\) Chronic rejection is characterized by accelerated atherosclerotic plaque development, termed transplant vasculopathy,\(^1,4–6\) with diffuse occlusive narrowing of the coronary vessels. The incidence of intimal disease by angiography was recently reported to be 42% at 5 years,\(^1,2,7\) with a further 40% of normal angiograms having demonstrated intimal thickening by intravascular ultrasound.\(^8,9\)

Although the term chronic rejection is used to describe this vasculopathy, correlation with the severity of acute rejection episodes is variable\(^10,11\) and, in fact, patients with no biopsy evidence of rejection can develop progressive coronary disease. Traditional immunosuppressive protocols have been directed at inhibiting T-cell and B-cell clonal expansion and cytokine production,\(^3,4,10,12\) but these protocols do not prevent vasculopathy development. Both antigen-dependent and antigen-independent mechanisms thus contribute to the development of transplant-associated coronary disease.\(^2–4\)

Antigen-independent factors, including brain death,\(^13,14\) ischemia-reperfusion injury,\(^15,16\) hyperlipidemia,\(^17,18\) diabetes,\(^18\) elevated homocysteine,\(^19\) and viral infections\(^9,20\) activate nonspecific inflammatory responses that can initiate vascular damage.\(^4,21,22\) These responses are designed to heal the arterial wall under normal conditions.\(^23\) Both the thrombotic and inflammatory cascades have pivotal roles in the regulation of vascular wound healing responses,\(^24–29\) stimulating cellular migration, invasion, and proliferation.\(^24–26,29–31\)

Myxoma virus and other large DNA viruses have evolved successful strategies for survival in the presence of active host
inflammatory responses. Among these diverse strategies is a secreted myxoma viral 55-kDa serine proteinase inhibitor, a serpin known as Serp-1 that binds and inhibits tissue-type plasminogen and urokinase-type plasminogen activators and plasmin. We have previously demonstrated in rabbit models that purified Serp-1 profoundly reduces monocyte cell infiltration and subsequent atherosclerotic plaque growth after balloon injury and reduces joint inflammation in a model of collagen-induced arthritis, again implicating the thrombolytic proteinases in local tissue responses to injury. The antiatherosclerotic activity of Serp-1 was abrogated by mutation of the Serp-1 active site to an inactive sequence, indicating that this activity is that of a bona fide serpin. Our hypothesis is that the thrombolytic cascade, and more specifically regulatory serpins such as plasminogen activator inhibitor (PAI)-1, act as central mediators in early events leading to transplant vasculopathy. To test this hypothesis we used a viral serpin that inhibits thrombolytic proteinase enzymes.

Methods

Aortic Allograft Transplantation

Plaque growth and mononuclear cell invasion were examined in 2 rat aortic allograft models in a series of 3 studies. In study 1, Lewis (inbred, RT1/RT2a/RT3a) donor to Sprague-Dawley (outbred rats) recipient (L/SD) transplants were used as a first proof of principle. Although other researchers have used outbred SD rats in islet cell transplantation and human neuronal xenotransplantation models, there is potentially greater variability. Therefore in study 2, an aortic transplantation model with inbred ACI (RT1a/RT2b/RT3a) donor to Lewis recipient rats (A/L) was used. Rats were followed for 28 days after transplantation and then were euthanized for histological assessment. In study 3, the early effects (12 and 48 hours) of Serp-1 infusion on monocyte/macrophage, CD2-positive T lymphocytes, B lymphocytes, and smooth muscle cell invasion were examined in the L/SD model.

All surgical transplantation procedures were performed with the use of the same operative approach and general anesthetic: 0.1 mL/100 g pentobarbital (Somnotrol, MTC Pharmaceuticals) and 0.1 mg atropine by intraperitoneal injection. By use of sterile technique, the aorta was exposed below the renal arteries through an abdominal incision. A 2.0-cm section of Lewis (L/SD) or ACI rat infrarenal aorta (A/L) was removed. The recipient rats had identical abdominal incisions; a 1.0-cm-length aortic section was isolated by clips and excised, and half of each donor aortic section (1.0 cm) was transplanted end to end into the recipient aorta with the use of 10-0 nylon sutures (Surgical Specialties Corp).

