Perforin Mediates Endothelial Cell Death and Resultant Transplant Vascular Disease in Cardiac Allografts

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T cell-induced endothelial injury is an important event in the development of transplant vascular disease (TVD), the leading expression of chronic rejection of vascularized organ transplants. However, the precise contribution of perforin to vascular damage in allografts and resultant TVD has not been addressed *in vivo*. Minor histocompatibility antigen mismatched mouse heterotopic cardiac transplants were performed from 129J donors into C57BL/6 (wild-type (WT)) or perforin knockout (PKO) recipients. Perforin was abundant in immune infiltrates in the myocardium and vasculature of transplanted hearts in WT mice. Allograft coronary arteries in both WT and PKO mice had considerable vasculitis. There was also marked endothelial disruption, as well as TUNEL-positivity in the endothelial region, in coronary arteries of hearts transplanted into WT mice that was not evident in PKO recipients ($P = 0.05$). At 30 days post-transplantation, intimal thickening was assessed on elastic Van Gieson-stained ventricular sections. There was an average of $54.2 \pm 6.7\%$ luminal narrowing of coronary arteries in allografts from WT mice as compared to $13.4 \pm 5.1\%$ luminal narrowing in PKO counterparts ($P < 0.00002$). In summary, perforin plays a primary role in endothelial damage and the resultant onset and progression of TVD. (*Am J Pathol* 2004, 165:127-133)

Early endothelial cell death and dysfunction caused by alloreactive T lymphocytes is believed to contribute to the development of transplant vascular disease (TVD), a vascular disorder characterized by the intimal accumulation of vascular smooth muscle cells (VSMC), T lymphocytes, and macrophages in solid organ transplants.^{1,2} T lymphocytes induce cell death of allogeneic target cells through FasL and perforin/granzyme pathways. FasL is localized to the plasma membrane of activated T lymphocytes and induces apoptosis through the activation of

caspases. Perforin is a lytic protein and granzymes are cytotoxic serine proteases that are packaged together into cytotoxic granules of T lymphocytes.³ On activation, these granule components are released into the synapse between T lymphocytes and target cells whereupon perforin facilitates the entry of granzymes into target cells. Once delivered into the cytoplasm of target cells, granzymes induce apoptosis through either the direct or indirect activation of caspases, and can also cause non-apoptotic cell death through the cleavage of other intracellular substrates.⁴⁻⁷

Early immune-mediated endothelial disruption is apparent in cardiac transplants, and T lymphocyte-induced endothelial cell (EC) death has been suggested to be an important mediator of this event.⁸ Hruban et al⁹ originally observed the infiltration and accumulation of T lymphocytes in the subendothelial region of early TVD lesions in humans. Immunohistochemical analysis of these lesions indicated that several T lymphocytes underlying the endothelium expressed perforin-containing granules that polarize toward the endothelial cell surface.¹⁰ We have also reported that granzyme B is abundant in TVD and that it localizes to apoptotic cells in these lesions.¹¹ Combined, these observations suggest that the perforin/granzyme pathway may be an important mediator of vascular damage in allograft vessels.

T lymphocytes induce EC death primarily through a perforin/granzyme pathway *in vitro*.^{12,13} As such, T lymphocyte-induced EC death through a perforin regulated pathway may be an important event in inducing allograft arterial damage and in resulting TVD. To address this

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issue, we have performed heterotopic cardiac transplants into wild-type (WT) and perforin knockout (PKO) recipients. We demonstrate that perforin mediates early endothelial cell death *in vivo*, and that the reduction in endothelial disruption in PKO mice is accompanied by a significant and dramatic attenuation of TVD.

Materials and Methods

Animals

Minor histocompatibility antigen mismatched animals were used in this study. Hearts from 129J (H-2^b) mice were used as donors into either C57BL/6 (H-2^b, WT) or PKO (H-2^b) recipients (Jackson Laboratories, Bar Harbor, ME). The PKO mice are derived from a C57BL/6 background.

