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Viral Infection Induces De Novo Lesions of Coronary Allograft Vasculopathy through a Natural Killer Cell-Dependent Pathway

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Abstract

Viral infections including those due to cytomegalovirus (CMV) have been associated with accelerated cardiac allograft vasculopathy (CAV) in clinical trials and some animal models. Evidence demonstrating a direct causal relationship between such infections and de novo formation of coronary vascular lesions is lacking. Heterotopic murine cardiac transplants were performed in a parental to F1 combination in animals lacking both T- and B-lymphocytes ($RAG^{-/-}$). Coronary vasculopathy developed almost exclusively in the presence of recipient infection with lymphocytic choriomeningitis virus (LCMV) but not in uninfected controls. This process was also dependent upon the presence of NK cells as depletion of NK cells abrogated the process. These data show that a viral infection in its native host, and not previously implicated in the production of CAV, can contribute to the development of advanced coronary vascular lesions in cardiac allotransplants in mice. These data also suggest that virus-induced CAV can develop via an NK-cell dependent pathway in the absence of T- and B-lymphocytes.

Introduction

Coronary vasculopathy (CAV) remains a major limitation to long-term survival of cardiac allografts.(1–4). Cytomegalovirus (CMV) infection and seropositivity have been associated with accelerated coronary vasculopathy (CAV), however the mechanisms underlying development of CAV are incompletely understood(5,6). In clinical trials, patients receiving intensive anti-CMV prophylaxis after cardiac transplantation are less likely to experience transplant coronary artery disease than patients not receiving prophylaxis(1–4). In immunosuppressed rats undergoing allogeneic heart transplantation, formation of coronary vascular lesions is accelerated in the presence of infection with rat CMV with viral antigens expressed in myocardial tissues (7).

Studies by Maier et al. produced evidence that NK cells can contribute to heart transplant rejection in mice.(8) The role of innate immunity in the generation of CAV was supported by our observation that mice made fully tolerant of allogeneic antigens by neonatal exposure develop CAV in hearts transplants received in adulthood.(9) We subsequently found that

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parental to F1 heart transplants (C57BL/6 to (C57BL/6 × BALB/c)F1 also develop CAV. A combination of CD4 and NK cell depletion was required to prevent CAV.(10) To isolate NK cell functions from those of T and B cells, murine heart transplants were performed from parental donors to F1 strain RAG1$^{-/-}$ recipients lacking both T- and B-lymphocytes.. This system was selected to minimize the activation of acquired immune pathways, as donors present no MHC determined histocompatibility antigens to recipients (11) While the first group of RAG1$^{-/-}$ recipients developed CAV as expected, subsequent recipients from a pathogen-free facility failed to develop CAV lesions. Only sera derived from mice developing CAV were found to carry antibodies to lymphocytic choriomeningitis virus (LCMV).

LCMV, an arenavirus, produces a persistent but non-lethal infection in mice and is a causative agent linked to aseptic meningitis and other pathological processes in humans. LCMV infection has been shown to enhance NK cell activity in vitro(12). Recent studies have demonstrated the viral abrogation of graft tolerance in bone marrow transplantation(13).The present studies were designed, therefore, to investigate the effects of LCMV infection in the setting of an isolated NK cell response on the development of vascular lesions in cardiac allografts in an otherwise pathogen-free system.

These studies demonstrate a clear role for viral infection in the development of coronary vasculopathy due by a novel, NK cell-mediated mechanism. These observations have clinical implications for solid organ transplantation recipients.

Materials and Methods

Mice

C57BL/6.RAG1$^{-/-}$ (B6.RAG1$^{-/-}$) (14) and BALB/c.RAG1$^{-/-}$ (BALB/c.RAG1$^{-/-}$) (15) mice were purchased from the Jackson Laboratory. F1 mice (BALB/c.RAG1$^{-/-}$ × C57BL/6.RAG1$^{-/-}$)F1 {H-2^{bxd}} (designated CB6F1.RAG1$^{-/-}$ for simplicity) were bred in a pathogen-free, BL2 facility in filter top isolator cages. They remained healthy throughout the experiments. All animals were cared for according to methods prescribed by the American Association for the Accreditation of Laboratory Animal Care and all protocols were approved by the Institutional Committee for Research Animal Care.

