Transplant Coronary Artery Disease
A Novel Model Independent of Cellular Alloimmune Response

Bernard Cantin, MD, PhD; Peizhong Wen, MD; Dening Zhu, MD; Michelle Dai, MSc; Shyam N. Panchal, BSc; Margaret E. Billingham, MD; Judith K. Gwathmey, VMD, PhD; Hannah A. Valantine, MD

Background—Allograft coronary atherosclerosis (TxCAD) is the leading cause of death after the first year after transplantation. TxCAD is believed to be a form of chronic rejection of the cardiac allografts. This study was undertaken to determine whether TxCAD could develop in the absence of a cellular alloimmune response.

Methods and Results—Inbred lean Zucker rats (>26 generations) served as donors and recipients of the cardiac grafts. Donor hearts were explanted at 60 or 90 days. Explanted hearts were processed for coronary artery histological analysis. Cytokine expression was determined by reverse transcription–polymerase chain reaction, and the presence of T cells within the explanted hearts was evaluated by immunohistochemistry. Forty-six transplantations were made, and TxCAD developed in all but one of the transplanted hearts. Overall, one third of the vessels examined were affected by TxCAD, and in roughly half of these vessels, the disease was severe. Native hearts were free of atherosclerosis. Interleukin-2 was absent from the transplanted hearts, and T cells were present in minimal amounts (<1 per low-power field).

Conclusions—TxCAD developed in the absence of a cellular alloimmune response in these genetically similar donors and recipients. The observed TxCAD was significant and comparable to what is found in rat allografting models.

Key Words: transplantation ■ immunology ■ interleukins ■ coronary disease ■ immunohistochemistry

Cardiac allografts may develop an accelerated form of atherosclerosis that has different characteristics from the more traditional atherosclerosis. It is a diffuse disease that is not as easily amenable to standard revascularization techniques. Transplant coronary artery disease (TxCAD) is the leading cause of death after the first year of transplantation.1 The diagnosis of TxCAD in transplant recipients is an ominous sign, with an associated mortality rate of >40% within 2 years.2 TxCAD is widely believed to be a chronic form of rejection, most likely mediated by both major and minor histocompatibility antigens present on the endothelium of the allograft.3 Many animal models of TxCAD have been developed across both major and minor histocompatibility determinants.3–5 Although TxCAD and rejection have developed in these models, the impact of immunosuppressive drugs has been beneficial only on acute rejection, with little impact on TxCAD.6,7 In both experimental and clinical settings, metabolic abnormalities have been shown to affect the development of TxCAD,5,9 leading to the hypothesis that these abnormalities modulate the response to vascular injury induced by ischemia or immunological factors.

We report here on a novel animal model of TxCAD occurring independently of a T cell–mediated alloimmune response. Inbred lean Zucker rats developed significant TxCAD of the isograft without evidence of native heart coronary artery disease or immune activation. This model lends support to the hypothesis that factors other than alloimmunity may be involved in the initiation and evolution of TxCAD.

Methods

Animals and Transplantation Techniques

The experimental protocol was approved by the Stanford University School of Medicine ethics committee. Ninety-two adult male and female inbred (F >26) Zucker lean rats weighing 200 to 300 g were obtained from Genetic Models Inc (Indianapolis, Ind). These rats differ from the traditional Zucker diabetic fat (ZDF) rat in that they do not express the fat phenotype; 44 rats were heterozygous for the leptin receptor gene (fa/−), and the remaining 48 rats were of the wild type (fa/−/−). All animals were housed under conventional conditions and fed Purina rodent chow 5008 for ≥2 weeks before surgery. This diet was resumed as soon as possible after surgery and kept unchanged until euthanization.

Heterotopic abdominal cardiac isograft transplantations were performed by standard microsurgical technique.10 Donors and recipients were matched with regard to the fat genotype, and the matching was confirmed by polymerase chain reaction (PCR) DNA analyses. The ascending aorta and pulmonary artery of the donor heart were anastomosed to the recipient’s abdominal aorta and inferior vena cava, respectively. Surgical infection was controlled by injection of...
penicillin G (100,000 U IM) immediately after surgery. All recipient animals were weighed weekly, and graft function was assessed by daily palpation. Animals were euthanized on day 60 or 90 after transplantation.

**Blood Glucose and Lipid Measurements**

Fasting blood samples (0.5 to 1.0 mL) were obtained from each animal at baseline before surgery and at the time of death for the measurement of glucose, total cholesterol, and triglyceride levels. All animals were fasted for 6 to 7 hours before blood sampling.