Heterotopic Cardiac Transplant Model

Cardiac transplantation was performed as described previously.^{8,14} Hearts from 129J donors were implanted into the abdomen of 8- to 12-week-old WT or PKO recipients (five transplants were performed for each group at each time point). Animals were anesthetized with 4% halothane and anesthesia maintained with 1% to 2% halothane (Halocarbon Laboratories, River Edge, NJ). Donor mice were infused with heparinized saline and their hearts excised. The recipient's abdominal aorta and inferior vena cava were located and clamped. The donor's aorta and pulmonary artery were anastomosed to the recipient's abdominal aorta and inferior vena cava, respectively, in an end-to-side manner. One dose of buprenorphine (Buprenex Injectable; Reckitt and Colman Pharmaceuticals, Richmond, VA) (0.01 mg/kg i.m.) was administered after surgery. Implantation was performed within 30 to 40 minutes of removal of the donor heart. All experiments were approved by the University of British Columbia Animal Care Committee.

Tissue Harvesting and Morphometry

At 12 and 30 days post-transplantation, mice were anesthetized by injection with ketamine/xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The native and transplanted hearts were perfused with sterile saline at 2 ml/minute followed by 4% formalin (Fisher Scientific, Fairlawn, NJ) at the same flow rate. Subsequent to perfusion-fixation, hearts were rapidly removed and immersion-fixed in 10% formalin for 24 hours. Ventricular transverse-sections were then paraffin-embedded.

Paraffin-embedded sections were cut serially (4 μ m) and stained with hematoxylin and eosin (H&E) and elastic Van Gieson (EVG). Vasculitis in all five transplanted hearts per group was scored on H&E-stained sections (0 to 6+ scale) by a registered pathologist blinded to the data and experimental protocol. To evaluate TVD, all visible medium to large coronary arteries from five transplanted hearts per group were photographed at $\times 400$ magnification using a Spot digital camera ($n = 31$ arteries

for WT recipients and $n = 27$ arteries for PKO recipients). ImageProPlus was used to quantitate intimal and luminal areas, and percent luminal narrowing was calculated using the following formula described by Armstrong et al.¹⁵

% Luminal narrowing =

$$\frac{\text{Intimal area}}{(\text{Intimal area} + \text{Lumen area})}$$

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections as described previously.¹¹ Briefly, sections were de-paraffinized and rehydrated in xylene and decreasing concentrations of ethanol. Antigen retrieval was performed by autoclaving slides in citrate buffer (pH 6.0) for 10 minutes. Background staining was blocked by incubation of sections in 10% goat or horse serum (depending on the source of the secondary antibody) for 30 minutes. Sections were incubated in a 1:100 dilution of either rabbit anti-von Willebrand factor (vWF; Dako, Carpinteria, CA) or goat anti-perforin (Research Diagnostics, Flanders, NJ) overnight, followed by incubation in the appropriate secondary antibody for 1 hour. Staining was visualized with the chromagen Vector Red, which possesses both colorimetric and fluorescent properties (Vector Laboratories, Burlingame, CA), and nuclei were counter-stained with hematoxylin. Staining of cardiac ventricular transverse sections with irrelevant isotype-matched antibodies in the place of the primary antibodies was performed with each immunohistochemical run to ensure the specificity of the procedure. There was never any immunopositivity in these negative controls.

In Situ TUNEL

TUNEL was performed on formalin-fixed, paraffin-embedded sections as described previously to detect apoptotic cells.¹¹ The number of TUNEL-positive cells in the endothelial region of coronary arteries from five hearts per group ($n = 16$ arteries for each group) was counted manually and expressed as the average number of TUNEL-positive cells in the endothelial region/artery. TUNEL can also occasionally label necrotic cells as a result of uncontrolled DNA fragmentation that occurs during this form of cell death. Because the nuclear membrane is disrupted during necrosis but remains intact during apoptosis, TUNEL localization to the nucleus can be used to distinguish between apoptotic and necrotic cells under the microscope. In the allografts analyzed in this study, TUNEL-positivity was localized to the nucleus in the very large majority of studied sections. Only TUNEL-positivity that displayed this localization was included in the quantitative analysis.

Statistical Analysis

Statistical differences between two groups were determined using a Student's *t*-test. A chi-square test was

used to determine the statistical significance between the number of arteries with TVD in WT and PKO mice. For both tests, a *P* value (alpha error) of 0.05 or less was considered significant.