Heart Transplantation

Mouse hearts were transplanted to a heterotopic abdominal location with appropriate microsurgical anastomoses according to our previously described technique.(15) The continuing status of transplanted hearts was determined by direct palpation at least twice per week with the vigor of contractions of the transplants being recorded on a scale of 0–3+. Only hearts with palpation scores of 2 or greater were included in this study.

Viral Inoculation

LCMV Armstrong strain was a generous gift of Drs. Robert W. Finberg and Shenghua Zhou (University of Massachusetts Medical Center, Worcester, MA). Virus was recovered from supernatants of infected BHK-21 cells, filtered to 0.22μm and frozen at −80°C. Virus stock was quantified by plaque assay using Vero cells as targets.

CB6F1.RAG1$^{-/-}$ recipients of B6.RAG1$^{-/-}$ or CB6F1.RAG1$^{-/-}$ hearts received inocula of 2.25×10^5 PFU of LCMV Armstrong strain i.p. on the first day after cardiac transplantation. Control animals received virus-free vehicle injections containing MEM supplemented with FCS to 10% and antibiotics (penicillin 100units/ml and streptomycin 100ug/ml). Subsequent to inoculation, recipients were housed in the BL2 filtered isolation facility to prevent

adventitious exposures. Transplanted hearts were generally removed on the 56th day after transplantation for histologic and microbiologic evaluation. Some hearts were removed at 28 days to examine the character of coronary lesions at an earlier time.

NK Cell Depletion

Anti-mouse NK 1.1 mAb (PK136, a mouse IgG2a) was purchased from BioXCell, West Lebanon, NH. Antibody (200μg) was injected i.p. to selected recipients. Peripheral blood lymphocytes were prepared after brief erythrocyte water lysis and re-suspension with 1%BSA-PBS. NK cell depletion was confirmed by flow cytometry (FACScan BD Biosciences; San Jose, CA) of blood samples incubated with 0.5 μg antibody for 30 min at 4° C, with PE-conjugated anti-mouse CD49b (DX5), an NK cell marker. All reagents used for staining were obtained from BD Pharmingen; San Diego, CA.

Histological Techniques

Transplanted and native hearts were removed from recipients and frozen sections cut at 4μm were prepared. To determine the presence and severity of CAV formation, Weigert's elastic tissue stain was used as described previously (16). Sections were also stained for NK cells with anti-Ly49G2 (Clone 4D11; BD Pharmingen), for smooth muscle actin (SMA) {Clone 1-A4, Dako} and macrophages using anti-CD16/32 (Clone 93, eBioscience). Intimal proliferative changes were quantified by morphometric analysis as described below.

Morphometric analysis

Morphometric analysis was performed on images of coronary arteries near the aortic ostia on tissue sections stained with Weigert's elastin stain. This is the preferential and earliest site of CAV formation in the mouse. An image of the most representative section was captured digitally by light microscopy at 40× magnification. Image J software (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA.) was used to demarcate the borders of the lumen and then to identify the intima of the artery in question. Available software permitted quantification of the area of neointima within the internal elastic lamina. From these values a *neointimal index*, defined as {(intimal area - luminal area) / intimal area} × 100, was calculated in a manner described previously (17). Where two coronaries were identified, their respective neointimal indices were calculated and averaged for statistical analysis.

Quantification of cardiac LCMV infections

Native and transplanted apical heart tissues were harvested directly to liquid nitrogen. While frozen, tissues were coarsely ground and then homogenized. Total RNA was recovered from cardiac tissues using the RNeasy Kit (Qiagen, Valencia, CA) following the Qiagen protocol for fibrous tissues. RNA was reverse transcribed with Omniscript RT (Qiagen, Valentia, CA). A quantitative PCR was developed using primers and probe targeting the LCMV nucleoprotein gene. Forward LCMV primer 5'GCA TTG TCT GGC TGT AGC TTA3' and reverse LCMV primer 5'TTT GCC TTT CAG GTG AAG GAT GGC3' and probe 5'FAM-TGA CTG CAG GTT TCT CGC TTG TGA GA-TAMRA3' were employed. Quantitative viral data were normalized to total RNA isolated from each tissue sample.