Serp-1 or control saline was infused immediately after transplantation as a single dose given by injection into the tail (study 1) or penile vein (studies 2 and 3) after blood flow return was confirmed by visible aortic pulsation. After surgery, buprenorphine analgesic (0.02 mg/kg) was given by subcutaneous injection. For study 1, Serp-1 was infused at doses of 1 pg/g (6 rats), 10 pg/g (11 rats), 100 pg/g (5 rats), and 1000 pg/g (6 rats), or saline control was used (11 rats). In study 2, a wider range of Serp-1 doses was tested with infusion of Serp-1 at 0.01 pg/g (6 rats), 0.1 pg/g (6 rats), 1.0 pg/g (6 rats), 10 pg/g (6 rats), 100 pg/g (6 rats), 1000 pg/g (6 rats) or saline control (8 rats). For studies 1 and 2, 5 Sprague-Dawley to Sprague-Dawley (SD/SD) and 10 Lewis to Lewis (L/L) isograft controls were given 1.0 mL of saline through tail vein injection immediately after transplantation. For study 3, Serp-1 (30 ng, 12 rats) or control saline (12 rats) was infused, with 6 euthanized at 12 hours and 6 at 48 hours per group.

Rats were euthanized with 1.0 mL of pentobarbital sodium per kilogram (114 mg/kg pentobarbital; MTC Pharmaceuticals, Canada Packers Inc). All research protocols and general animal care were approved by university laboratory animal ethics committees and conform to national guidelines.

Histology and Morphometric Analysis

Each arterial specimen was fixed in 10% sodium phosphate-buffered formalin, processed, impregnated, embedded in paraffin, and cut into 5-μm sections as previously described. Harvested 3.0-cm transplantation segments were then divided into three 1.0-cm lengths, and 3 sections per segment were stained with hematoxylin and eosin for light microscopic and morphometric examination (9 stained sections per specimen). Intimal area and invading mononuclear cell area were measured by morphometric analysis, with the use of the largest detectable atherosclerotic plaque. Histological sections in study 1 were independently assessed by a pathologist.

Immunohistochemistry

Tissue sections from the top and mid transplanted aortic specimens were incubated with primary antibodies and immunostained with the indirect peroxidase-labeled antibody technique. The primary antibodies used were mouse monoclonal anti-α smooth muscle cell actin (IgG2) diluted 1:400 (Sigma), anti-rat macrophage antibody (IgG specific to ED2-like antigen) diluted 1:100 (Pharmingen), anti-rat CD2 (IgG) thymocyte for natural killer (NK) cells and maturing T lymphocytes (Cedarlane Laboratories Ltd), and anti-rat CD45RA (IgG antibody to a subfraction of 240-kDa rat CD45) pan B-cell stain diluted 1:200 (Cedarlane Laboratories). Sections were incubated with 20% normal horse serum followed by primary antibody for 30 minutes to 2 hours (dependent on effective incubation times), biotinylated anti-mouse IgG diluted 1:250 (Vector Laboratories) for 20 minutes, and avidin-biotin-peroxidase complex for 40 minutes. For polyclonal leukocytes, mouse anti-rat IgM (1:100 dilution) was the primary antibody with biotin-conjugated goat anti-mouse (rat adsorbed) immunoglobulin-specific polyclonal secondary antibody (Pharmingen). Color was developed with 3,3'-diaminobenzidine (5 minutes) counterstained with hematoxylin. Control stains lacking primary or secondary antibodies were performed. The number of positively staining cells was measured in 3 high-power fields (sections with the largest number of invading cells) and divided by the area examined.

Viral Anti-Inflammatory Protein Purification and Source

Serp-1 was produced from recombinant Chinese hamster ovary cells (Biogen, Inc) by affinity chromatography with monoclonal antibody AXB7.9 (courtesy of Dr L. Ling, Biogen, Inc). The purified Serp-1 protein was >95% pure as judged by loaded Coomassie-stained SDS-PAGE gels and a single peak on reverse-phase high-performance liquid chromatography.

Statistical Analysis

The mean value for plaque areas or positively stained cells for each experimental animal was used for all statistical analyses. Correlations with treatment were assessed by ANOVA and Student’s t test.

Results

Intimal Hyperplasia After Aortic Transplantation

Diffuse areas of intimal hyperplasia, mononuclear cellular invasion, and local connective tissue deposition were detected at 28 days in control saline-treated L/SD and ACI to Lewis A/L aortic allografts (Figure 1, top left). In contrast, a single infusion of Serp-1 at ≥10 pg/g markedly reduced aortic allograft plaque area and cellular invasion (Figure 1, top right) (P<0.0016) for L/SD (Figure 2A) and A/L transplants (P<0.0001) (Figure 2B). There was a clear dose-dependent effect on plaque area in the A/L transplantation model (Figure 2B). A significant reduction in intimal area was detected in mean plaque area either measured with both central trans-
planted aortic segments and adjacent anastomotic sites or sections taken from the middle of the transplanted specimen alone.