Results

Perforin Does Not Affect Immune Infiltration into Allograft Vessels

Cardiac contractions in hearts transplanted into WT recipients were not detectable past an average of 19 days, although contractions were evident until at least 12 days post-transplantation in all animals and were evident in two animals at 30 days post-transplantation. Contractions in hearts transplanted into all PKO mice were evident at 30 days post-transplantation. Perforin has been shown previously to be abundantly expressed in transplanted hearts, and is localized to immune infiltrates in the myocardium.^{16,17} However, these reports did not examine the localization of perforin in and around blood vessels. Consistent with these reports, we observed perforin in immune infiltrates in the myocardium of hearts transplanted into WT mice. Perforin was also abundant in and around several coronary arteries at 12 days post-transplantation, whereupon this cytotoxic protein localized primarily to infiltrating mononuclear leukocytes in the perivascular space. Occasional perforin-positive cells were also observed in the subendothelial space of allograft vessels (Figure 1A). No perforin-positivity was observed in hearts transplanted into PKO mice.

To assess the contribution of perforin to vascular inflammation, histology of H&E-stained ventricular transverse-sections was assessed. At both 12 and 30 days post-transplantation there was equivalent immune infiltration in the myocardium of hearts transplanted into WT and PKO recipients. Vasculitis, which is characterized by medial swelling, mononuclear leukocyte infiltration into the adventitia and media, and leukocyte involvement with the surface endothelium was similar in hearts transplanted into WT and PKO mice (Figure 1B). Semi-quantitation of vasculitis on a 0 to 6+ scale also indicated no differences in vasculitis in hearts transplanted into WT as compared to PKO mice, suggesting that perforin does not affect the infiltration of mononuclear leukocytes into allograft vessels (Figure 1C).

Perforin Mediates Endothelial Damage in Transplanted Hearts

We have previously shown that there is early immune-mediated endothelial disruption in cardiac allografts.⁸ To determine whether perforin contributes to this early en-

Figure 1. Histology of the vasculature in transplanted hearts. **A:** Hearts transplanted into WT and PKO recipients were stained immunohistochemically for perforin. Bright-field and dark-field photomicrographs were obtained from the same optical field to visualize perforin localization. In WT mice, perforin is abundant in immune infiltrates both within the myocardium (arrowhead) and in the perivascular space (arrows). There is also perforin-positivity in infiltrating mononuclear cells in the subendothelial space of allograft arteries (arrows). No perforin-positivity is apparent in PKO recipients (magnification, $\times 400$). **B:** Ventricular transverse-sections were stained with H&E and vasculitis in transplanted hearts assessed. In both WT and PKO recipients, there is extensive vasculitis (magnification, $\times 400$). There is no vasculitis in syngraft controls (inset). **C.** Vasculitis in both WT and PKO recipients was scored on a 0 to 6+ scale. There is equivalent vasculitis in both WT and PKO mice.

Table 1. Characteristics of Allograft Coronary Arteries 30 Days Post-Transplantation

	WT	PKO	<i>p</i> value
Average diameter of coronary arteries (μ m)	200.0 $\pm$ 16.4	229.4 $\pm$ 18.2	0.23
% of vessels with luminal narrowing	83.9	38.5	0.001

endothelial damage, we stained ventricular transverse sections for vWF to visualize the integrity of the endothelium at 12 days post-transplant. Intact endothelium was observed to be lining the luminal surface of coronary arteries of native hearts in both WT and PKO mice. There is also vWF-positivity in the myocardium of all hearts that corresponds to the abundant microvasculature in this organ. At 12 days post-transplantation, there was marked endothelial disruption in allograft arteries in WT mice. This was characterized by a reduction in the amount and consistency of endothelium lining the lumen of coronary arteries (Figure 2A). These changes were not apparent in coronary arteries in hearts transplanted into PKO mice. In these animals the endothelium in transplanted hearts remained intact, indicating that perforin mediates endothelial disruption early in TVD. We also assessed apoptosis in hearts transplanted into both WT and PKO mice. At 12 days post-transplantation, TUNEL-positivity was apparent in the endothelial region of allograft coronary arteries in WT recipients, but there was limited TUNEL-positivity in the endothelial region of hearts transplanted into PKO mice (Figure 2B). Quantitation of the number of TUNEL-positive cells in allograft coronary arteries indicated that there was significantly less TUNEL-positivity in the endothelial region of arteries in hearts transplanted into PKO as compared to WT recipients (Figure 2C, $P = 0.05$).