**Processing of Cardiac Tissues**

Cardiac grafts that survived to the targeted 60- or 90-day end points were excised. At the time of harvest, donor hearts were snap-frozen in liquid nitrogen and stored at −70°C for histological analysis, immunohistochemical analysis of T cells, and reverse transcription (RT)–PCR analysis of cytokine mRNA.

Elastin–van Gieson’s stain was used for morphological assessment of TxCAD. All vessels in each section that contained an identifiable elastin layer were evaluated for TxCAD. Each section was examined and graded for the severity of TxCAD by a cardiac pathologist (M.E.B.) blinded to the surgical groups and using a scale previously described. Quantitative measurements of the severity of TxCAD in affected vessels were obtained by computerized morphometric analysis using an image analysis system (SIMPLEX, Compix Inc). Tissues were assessed qualitatively and graded for the presence of mononuclear cell infiltrate and myocyte damage according to theBillingham criteria for histological rejection.

**Immunohistochemistry**

The presence of T cells within the isografts was evaluated with an indirect immunoperoxidase technique. Cryostat sections (7 μm) cut from the frozen grafts and embedded in O.C.T. (Sakura Finetechical) were incubated with primary monoclonal antibodies for CD4 and CD8 (Serotec) for 30 minutes. Sections were then washed and incubated with a secondary antibody (Sigma Chemical Co) for 30 minutes. Stained tissues positive for CD4 or CD8 were examined by light microscopy with a ×25 objective. The entire area of each histological section was scanned for positively stained cells. The counts per low-power field (×25) of 8 to 10 randomly selected regions were then averaged and reported as the mean number of CD4- or CD8-positive cells per low-power field for each heart.

**Cytokine Measurements**

Cytokine mRNA levels were determined by measuring levels of cDNA obtained with an RT-PCR technique. Primers for tumor necrosis factor (TNF)-α, interferon (IFN)-γ, transforming growth factor (TGF)-β, interleukin (IL)-2, and IL-6 were obtained from Biosource. Total RNA was extracted from ∼25 to 50 mg of frozen cardiac tissue. PCR products were analyzed on 1.8% agarose gels, and the bands were visualized by ethidium bromide staining. Gel bands were digitized by densitometry (Alpha Imager, Alpha Inno-tech Corp).

**Statistical Analyses**

Differences in the percentage of affected vessels at days 60 and 90 were tested by χ² analyses. Differences in intima thickness, intima index, cytokine levels, T-cell markers, plasma lipids, and glucose were tested by ANOVA. All statistical analyses were carried out with the SAS statistical package.

**Results**

Because there were no differences in metabolic parameters, cytokines, T cells, rejection, and graft vasculopathy between the heterozygous (fa/−) and wild-type rats (−/−), they were pooled for analyses.

The heart transplantations were performed at an average age of 11 ± 2 weeks. The average weight of the rats at the time of operation was 262 ± 44 g. Ischemic time was 30 ± 10 minutes for all transplants. All animals completed their scheduled experimental course without any reduction in isograft function as estimated by daily palpation. The animals euthanized at 60 days had a mean weight of 385 ± 32 g, and those euthanized at 90 days weighed 424 ± 30 g.

**Graft Vasculopathy**

All but one of the transplanted hearts developed TxCAD (Figure). There was no sign of cellular rejection in any of the explanted hearts. The number of affected vessels varied; overall, one third of the vessels examined were affected by TxCAD, and in roughly half of these vessels, the disease was of grade 3 or 4 (Table 1). The percentage of affected vessels and the frequency of vessels with severe disease (grade 3 or 4) were similar among hearts explanted at 60 versus 90 days. Morphometry analyses, however, showed that TxCAD progressed between 60 and 90 days, as reflected by the significant increases in intimal thickness and index (Table 1). The native hearts were also examined according to the same qualitative histological criteria and were found to be free of coronary intimal thickening. Intimal thickness measurements were not performed on native hearts.

**Immunohistochemistry and RT-PCR of Cytokines**

Table 2 shows the results of RT-PCR and immunohistochemical analyses on explanted hearts. Semiquantitative cytokine determinations by RT-PCR showed that IL-2 mRNA was absent from all the transplanted hearts. Over the course of the study, there was a significant increase in IFN-γ and TGF-β. Levels of TNF-α and IL-6 were not different from 60 to 90

<table>
<thead>
<tr>
<th>Days</th>
<th>% Affected Vessels</th>
<th>% Severe Disease</th>
<th>Intima Thickness, μm</th>
<th>Intima Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>39.5 ± 21.8</td>
<td>15.1 ± 19.6</td>
<td>13.19 ± 6.64</td>
<td>0.74 ± 0.20</td>
</tr>
<tr>
<td>90</td>
<td>31.4 ± 17.3</td>
<td>14.5 ± 13.9</td>
<td>20.48 ± 9.85</td>
<td>0.76 ± 0.17</td>
</tr>
</tbody>
</table>

*P* values: *NS* indicates not significant.
days, T-cell markers, as examined by immunohistochemistry, were increased over the course of the study, but the amounts of T-cell markers found remained low, averaging <1 positive cell per low-power field.

