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Citation

Published Version
doi:10.1172/JCI119564

Accessed
April 1, 2017 1:14:03 PM EDT

Citable Link
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Interferon-γ Deficiency Prevents Coronary Arteriosclerosis but not Myocardial Rejection in Transplanted Mouse Hearts

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Abstract

We have hypothesized that T cell cytokines participate in the pathogenesis of graft arterial disease (GAD). This study tested the consequences of IFN-γ deficiency on arterial and parenchymal pathology in murine cardiac allografts. Hearts from C-H-2^b^m12 KhEg (bm12, H-2^b^m12) were transplanted into C57/B6 (B6, H-2^b^), wild-type, or B6 IFN-γ-deficient (GKO) recipients after immunosuppression by treatment with anti-CD4 and anti-CD8 mAbs. In wild-type recipients, myocardial rejection peaked at 4 wk, (grade 2.1 ± 0.3 out of 4, mean ± SEM, n = 9), and by 8–12 wk evolved coronary arteriopathy. At 12 wk, the GAD score was 1.4 ± 0.3, and the parenchymal rejection grade was 1.2 ± 0.3 (n = 8). In GKO recipients of bm12 allografts, myocardial rejection persisted at 12 wk (grade 2.5 ± 0.3, n = 6), but no GAD developed (score: 0.0 ± 0.0, n = 6, P < 0.01 vs. wild-type). Mice treated with anti–IFN-γ mAbs showed similar results. Isografts generally showed no arterial changes. In wild-type recipients, arterial and parenchymal cells showed increased MHC class II molecules, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 compared to normal or isografted hearts. The allografts in GKO recipients showed attenuated expression of these molecules (n = 6). Thus, development of GAD, but not parenchymal rejection, requires IFN-γ. Reduced expression of MHC antigens and leukocyte adhesion molecules may contribute to the lack of coronary arteriopathy in hearts allografted into GKO mice. (J. Clin. Invest. 1997, 100:550–557.) Key words: arteriosclerosis • cardiac transplantation • IFN-γ knockout mouse

Introduction

Graft coronary arteriosclerosis currently constitutes the single major limitation to long-term survival of cardiac allograft recipients (1, 2). Concentric intimal thickening composed of variable numbers of T cells, macrophages, and smooth muscle cells beneath MHC class II bearing endothelium characterizes the hallmark lesion of transplant arteriosclerosis (3, 4). Although the precise molecular and cellular events that produce this artieriopathy remain conjectural, they presumably involve multiple factors. For example, immunological differences between host and donor tissues (with resultant cellular and/or humoral immunity) probably contribute to the pathogenesis, although ischemic, infectious, and other etiologies have also been implicated (5). We have hypothesized that graft coronary arteriosclerosis results in part from a persistent delayed-type hypersensitivity response mediated by helper T lymphocytes activated by encountering allogeneic class II MHC antigens (6). Endothelial cells can stimulate such an allogeneic lymphocyte response in vitro (6). T cells that encounter endothelial cells bearing the foreign MHC class II molecules release cytokines that can in turn modulate endothelial cell expression of MHC antigens as well as macrophage activation. Activated T cells hence orchestrate an elaborate cytokine cascade that may regulate the growth, migration, and extracellular matrix metabolism of vascular smooth muscle cells, promoting the development of the fibroproliferative intimal lesions characteristic of graft coronary arteriosclerosis. IFN-γ, a cytokine produced by certain T cell subsets, would presumably occupy a proximal position in this sequence by directly activating macrophages, and by enhancing the expression of MHC products, other components of the antigen presentation pathway, and adhesion molecules (7–10). The present study tested the hypothesis that IFN-γ contributes to the development of graft arterial disease (GAD) in vascularized cardiac allografts, using mice with an IFN-γ deficiency due either to targeted disruption of the IFN-γ gene, or treatment with neutralizing antibody.

Methods

Animals. Inbred mice of several strains were used. C-H-2^b^m12 KhEg (bm12, H-2^b^m12) were used as allograft donors. C57BL6 (B6, H-2^b^) and B6 IFN-γ knockout mouse (B6GKO, H-2^b^) were used as recipients. B6 to B6 isografts were used as controls. The original GKO mice were generated by disrupting the IFN-γ gene; these mice were interbred, and the progeny were selected for IFN-γ-deficiency due either to targeted disruption of the IFN-γ gene, or treatment with neutralizing antibody.

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Received for publication 12 February 1997 and accepted in revised form 2 May 1997.