TVD Is Reduced in PKO Recipients

Ventricular cross-sections were stained with EVG to assess intimal thickening at 30 days post-transplantation. There was no difference in allograft arterial diameter in WT and PKO recipients (Table 1). In WT recipients, inti-

Figure 2. Assessment of endothelial damage in transplanted hearts. **A:** Ventricular transverse-sections were stained for vWF to visualize the endothelium. In native hearts from both WT and PKO mice, the endothelium is intact and lines the luminal surface of all coronary arteries. vWF-positivity is also observed in the microvasculature of the myocardium. In hearts transplanted into WT recipients, there is marked endothelial disruption at 12 days post-transplantation, as determined by the absence of endothelial cells lining the luminal surface of allograft coronary arteries (arrows). This endothelial disruption is not apparent in PKO recipients. The endothelium of allograft coronary arteries in PKO recipients is intact and resembles that observed in native hearts (magnification, $\times 400$). **B:** Ventricular transverse-sections were also stained with *in situ* TUNEL to detect apoptotic cells. In coronary arteries transplanted into WT mice, there is TUNEL-positivity in several cells within the endothelial region (arrow). This pattern of TUNEL-positivity is not observed in PKO recipients (magnification, $\times 400$). **C:** TUNEL-positivity in the endothelial region of allograft coronary arteries was quantitated in both WT and PKO mice (five hearts per group, $n = 16$ arteries for each group). There is a significant reduction in the number of TUNEL-positive cells in this region of allograft vessels in PKO recipients as compared to WT counterparts ($*P = 0.05$).

Figure 3. TVD in allograft arteries in PKO and WT recipients. **A:** Ventricular transverse-sections from hearts 30 days post-transplantation were stained with EVG to assess TVD. In allograft arteries from WT recipients, there is considerable luminal narrowing that is not apparent in PKO mice (magnification, $\times 400$). There is no luminal narrowing in arteries from syngraft controls (inset). **B:** Luminal narrowing in allograft coronary arteries was quantitated in ImageProPlus (five hearts per group; $n = 31$ arteries for WT recipients and $n = 27$ arteries for PKO recipients). There is a significant reduction in the extent of TVD in allograft coronary arteries from PKO recipients as compared to WT counterparts (*, $P < 0.00002$).

mal thickening of allograft arteries was not evident at 12 days post-transplant, but was prominent at 30 days post-transplant (Figure 3A). The developed TVD lesions were cellular in nature, consisting mainly of VSMC and leukocytes (data not shown). However, both the extent of intimal thickening and the number of vessels containing TVD were reduced in hearts transplanted into PKO recipients. In WT recipients, 83.9% of coronary arteries developed TVD, as compared to only 38.5% in PKO recipients (Table 1, $P = 0.001$). Also, there was a significant reduction in the extent of luminal narrowing in PKO as compared to WT recipients. In WT recipients, there was an average of $54.2 \pm 6.7\%$ luminal narrowing of coronary arteries in cardiac allografts as compared to $13.3 \pm 5.1\%$ luminal narrowing in PKO recipients (Figure 3C, $P < 0.00002$). There was no luminal narrowing in syngraft controls.

Discussion

Perforin has been implicated in cardiac transplant rejection and specifically in the pathogenesis of TVD for over

a decade. Although several reports have documented the expression of perforin in alloreactive T cells within TVD lesions,¹⁰ and shown that T cells kill allogeneic endothelial cells mainly through a perforin- and granzyme B-dependent mechanism *in vitro*,^{12,13} to our knowledge this is the first report evaluating the role of perforin in vascular damage in cardiac allografts. In this study we show that perforin induces endothelial cell death *in vivo* and that TVD is reduced in PKO recipients. Moreover, although there is a reduction in endothelial damage and TVD in PKO mice as compared to wild-type recipients, the observation that vasculitis (a representation of vascular inflammation) is equivalent in both groups indicates that the effects of perforin on vascular damage occur downstream of immune infiltration into cardiac transplants. This is consistent with the cytotoxic T cell effector function ascribed to perforin and indicates that the specific inhibition of the perforin pathway can prevent vascular damage while minimally affecting leukocyte migration and infiltration. This finding also suggests that cell death induced by the granule pathway is an important contributor to vasculitis-mediated vascular damage and its sequelae.