Metabolic Measurements

Lipid parameters and fasting glucose levels at the time of transplantation and at the time of death are shown in Table 3. Cholesterol and triglyceride levels did not change over the course of the experiment, but there was a significant increase in fasting plasma glucose at both 60 and 90 days. None of these values were significantly different between animals euthanized at 60 versus 90 days.

Discussion

Immune Mechanisms

Heterotopic cardiac isografting has on occasion led to mild TxCAD, especially in the Lewis-to-Lewis model.13,14 This finding has not been consistent, however, and isografts are frequently used as a control for mismatched models.6–8,11,15 The inbred lean Zucker rats (−/− and fa/−) used in the present experiment are genetically related to the better-known ZDF (Fa/Fa) rat but do not express the fat phenotype or diabetes. The lean Zucker rats developed significant TxCAD of the isograft that was comparable in incidence and severity to several models of transplantation across minor histocompatibility barriers.13,15 On the basis of immunohistochemical and cytokine analysis, however, there was no evidence of cellular alloimmune response in this model.

Immunological mechanisms are believed to play a major role in the development of TxCAD. Lending credence to this hypothesis is the fact that TxCAD affects the whole heart but never progresses beyond suture lines. Conflicting results have been obtained, however, with interventions aimed at modulating the immune response. Some experimental reports on immune mediators and effectors in the different layers of aortic isografts and allografts, Plissonnier et al demonstrated that in nonpresensitized Brown Norway and Lewis isografts, vasculopathy was absent, as were humoral markers other than anti–endothelial cell antibodies. Allografts did develop vasculopathy and have humoral markers and mediators, but these were always accompanied by T cells. To date, the role of humoral immunity in TxCAD remains controversial and poorly documented.25

The data obtained in our study cannot completely exclude the presence of a humoral immune mechanism in TxCAD. Many features of our study and data, however, make the presence of such a mechanism unlikely. Analysis of the vessels affected by TxCAD yielded minimal amounts of effector T cells (<1 per low-power field), and these cells are mediators involved in both cellular and humoral rejection.26 Furthermore, the progression of intima thickness and luminal occlusion over the course of the study suggests an active phenomenon that should have recruited T cells if humoral rejection were still going on. Furthermore, if antibody-mediated mechanisms were involved, we would have expected to find a certain level of disease in the native hearts. We used an inbred model that has minimal immunogenicity, mismatch,16,17 these observations failed to be confirmed by the multicenter observational Cardiac Transplant Research Database in 3837 patients.18 Furthermore, introduction of cyclosporine to the immunosuppressive regimen of heart transplant recipients did not alter the incidence of TxCAD,19 nor have the modern multiple-drug immunosuppressive regimens.20

The role of humoral immunity and anti–endothelial cell antibodies in the genesis of TxCAD has received some attention. Anti–endothelial cell antibodies have been widely documented in transplant recipients. They appear early after transplantation and could lead to some kind of endothelial injury.21 Such antibodies have been documented in human transplant recipients and linked to TxCAD.17,22 Conflicting results have been obtained, however, as to which immunological pathway would be solicited by such antibodies to create TxCAD. Fredrich et al22 suggested that the humoral pathway may be the effector, whereas Hosenpud et al17 showed that this effect was independent of humoral immunity.

It appears that in human heart transplant recipients, the presence of humoral rejection leads to increases in TxCAD and reduction in allograft survival.23 Russell et al23 showed that in presensitized mice, serum transfusions lead to TxCAD. They also showed that the histological features of the TxCAD obtained are distinct from those obtained in models in which both the cellular and humoral activities are present.24 In a systematic study of immune mediators and effectors in the different layers of aortic isografts and allografts, Plissonnier et al demonstrated that in nonpresensitized Brown Norway and Lewis isografts, vasculopathy was absent, as were humoral markers other than anti–endothelial cell antibodies.