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/97/08/0550/08 $2.00

Volume 100, Number 3, August 1997, 550–557

http://www.jci.org

1. Abbreviations used in this paper: ECM, extracellular matrix; GAD, graft arterial disease; GKO, IFN-γ knockout; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
run for 40 cycles (thermocycler; Perkin Elmer Corp., Norwalk, CT) using Taq DNA polymerase (BRL, Burlington, Ontario, Canada) at 55°C annealing temperature. The PCR products were run on an ethidium bromide gel; the GKO homozygous mice show a single (neo’) band, the wild-type mice show a single IFN-γ band, and the heterozygotes showed both bands. The heterozygotes were intercrossed, and the homozygous GKO mice were identified by tail DNA testing. Homozygosity of the offspring was confirmed by further tail testing, and by testing each mouse for IFN-γ mRNA expression by reverse transcriptase PCR on spleen RNA at the time of experimental use. All GKO mice used were confirmed homozygotes, and were from the eighth generation (or greater) backcross into the B6 parental strain. B6 with wild-type IFN-γ genes and bm12 mice aged 8–12 wk were obtained from Taconic Farms, Inc. (Germantown, NY) and The Jackson Laboratory (Bar Harbor, ME). The mice were maintained in the Harvard Medical School animal facilities on acidified water. Sentinel animals surveyed serologically for viral pathogens were negative in the room in which these mice were housed. All experiments conformed to approved animal care protocols approved by the institutional review group.

Heart transplantation and immunosuppression. Heterotopic cardiac transplantation was performed using a modification of the method described by Corry et al. (12). In brief, donors and recipients were anesthetized with Metofan (Pittman-Moore, Mundelein, IL). Donor hearts were perfused with chilled and heparinized saline via the inferior vena cava, and were harvested after ligation of the vena cava and pulmonary veins. The aorta and pulmonary artery of donor hearts were anastomosed to the abdominal aorta and inferior vena cava, respectively, of recipients using a microsurgical technique. Ischemic time was routinely 25 min, with a success rate of 90%. The viability of the cardiac allograft was assessed by daily abdominal palpation. Histological evaluation and statistical analysis. Grafts were analyzed by standard hematoxylin and eosin and elastin stains, and the sections were stained with rat monoclonal antibodies against CD45, CD4, CD8, Mac-3, a-actin, MHC class II, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) using standard techniques. The sections were stained using rabbit anti- rat IgG by the avidin–biotin complex method (15), and were counterstained with hematoxylin. Parietal sections were stained with hematoxylin and eosin and the elastic fiber stain using Weigert’s method.

Monoclonal antibodies. 2.43 (anti-CD4) and GK1.5 (anti-CD8) antibodies for immunosuppression were prepared from hybridoma clones (American Type Culture Collection, Germantown, MD) and used as ascs preparations, or from comparable concentrations of antibody prepared from serum-free supernatants in an artificial capillary system (Cellmax; Celluco Inc., Rockville, MD); antibodies for ICAM-1, VCAM-1, CD4, CD8, CD45, Mac-3, and MHC class II were purchased from Pharmingen; antibody to a-actin (IAA) was from Sigma Chemical Co. (St. Louis, MO).

Table I. Parenchymal Rejection and GAD Score

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PR</td>
<td>GAD</td>
<td>PR</td>
</tr>
<tr>
<td>bm12-B6</td>
<td>Anti-CD4 mAb</td>
<td>2.1±0.3</td>
<td>0.4±0.1</td>
<td>2.0±0.5</td>
</tr>
<tr>
<td></td>
<td>Anti-CD8 mAb</td>
<td>(n = 9)</td>
<td></td>
<td>(n = 9)</td>
</tr>
<tr>
<td>bm12-B6GKO</td>
<td>Anti-CD4 mAb</td>
<td>2.0±0.5</td>
<td>0±0.5</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td></td>
<td>Anti-CD8 mAb</td>
<td>(n = 6)</td>
<td></td>
<td>(n = 6)</td>
</tr>
<tr>
<td>bm12-B6</td>
<td>Anti-CD4 mAb</td>
<td>1.4±0.3</td>
<td>0.6±0.5</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td></td>
<td>Anti-CD8 mAb</td>
<td>(n = 4)</td>
<td></td>
<td>(n = 4)</td>
</tr>
<tr>
<td>bm12-B6</td>
<td>Anti-IFN-γ mAb (H-22)</td>
<td></td>
<td></td>
<td>2.8±0.2</td>
</tr>
<tr>
<td></td>
<td>Hamster control IgG</td>
<td>(n = 2)</td>
<td></td>
<td>(n = 2)</td>
</tr>
<tr>
<td>B6-B6</td>
<td>Anti-CD4 mAb</td>
<td>0±0</td>
<td>0±0.5</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>Anti-CD8 mAb</td>
<td>(n = 5)</td>
<td></td>
<td>(n = 3)</td>
</tr>
<tr>
<td>B6-B6</td>
<td>(−) Anti-CD4 mAb</td>
<td>0±0</td>
<td>0±0.5</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>(−) Anti-CD8 mAb</td>
<td>(n = 3)</td>
<td></td>
<td>(n = 3)</td>
</tr>
</tbody>
</table>