Studies 1 and 2 (L/SD and A/L allografts) revealed comparable pathomorphological changes in intimal plaque development (Figure 2). Lipid-filled areas, cholesterol crystals, and thrombosis were not observed. Intimal reaction and increased cellular hyperplasia and connective tissue scar formation were seen at suture sites (Figure 1, bottom left) but with minimal mononuclear cell invasion. The isograft controls (L/L and SD/SD) had small focal areas of intimal hyperplasia consistent with marked reduction in inflammatory reactions expected with MHC-matched strains (Figure 1, bottom right). Mean plaque areas (mm²) for saline-treated allografts and isografts at 28 days were as follows: L/SD 0.16±0.035, SD/SD 0.024±0.003, A/L 0.125±0.014, and L/L 0.025±0.002.

There was minimal surgical loss in either saline-treated or Serp-1–treated rats after transplantation (7 of 98 rats). Three Serp-1–treated rats and 3 saline-treated rats (1 L/SD, 1 SD/SD, and 1 L/L) died at 24 to 48 hours as the result of surgical complications (hemorrhage). One Serp-1–treated rat died with aneurysmal hemorrhage at 3 weeks. There was no significant difference in the incidence of infections or thrombosis after Serp-1 treatment.

Late Cell Invasion After Aortic Transplantation
Large areas of invading mononuclear cells were seen in saline-treated L/SD allograft controls at 28 days. Mononuclear cell infiltrates into the intimal (P<0.03 for L/SD, P<0.02 for A/L) and adventitial (P<0.0001 for L/SD, P<0.0002 for A/L) layers were markedly reduced after Serp-1 infusion compared with saline controls (Figure 2, C and D, respectively). Similar reductions in cell invasion were seen in the isografts compared with Serp-1 treatment (P=NS), with the exception that larger infiltrates were detected in outbred SD/SD than L/L isografts (data not shown).

All cell types (Figure 1, top), macrophages (Figure 3, top), smooth muscle cells, CD2-positive lymphocytes, and B cells were reduced with Serp-1 infusion in transplanted sections at 28 days (data not shown). Significant reductions in macrophage (Figure 3, top; P<0.0018), CD2-positive (P<0.0001), and smooth muscle cells (P<0.0374) were seen in the intima at 28 days with Serp-1 infusion (300 ng). Parallel reductions

Figure 1. Hematoxylin and eosin–stained ACI to Lewis allograft sections (28 days). I indicates intima; M, media; and A, adventitia. Top left, Saline-infused allograft intimal hyperplasia, connective tissue deposition, and mononuclear leukocyte invasion. Magnification ×400. Top right, Marked reduction in intimal vasculopathy with Serp-1 treatment (100 pg/g). Magnification ×400. Bottom left, Smooth muscle cellular (SMC) proliferation and connective tissue deposition at sites of graft anastomosis. S indicates suture. Magnification ×1000. Bottom right, L/L isografts with reduced intimal hyperplasia and cellular infiltrates. Magnification ×400.
in smooth muscle ($P<0.00004$), macrophage ($P<0.0001$), and CD2-positive cells ($P<0.0001$) were seen in the adventitia. Significant decreases were not seen for macrophage or CD2-positive cell invasion in the media. With Serp-1 treatment there was a significant increase in medial smooth muscle cell staining ($P<0.0001$) with an associated reduction in the intima ($P<0.0374$) and adventitia ($P<0.0001$), indicating a decrease in cellular migration out of the media. The invading mononuclear cells were predominantly macrophage and CD2-positive cells, with very few B cells or neutrophils (data not shown). Aortic isografts stained for macrophages, CD2-positive T lymphocytes, B cells, neutrophils, and smooth muscle cells demonstrated a reduced inflammatory response at 4 weeks ($P=NS$ compared with Serp-1–treated allografts), suggesting that the reaction is non–alloantigen dependent.

The effects of Serp-1 on cellular invasion were similar for the L/SD allograft and the A/L models at 4 weeks after surgery and did not reach significance at equivalent doses (1.0 to 1000 pg/g, $P=NS$). Intimal and adventitial mononuclear cell infiltrates were significantly larger in the saline-treated L/SD compared with A/L grafts ($P<0.009$ for intima, $P<0.0001$ for adventitia).

**Selective Inhibition of Early Cellular Invasion**

Significant early reductions in macrophages ($P<0.0016$) (Figure 3, center, and Figure 4A) and CD2-positive (Figure 4B) cells ($P<0.018$) (histology not shown) were detected with Serp-1 infusion at 12 and 48 hours. There was minimal positive staining for B cells and neutrophils in the medial or adventitial layers with either Serp-1 or saline infusion at 12 or 48 hours, with overall 10$^3$-fold fewer cells compared with other selectively stained cells. An associated increase in smooth muscle cells in the media and a decrease in the adventitia (Figure 3, bottom) ($P<0.0003$) was detected in Serp-1–treated rats at 48 hours (Figure 4C), consistent with a reduction in early smooth muscle cellular migration out of the media.