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![Table 2. Cytokine and T-Cell Profiles](image-url)

<table>
<thead>
<tr>
<th>IFN-γ</th>
<th>TGF-β</th>
<th>TNF-α</th>
<th>IL-6</th>
<th>IL-2</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 days</td>
<td>0.86±0.37</td>
<td>2.20±0.43</td>
<td>0.78±0.41</td>
<td>0.43±0.30</td>
<td>Absent</td>
<td>0.24±0.16</td>
</tr>
<tr>
<td>90 days</td>
<td>1.56±0.85*</td>
<td>5.06±2.51*</td>
<td>0.56±0.63</td>
<td>1.32±2.41</td>
<td>Absent</td>
<td>0.70±0.35*</td>
</tr>
</tbody>
</table>

Results are average mimic ratio ± SD for cytokines and average of cells per low-power field ± SD for T-cell markers.

*P<0.05 vs 60 days.

![Table 3. Fasting Plasma Lipids and Glucose](image-url)

<table>
<thead>
<tr>
<th>n</th>
<th>Total Cholesterol, mg/dL</th>
<th>Total Triglycerides, mg/dL</th>
<th>Glucose, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>46</td>
<td>66±9</td>
<td>35±13</td>
</tr>
<tr>
<td>60 days</td>
<td>23</td>
<td>64±10</td>
<td>42±15</td>
</tr>
<tr>
<td>90 days</td>
<td>23</td>
<td>72±26</td>
<td>39±8</td>
</tr>
</tbody>
</table>

All values are mean±SD.

*P=0.0001 vs baseline.
as demonstrated by the absence of heart rejection without any kind of immunosuppressive therapy. Furthermore, transplanted hearts remained healthy during the experiment, and none of the animals had to be euthanized for graft failure before the completion of their scheduled course. The possibility of an overlooked minor mismatch is minimal, considering the degree of inbreeding (exceeding 26 generations). Furthermore, minor histocompatibility mismatches are very unlikely in this model, because these antigens trigger reactions that involve the presence of cytotoxic T cells, which were present in only minimal amounts in the transplanted hearts (Table 2). In contrast, Jaques et al., using a minor mismatch model, found significant levels of IL-2 and T cells within transplanted hearts, even in those that were not rejecting. The increases in TNF and TGF may thus be a reflection of the inflammatory process associated with TxCAD. The source of these 2 cytokines in the present model remains unknown, however, because we did not conduct in situ hybridization studies.

Metabolic Influences
We can only speculate as to the exact nature of the trigger of TxCAD we found in the inbred lean Zucker rats. The absence of native coronary artery disease suggests that a factor specific to the transplantation was involved. The possibility of ischemic injury at the time of transplantation as an important trigger of TxCAD has been evoked previously. It has been shown, however, that even 3 hours of ischemic cold storage may not induce significant TxCAD in an isografting model using preserving solutions. Prolonged storage does, however, increase the extent of disease in cardiac allografting models. Tori at al. recently showed, however, that despite retransplantation (thus increased ischemic time), TxCAD does not develop in the absence of function T cells. In the same model, they established that a T-cell reaction, most likely between 3 and 5 days from transplantation, was the triggering event leading to TxCAD. Because we did not examine the transplanted hearts during the initial 30 days, it is possible that a transient T cell–mediated response occurred. The absence of any evidence of myocyte damage, however, suggests that this explanation is unlikely and that a cytotoxic response was not involved in the process of TxCAD in this model.

The clustering of metabolic abnormalities associated with insulin resistance has been shown to be a significant risk factor for coronary artery disease in the general population. In heart transplant recipients, these abnormalities are also found and may play an important role in the progression of TxCAD. We have previously shown that the presence of diabetes is sufficient to induce TxCAD in rat isografts and increases TxCAD in allografts. The lean Zucker rats used in our experiments do not express the profound metabolic abnormalities found in their obese counterparts. Our results, however, show that there was a significant increase in glucose levels over the course of the experiment, raising the possibility that metabolic abnormalities were indeed present and may have contributed to the presence of TxCAD (Table 3). It has been shown previously that, despite quite different lipid profiles, lean and obese Zucker rats have a similar level of insulin resistance. It is thus possible that after heterotopic heart transplantation, these metabolic abnormalities were expressed to a higher level and contributed significantly to a process initiated by some endothelial injury at the time of transplantation. Alternatively, an undefined genetic predisposition to vascular disease may exist in this particular strain of rats.

This is the first rat model that shows the presence of severe TxCAD without major or minor histocompatibility mismatches, thus supporting the concept that it is possible to have TxCAD without an immune response. The exact nature of the triggering event leading to TxCAD is not known, but it is very likely that the metabolic abnormalities present in Zucker rats were sufficient to contribute to the expression of the disease. This unique model will certainly be helpful in studying factors and interventions that could potentially modulate TxCAD in humans.

Acknowledgments
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References
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