Statistical analyses

The statistical significance of differences between group means was determined using Fisher's exact test. The Mann Whitney Test was used to determine the degree of difference between the neointimal indices in different groups of recipients. Comparisons of tissue viral loads were made by Student's t-test. P values of <0.05 were considered significant.

Results

Parental to F1 hybrid heart transplants did not develop CAV in B and T cell deficient mice

Parental B6.RAG1$^{-/-}$(H-2^{b}) hearts were transplanted to uninfected CB6F1.RAG1$^{-/-}$(H-2^{bxd}) mice and to mice receiving injections of uninfected culture medium. Transplanted and native hearts were removed at 56 days after transplantation. All hearts continued to beat actively throughout the period of observation. Evidence of CAV lesions was found in 2 of 11 hearts transplanted to uninfected recipients and in none of 4 recipients receiving injections of uninfected viral culture medium (Table 1; Group 1 and 2, Figures 1a and 1b). Lesions did not develop in native hearts.

Inoculation of LCMV results in advanced CAV

At day 56 after transplantation to infected animals, LCMV was detected in all native and transplanted hearts (Table 1). The intensity of infection was consistently greater in transplanted hearts, both isografts and allografts, than in native hearts in all groups (P=0.0149) (Figure 2). However, viral loads did not differ significantly between animals related to the development of CAV or based on treatments received in any of groups 1–6 (Figure 2).

Distinct CAV lesions developed in 7 of 9 parental to F1 transplants removed on day 56 from recipients infected with LCMV and in none of their native hearts (Table 1; Group 3, Figure 1e). In parental to F1 hearts examined on day 28 CAV lesions were also observed at this earlier time in 7 of 8 hearts. (Table 1; Group 6, Figure 1d).

On day 28 after transplantation, pathological analysis of the proximal coronary arteries revealed infiltration of mononuclear cells in the intima that included Ly49G2 + cells (NK cells) and CD16/32+ cells (macrophages and NK cells) (Figure 1g and h). These cells also infiltrated the arterial adventitia, where the CD16/32+, Ly49G2-macrophages predominated. Conversely, ∝SMA + cells (smooth muscle cells or myofibroblasts) were abundant in the intimal lesions (Figure 1h). While the majority of the coronary lesions consisted of smooth muscle cells, immunohistochemical evaluation also revealed many macrophages (Figure 1i) (18). At later times (56 days) the mononuclear infiltrate was diminished and the intima had become fibrous. No lesions were seen in the arteries of the native hearts and no myocardial inflammation was evident.

NK cell depletion results in the abrogation of CAV lesions in parental to F1 transplants to RAG1$^{-/-}$ mice

To deplete NK cells CB6F1.RAG1$^{-/-}$ recipients of B6.RAG1$^{-/-}$ heart transplants received i.p. injections of 200 μg of PK136 mAb (anti-NK1.1) on days −6, and +1 relative to cardiac transplantation, then once weekly until post-operative day 56. NK cell depletion of greater than 80% from peripheral blood was confirmed by flow cytometry after the initial administration of NK 1.1 mAb. Only 1 of the 7 mice infected with LCMV and treated with anti-NK1.1 mAb developed CAV (Table 1; Group 5, Figure 1f).

Morphometric analysis of intimal luminal encroachment

Morphometric analysis was performed on all samples using elastic tissue stained sections (Figure 3). The median neointimal index for LCMV-infected parental into F1 cardiac recipients was 67%, while the median for NK-cell depleted (anti-mouse NK 1.1 treated) and LCMV-infected, parental to F1 mice was 6%. This difference was statistically significant ($p<0.017$).

Discussion

Multiple components of the innate and adaptive immune system participate in the pathogenesis of CAV after cardiac transplantation. In these studies we have developed a set of conditions in which viral infection was responsible for the development of CAV in the absence of T- and B-lymphocytes. In previous reports, observations on the influence of viral infection on the development of arteriopathy were made in the presence of elements of the adaptive immune system which would have caused the development of CAV in the absence of viral infection. In the present experiments, transplanted hearts remained virtually free of CAV in the absence of viral infection. Thus, we present evidence that this viral infection can be the direct precursor of vascular disease rather than simply an accelerator or amplifier of its formation.