PR, parenchymal rejection; *P < 0.01 vs. bm12-B6 with anti-CD4 and -CD8 mAb treatment; †P < 0.05 vs. bm12-B6 with anti-CD4 and -CD8 mAb treatment.

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The cellularity versus extracellular matrix (ECM) content of GAD was assessed blindly by one observer (R.N. Mitchell) using scanned photographic images of all GAD lesions (×66–100) and image analysis (5.2 software; Optimas Corp., Bothell, WA). Thresholds for nuclear versus nonnuclear staining were set using two-pixel averages; intimal lesions delimited by the internal elastic lamina were scored independently for percentage of area encompassed by nuclei (as an approximate measure of cellularity), versus nonnuclear staining (as an approximation of ECM). Areas of nuclear plus nonnuclear staining were normalized to 1. All results are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. *P < 0.05 was considered statistically significant.

Results

All of the bm12 allografts immunosuppressed by treatment with preoperative anti-CD4 and anti-CD8 mAbs continued to beat until harvested (up to 12 wk) in both the wild-type and GKO recipients, although all exhibited substantial myocardial rejection. In the wild-type recipients, rejection was maximal at the earliest time point examined (4 wk), and diminished at later times; the coronary arteriopathy progressively increased with time (Figs. 1 and 2, Table I). When present at 4 wk, the arterial lesions in the transplanted coronary arteries predominantly consisted of accumulations of CD45+ mononuclear cells. At later time points, the lesions became markedly less cellular, with a relative increase in ECM. These later lesions consisted mainly of smooth muscle cells and MAC-3+ macrophages, and morphologically resembled typical human transplantation–associated arteriosclerosis. Some of these fibroproliferative lesions resulted in virtually complete occlusion of the coronary arteries. Many cells in the lesions displayed increased expression of MHC class II, ICAM-1, and VCAM-1 (see Fig. 4, Table II). Except for a single isograft with extensive ischemic injury, isografts showed neither myocardial rejection nor arteriopathy with (n = 13) or without (n = 9) anti-CD4 and anti-CD8 mAb pretreatment (Table I).

In comparison, and despite ongoing parenchymal rejection, allografts in IFN-γ–deficient mice developed little or no arteriopathy (Figs. 1 and 3, Table I). Even at time points up to 12 wk, hearts in the IFN-γ–deficient recipients showed negligible coronary arterial intimal thickening. In addition, the GKO recipient allografts showed markedly attenuated expression of VCAM-1 and ICAM-1 in arterial cells, and of MHC class II in arterial cells and infiltrating leukocytes (Table II, Fig. 4).

Congenital absence of the immunostimulatory cytokine IFN-γ might conceivably alter the ontogeny of the immune response in ways that could influence the development of arterial pathology. Therefore, to verify that deficiency of IFN-γ itself interrupts the development of graft vascular disease, we administered a neutralizing antibody to IFN-γ after transplants into wild-type mice. As in the IFN-γ–deficient animals, hearts of bm12 allografts in B6 wild-type recipients treated

![Figure 1](image-url)
Figure 2. (A) A representative section of an allograft from bm12 to C57/B6, 4 wk after transplantation. The recipient was pretreated with anti-CD4 and anti-CD8 mAbs (elastin stain, ×40). Note the focal mononuclear inflammatory cell infiltration in the parenchyma. Parenchymal rejection grade: 3–4. GAD grade: 1; the cellularity is assessed as 64.4% and the ECM as 35.6%. (B) A representative section of an allograft from bm12 to C57/B6, 8 wk after transplantation (elastin stain, ×50). Extensive infiltrate expanding the intima and compromising the lumen. Parenchymal rejection grade: 3; GAD grade: 4; the cellularity is assessed as 54.8% and the ECM as 45.2%. (C) A representative section of an allograft from bm12 to C57/B6, 12 wk after transplantation (elastin stain, ×33). Marked fibrointimal expansion and luminal stenosis, morphologically resembling typical human graft arteriosclerosis. Parenchymal rejection grade: 2; GAD grade: 3; the cellularity is assessed as 31.8%, and the ECM as 68.2%.