**Discussion**

There is increasing evidence that the initiating event that leads to allograft vasculopathy formation is produced by inflammatory cells responding to alloantigen-independent factors. The thrombolytic cascade has very recently been implicated in early activation of inflammatory responses that lead to atherosclerosis. In this work we have demonstrated that the viral serpin Serp-1, when given at the
time of aortic allograft transplantation, blocks early inflammatory cell invasion and subsequent transplant vasculopathy development in a rat aortic allograft model.

Acute inflammatory responses provide nonspecific early defenses to injury with rapid mobilization after organ harvest.\textsuperscript{4,16} This early inflammatory response is mediated by mononuclear phagocytes, polymorphonuclear leukocytes, and non–alloantigen-specific lymphocyte responses.\textsuperscript{4,21,22} Macrophages and T cells are also central to antigen presentation and B-cell activation in immune-mediated responses and therefore are associated with both acute and chronic rejection.\textsuperscript{4,10} We have demonstrated early reductions in macrophage and CD2-positive lymphocytic (NK and maturing T cells) invasion 24 to 48 hours after transplantation. At these

Figure 3. Immunohistochemical staining for invading mononuclear cells in the adventitial layer at early and late follow-up (L/SD model). With Serp-1 treatment there is reduction in all invading cells assessed at 4 weeks. Top left, Macrophage (MC) invasion, saline infusion (28 days). Top right, Macrophage invasion, Serp-1 infusion (28 days). Center left, Macrophage invasion adventitia, saline infusion (12 hours). Center right, Macrophage invasion adventitia, Serp-1 infusion (48 hours). Bottom left, Smooth muscle cell (SMC) migration adventitia, saline infusion (48 hours). Bottom right, Smooth muscle cell migration adventitia, Serp-1 infusion (48 hours). Magnification $\times 1000$. 

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very early times it is likely that the T cells staining positive for CD2 antigen are NK cells, that is, non-alloantigen-specific responders whose role in transplant rejection has not been fully defined but that have been detected at early times after transplantation.45

The marked acute (12- to 48-hour) and chronic (4-week) adventitial response to aortic allograft transplantation is consistent with what other groups have reported,46 which supports the hypothesis that the adventitia may play a prominent role in the development of transplant atheroscle-
rosis. The fact that the invading cellular populations in the isografts was similar to the cells invading the allografts also suggests that allograft vasculopathy is an accelerated sequel to an initial inflammatory response.

The thrombolytic serine proteinase target enzymes, their natural serpin inhibitors such as plasminogen activator inhibitors (PAI-1 and PAI-2), and the urokinase-type plasminogen activator receptor are acute phase reactants found throughout the vascular system bound to the cell surface of endothelial, smooth muscle, and mononuclear cells.\(^{24-26,29}\) PAI-1 knock-out mice have exacerbated intimal hyperplasia after arterial injury. PAI-1 overexpression by adenovirus vector reduces plaque growth in the PAI-1 knockout model, suggesting a protective role for PAI-1 against plaque development.\(^{30}\) The inhibitory effects of Serp-1 may be the result of direct blockade of thrombolytic enzyme activity or an as-yet undiscovered serine protease regulator of inflammation. Prior work on Serp-1 kinetics in vitro has demonstrated that Serp-1 inhibits tissue-type plasminogen activator, urokinase-type plasminogen activator, and plasmin with second-order association rate constants in the order of \(10^2 \text{ mol(L)}^{-1} \cdot s^{-1}\) and inhibition constants \(<100 \text{ pmolL}^{-1}.^{27-31,36,42}\) In this study, we made use of a model in which onset of the inflammatory response is defined at the time of aortic allograft transplantation. We observed similar efficacy for inhibition of plaque development, as was seen with infusion of Serp-1 after angioplasty injury in cholesterol-fed rabbits, where the use of an active site mutant resulted in a loss of antiatherogenic Serp-1 activity. This indicated that early activation of the thrombolytic cascade was involved in plaque growth.\(^{27}\)

In summary, we detected significant reductions in early macrophage and lymphocytic (possible NK cell) invasion in aortic allografts after a single infusion of Serp-1 at the time of transplantation. We also detected an associated reduction in subsequent cellular invasion and plaque growth. This work suggests that further investigation into the role of the thrombolytic cascade and vascular serpins may provide new insight into early inflammatory responses associated with the genesis of transplant vasculopathy, potentially allowing the development of improved therapeutic approaches.

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