We demonstrate further that virally induced CAV, in the absence of T- and B-lymphocytes, requires the participation of NK cells. NK cells are known to be essential to the control of LCMV infection. The stimulation of NK cells by LCMV infection and the NK cell-dependent control of virus appear to occur independently of T and B cell function(19). It is not known whether the activation of NK cells is mediated via antigens (viral or cellular) expressed on cardiac tissues as a result of viral infection, or via stimulation of other cells (e.g., dendritic cells, macrophages) with the subsequent elaboration of stimulatory cytokines, growth factors, and other inflammatory mediators that could activate NK functions. In affected animals, NK cells were observed to be associated with CAV lesions, supporting their role in this pathological process (Figure 1g). INFγ has been shown to be elaborated by activated NK cells and has been associated with CAV in other systems. Given the presence of macrophages in evolving CAV lesions at 28 days after transplantation, it is likely that multiple components of the innate immune system contribute to the pathogenesis of virally-mediated coronary disease after transplantation. The absence of CAV in isografts and in native hearts of transplant recipients suggests that although viral infection is necessary, it is not sufficient for the targeting of cardiac endothelium in this setting. It is possible that viral infection enhances an NK response in the parental to F1 combination.

NK cells recognize targets that express reduced levels of MHC class I molecules or antigens due to infection, inflammation or “stress” (20,21). MHC-I antigens normally bind inhibitory NK receptors. The net level of activation of NK cells reflects the cytokine milieu and a balance between inhibitory and activating cell surface receptors (22–24). Viral infections may reduce the cellular expression of MHC-1 molecules while increasing expression of cellular ligands for NK cell activating receptors. This is best described for CMV infection in which MHC-I expression is reduced, pro-inflammatory cytokines are released and NK activating (NKG2D) ligands are induced (25–27). The activation of NK cells is further enhanced by direct recognition of virally-encoded proteins such as the activating Ly49H NK receptor which binds the MCMV m157 viral protein. While possibly important to our experiments, reduced expression of MHC-1 is not a prerequisite for NK cell activation. Many of the cytokines known to induce NK cell proliferation, survival and, presumably, activation are produced both by infected cells and by dendritic cells (DC) in response to viral infection (28–30). DC-derived cytokines (e.g., IFN-α, IFN-β, IL-12, IL-15, IL-18) have been shown to promote IFN-γ, TNF-α, granulocyte-macrophage colony stimulating factor, and macrophage inflammatory protein (MIP-1α) production by NK cells (31,32). These factors are important in controlling viral replication and in recruiting inflammatory cells to the sites of infection. Of interest, production of type I interferon’s by DC are thought to be essential for NK cell activation, including the plasmacytoid DC (pDC) subset. In contrast to CMV infection, recent data suggest that acute and chronic infections due to LCMV reduce the release of type I interferons by pDC but not by DC from spleen and bone marrow (33). LCMV also blocks DC development and changes the profile of secreted cytokines.

These data demonstrate that the induction of coronary vasculopathy in murine cardiac allografts can occur in the absence of T- and B-lymphocytes in the parental to F1 $RAG^{-/-}$ combination. This process appears to be dependent upon MHC class I deficiency in the target as well as the presence of infection due to LCMV and NK cells. Further exploration of the mechanisms underlying formation of CAV lesions may clarify the role of NK cells and of viral infections. Such studies may lead to clinically applicable approaches to coronary vasculopathy.

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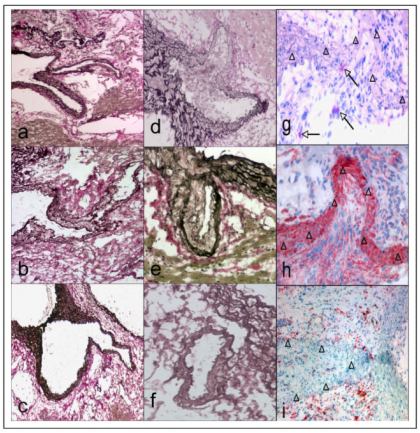

Figure 1.