Figure 3. (A) A representative section of an allograft from bm12 to B6 GKO, 4 wk after transplantation (elastin stain, ×40). Although a multifocal mononuclear inflammatory cell infiltration persists in the parenchyma (parenchymal rejection grade: 3), the artery did not have intimal disease (GAD grade: 0). (B) A representative section of an allograft from bm12 to B6 GKO, 8 wk after transplantation (elastin stain, ×50). There is no GAD (parenchymal rejection grade: 3–4; GAD grade: 0). (C) A representative section of an allograft from bm12 to B6 GKO, 12 wk after transplantation (elastin stain, ×33). The coronary artery lacks intimal thickening (parenchymal rejection grade: 3; GAD grade: 0).
with anti–IFN-γ mAb showed little (one of four recipients) or no (three of four recipients) arteriopathy (score: 0.6±0.5, n = 4 at 8 wk). These anti–IFN-γ antibody-treated mouse heart allografts did, however, develop significant myocardial rejection (grade: 1.4±0.3, n = 4 at 8 wk) (Table I, Fig. 5). As in GKO recipients, there was diminished MHC class II, ICAM-1, and VCAM-1 expression in transplant recipients treated with anti–IFN-γ antibodies. In comparison, grafts in mice treated with control nonspecific IgG showed typical rejection and GAD (scores of 3–4) comparable to untreated bm12 to B6 allografts.

**Discussion**

This study used vascularized cardiac transplantation in mice to probe the pathogenesis of the accelerated arteriosclerosis that develops in allografted organs. In the MHC class II–disparate donor–recipient combination studied here, coronary arterial lesions develop that morphologically and immunopathologically resemble those seen in human transplantation arteriosclerosis (6, 17). Although it has not been possible to examine histologically the sequence of events during the development of GAD in human transplants, the mouse model clearly lends itself to such an analysis. 4 wk after transplantation, arteries exhibit a vasculitis characterized by perivascular cuffing and intraluminal accumulations of mononuclear leukocytes (CD45+ cells). By 8 wk, cell-rich intimal lesions have formed. At this stage, the luminal surface of the arteries displays focal thickening developed. Administration of a neutralizing antibody to IFN-γ after heart allografting into wild-type mice verified that absence of IFN-γ rather than some developmental anomaly in the immune response in genetically IFN-γ–deficient mice accounted for the attenuated arteriopathy. These results agree with previous work using anti–IFN-γ antibodies (20).

IFN-γ regulates the proliferation and function of activated T lymphocytes (21–23), and plays a pivotal role in rejection by activating macrophages (24–26). In this study, T lymphocytes and macrophages infiltrated the myocardium and coronary arteries of allografts in the wild-type recipients. In contrast, IFN-γ-deficient recipients did not develop thickening of the arterial intima, despite a T lymphocyte and macrophage infiltrate in the parenchyma. IFN-γ powerfully stimulates the increased synthesis and cell surface expression of both class I and II MHC antigens in vivo (27, 28), including bone marrow–derived immunocompetent cells, as well as vessel wall cells (19, 29–33).

Induction of local tissue injury by LPS in GKO mice revealed an essential role for IFN-γ in the induction of MHC class I and II expression in arterial endothelium (34). Similarly, in the present study, endothelial cells and smooth muscle cells of grafts in GKO recipients displayed markedly diminished MHC class II expression relative to wild-type recipients.