The perforin/granzyme and FasL cytotoxic pathways are mainly used by CD8⁺ T cells and several groups have investigated the role of CD8⁺ T cells in the pathogenesis of TVD using different animal models. Specifically, Szeto et al¹⁸ have shown that elimination of direct alloantigen recognition prevents acute rejection, but that these allografts develop TVD. Depletion of CD8⁺ T cells from the recipients in this model does not affect the severity of TVD. In addition, in a complete major histocompatibility complex (MHC) mismatched aortic transplant model of TVD, there is no difference in intimal thickening of allograft arteries in WT recipients as compared to CD8⁺-deficient counterparts.¹⁹ However, Fischbein et al²⁰ have shown that CD8⁺ T cells augment the severity of TVD in a heterotopic cardiac transplant model of chronic rejection that utilizes the transplantation of hearts from donors that are immunologically distinct from recipients at one loci of their MHC class II molecules. Further, depletion of CD8⁺ T cells with neutralizing antibodies can reduce TVD in other animal models.²¹ As such, the differential contribution of CD8⁺ T cells to the pathogenesis of TVD in these reports may be a result of differing models and/or compensatory mechanisms in CD8⁺-deficient mice mediated through cytotoxic pathways induced by other effectors, such as CD4⁺ T cells or antibodies. Indeed, CD4⁺ T cells can develop into cytotoxic T lymphocytes and can induce target cell death through a perforin pathway.²² In a human-mouse arterial transplantation model, CD4⁺ T cells have been observed to differentiate into perforin-expressing cytotoxic cells.²³ Therefore, the development of cytotoxic CD4⁺ T cells in CD8⁺-deficient mice could contribute to TVD in these animals under certain conditions. The use of PKO recipients permits the evaluation of the role of this specific cytotoxic pathway in the development of TVD.

Elimination of perforin reduces TVD in our model by 75% as compared to WT recipients. Although striking, the reduction in TVD is not complete since there remains

significantly more intimal thickening in hearts transplanted into PKO mice as compared to syngraft controls and native hearts ($P < 0.05$). The residual 13% luminal narrowing observed in PKO recipients is likely mediated by endothelial damage induced by FasL or by cytokine-induced vascular cell changes. Dong et al²⁴ originally reported that Fas-positivity is associated with TUNEL-positivity in the endothelium of human TVD lesions, suggesting that a Fas-mediated pathway could be inducing apoptosis of endothelial cells in human heart transplants. Recently, inhibition of FasL signaling in a rat model of TVD was reported to partially reduce lesion severity.²⁵ In addition to this cytotoxic pathway, cytokines secreted by infiltrating T cells, such as γ -interferon, can cause TVD in the absence of cytotoxicity by indirectly inducing VSMC proliferation.²⁶

The immunopathological mechanisms that contribute to allograft vascular damage and resultant TVD may involve both acute and chronic rejection processes since acute rejection predicts the development of TVD in humans, and the induction of acute rejection episodes has been shown to markedly increase the severity of TVD in animal models.^{27–30} As such, the events studied in our model of cardiac transplant rejection relate to vascular damage and dysfunction induced by acute and chronic rejection.¹⁴ Although cardiac contractions were not evident in three of the five cardiac allografts from wild-type mice at 30 days post-transplantation, the cessation of cardiac contractions reflects parenchymal rejection and may provide limited insight into vascular changes.^{19,23,31} Importantly, we have shown that in non-immunosuppressed rodents receiving heterotopic cardiac transplants, myogenic tone and agonist-induced vasodilation are reduced but still apparent at 28 days post-transplantation.^{32,33} These findings are similar to the observed endothelial dysfunction that occurs in human cardiac allograft arteries and they indicate that although vascular dysfunction clearly occurs in non-immunosuppressed animals, the reactivity of the vasculature to a number of agonists remains apparent, signifying that the vasculature continues to be functional at the time points examined in this current study.^{34,35} In addition, the extent and morphology of intimal thickening observed in our current investigation is consistent with other models that prevent acute rejection by using cardiac transplantation of donors that are immunologically distinct from recipients due to a polymorphism in MHC class I or II molecules.^{20,36} Thus, the model used in this study provides valuable insight into the mechanisms through which the immune response damages the vasculature of cardiac allografts, and indicates that TVD induced by immune-mediated endothelial cell death in transplanted organs proceeds mainly through a perforin-dependent pathway.

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