Representative sections of proximal coronary arteries from transplanted hearts with elastic tissue stain. Group number refers to group labeling from Table I. (a,b,d,e,f) B6.RAG1$^{-/-}$ into CB6F1.RAG1$^{-/-}$, (c) CB6F1.RAG isograft. a) no treatment (grp1), normal artery; b) MEM alone (grp2), normal artery; d) LCMV (grp6), d.28, early lesion; e) LCMV (grp3), d. 56, late lesion; f) LCMV + anti-NK1.1 (grp5), d.56, artery clear; c) LCMV isograft (grp4), normal artery. Marked neointimal and adventitial hypercellularity is present at day 28 (d) while later lesion at day 56 (e) has increased fibrosis and reduced cellularity. Depletion of NK cells (f) blocked formation of intimal lesions. Immunoperoxidase stained proximal coronaries of B6.RAG$^{-/-}$ → CB6F1.RAG$^{-/-}$ LCMV inoculated hearts sacrificed at day 28 using anti-Ly49G2 (g), anti-∝SMA (h) and anti-CD16/32 (i). Several Ly49G2+ cells (NK cells) are shown to be present within the neointima (arrows). SMA+ cells (smooth muscle cells or myofibroblast) comprise the major cell type in the neointima. The media smooth muscle is also positive. Numerous CD16/32+ cells with the appearance of macrophages in the adventitia and in the neointima. Coronary vessel walls indicated by △.

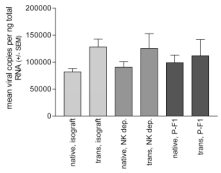

Figure 2.

Lymphocytic choriomeningitis virus quantitative molecular assay (viral copies per ng tissue total RNA). Transplanted hearts developed more intense viral infection (day 56 after infection) than native hearts (p=0.0149). No significant differences were observed between transplanted hearts (isografts and allografts) or between native hearts after various treatments. Trans: transplanted heart; native: native heart; P-F1: parental to F1; NK dep: NK cell depleted.

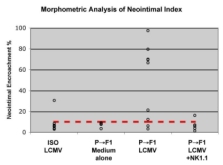

Figure 3.

Neointimal index (%) was calculated based on morphometric analysis of images of coronary arteries. The Mann Whitney Test was used to compare indices between various groups. ▪ ▪ ▪ Denotes a neointimal index of 10%.

p=0.019 when comparing ISO/LCMV to P→F1/LCMV; p=0.017 when comparing P→F1/LCMV to P→F1/LCMV + NK1.1.

Table I

Heart Transplants In RAG1$^{-/-}$ mice

Group	LCMV copies/μg total RNA (sd)		Donor	Recipient	Infection	Hearts with CAV
	Native	Graft				
1 P->F1	Neg	Neg	B6.RAG1$^{-/-}$	CB6F1.RAG1$^{-/-}$	None (56 days)	2 of 11[a]
2 P->F1	Neg	Neg	B6.RAG1$^{-/-}$	CB6F1.RAG1$^{-/-}$	Medium Only (56d)	0 of 4[b]
3 P->F1	98,997 (31,514)	111,735 (67,647)	B6.RAG1$^{-/-}$	CB6F1.RAG1$^{-/-}$	LCMV (56d)	7 of 9
4 Isograft	81,865 (18,814)	128,249 (42,839)	CB6F1.RAG1$^{-/-}$	CB6F1.RAG1$^{-/-}$	LCMV (56d)	1 of 9[c]
5 P->F1	90,684 (27,004)	125,584 (72,223)	B6.RAG1$^{-/-}$	CB6F1.RAG1$^{-/-}$	LCMV + NK1.1 (56d)	1 of 7[d]
6 P->F1	Nd	Nd	B6.RAG1$^{-/-}$	CB6F1.RAG1$^{-/-}$	LCMV (28d)	7 of 8

P->F1: Parental into F1

[a] $p < 0.02$ by Fischer's exact test compared with group 3.

[b] $p < 0.02$ by Fischer's exact test compared with group 3.

[c] $p < 0.02$ by Fischer's exact test compared with group 3.

[d] $p < 0.04$ by Fischer's exact test compared with group 3.