Endothelial cells increase their expression of surface adhesion proteins, including VCAM-1 or ICAM-1, in response to inflammatory cytokines (10, 35–39). In transplanted coronary arteries, medial smooth muscle cells as well as endothelial cells can express VCAM-1 (40). Vascular endothelia, in rejecting mouse and rabbit cardiac allografts, express VCAM-1 (41, 42). IFN-γ may contribute to this process (35, 36, 38). This adhesion molecule upregulation may be important, since treatment with anti–VCAM-1 antibody induces long-term acceptance of murine cardiac allografts (43). ICAM-1 (the receptor for the leukocyte surface molecule LFA-1) may also contribute to acute and/or chronic allograft rejection. Treatment with anti–ICAM-1 and anti–LFA-1 mAbs, for example, inhibits acute cardiac allograft rejection, and induces donor-specific allogeneic skin graft acceptance in mice (44). Coronary arteriosclerotic lesions in transplanted mouse hearts showed abundant ICAM-1, especially on the endothelial surface (40). Treatment with mAbs to ICAM-1 and LFA-1 limited coronary arteriosclerosis in transplanted mouse hearts (45), although the degree to which this finding results from diminished parenchymal rejection is not apparent. In the present study, endothelial and smooth muscle cells in wild-type recipients showed increased VCAM-1 and ICAM-1 expression during develop-
Figure 4. Immunohistochemical staining of representative sections from bm12 to B6 wild-type and bm12 to B6 GKO recipients, 8 wk after transplantation. B6 wild-type recipient grafts displayed increased MHC class II (A, ×50), ICAM-1 (C, ×50), and VCAM-1 (E, ×50). In contrast, B6 GKO recipients showed low levels of MHC class II (B, ×50), ICAM-1 (D, ×50), and VCAM-1 (F, ×50) expression.
The present study used an MHC class II disparate combination to examine the mechanism of development of graft vascular disease. CD4+ T cells interact preferentially with class II MHC molecules, rendering this choice appropriate for study of the effects of IFN-γ, a strong inducer of this class of MHC molecules (46) as well as in activating macrophages. Indeed, in a carotid interposition model, mice deficient in CD8+ T cells or natural killer cells develop significant allograft arterial lesions, while mice deficient in CD4+ T cells or macrophages show reduced intimal lesion formation (25). The possibility exists that the abrogation of GAD is attributable to a diminished MHC class II expression in a strain combination (bm12 to B6) where the immune response depends on class II difference. Similar results, however, were obtained in completely mismatched allografts (BALB/c (H-2b) to B6; data not shown).

In terms of the contribution of IFN-γ, our data show a clear difference in the pathogenesis of GAD versus parenchymal rejection. Although IFN-γ appears necessary for coronary intimal thickening, non–IFN-γ-dependent mechanisms suffice to generate parenchymal rejection. Indeed, in addition to the persistent diffuse mononuclear cell inflammatory infiltrate in the parenchyma, nonimmunosuppressed GKO recipients demonstrate accelerated cardiac allograft failure, indicating a more aggressive rejection (our own observation and reference 47). This mechanistic dichotomy illustrates why the term chronic rejection may be imprecise in context of cardiac allografts. Some transplantation pathologists use chronic rejection to refer to the proliferative intimal lesion of GAD, however, chronic rejection implies a mechanistic commonality with acute rejection, which may not pertain as shown herein. Moreover, acute rejection refers to a myocardial rather than to a vascular process. Because of this potential ambiguity, we advocate avoiding the term chronic rejection altogether. We prefer to use persistent myocardial rejection to describe the smoldering or indolent parenchymal process seen at endomyocardial biopsy, and graft arterial disease or graft vascular disease to describe the vascular pathology.

Our results support the view that the development of GAD results from a response of cells within the donor coronary arteries to stimuli released during a chronic, localized form of delayed-type hypersensitivity to alloantigens on graft-derived vessel wall cells. In such a reaction, host helper T cells activated by encountering foreign histocompatibility antigens secrete IL-2, IFN-γ, and TNF. The present data identify IFN-γ among these cytokines as an essential mediator of GAD. IFN-γ secreted by the T cells, activated by contact with foreign MHC, might trigger the next wave of responses within the transplanted arteries, and stimulate expression of MHC class II, VCAM-1, and ICAM-1 on the surface of vascular endothelial cells, culminating in an accentuated local immune response. IFN-γ might also activate macrophages, with subsequent increased production of other fibrogenic mediators including IL-1, IL-6, PDGF, and TNF-α.

These data establish a critical role for IFN-γ in the pathogenesis of coronary arteriosclerosis in transplanted hearts, but not in myocardial rejection. These data further indicate that reduced expression of class II histocompatibility antigens and leukocyte adhesion molecules may contribute to the mechanism of attenuated coronary arteriopathy in hearts allografted into IFN-γ-deficient mice.

**Acknowledgments**

We wish to express appreciation to Dr. Richard T. Lee (Brigham and Women’s Hospital) for his indispensable support and advice concerning image analysis. We also thank Ms. Eugenia Shvartz, Ms. Elena Rabkin, Ms. Sarah Murray, and Ms. Krista Condon, Brigham and Women’s Hospital, for their excellent technical assistance.

This work was supported by National Institutes of Health grant R01 HL 43364-07.
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