In Vivo Blockade of Tumor Necrosis Factor-α in Cholesterol-Fed Rabbits After Cardiac Transplant Inhibits Acute Coronary Artery Neointimal Formation

Nadine Clausell, MD, PhD; Silvana Molossi, MD; Suvro Sett, MD; Marlene Rabinovitch, MD

Background We previously identified in piglet cardiac allografts an immunoinflammatory response in coronary arteries in which increased fibronectin regulated by interleukin-1β was associated with early evidence of intimal thickening. In the present study, we used rabbits to assess whether acute neointimal formation after cardiac transplantation was reduced by blockade of tumor necrosis factor (TNF)-α, which modulates interleukin-1β, or by cyclosporine A.

Methods and Results Sixteen rabbits underwent heterotopic cardiac transplantation and were given saline, TNF-soluble receptor (sr), or cyclosporine A. In host hearts from saline- or TNFsr-treated groups, few coronary arteries (−13% to 16%) had intimal thickening, whereas values were higher in the cyclosporine A–treated group (−30%). In donor hearts from the saline-treated group, however, −68% of vessels had intimal thickening versus −32% in TNFsr- and −30% in cyclosporine A–treated groups (P<.01 for both). Severity of intimal thickening assessed quantitatively as percent vessel area was ~38% in the saline-treated group but reduced in TNFsr- and cyclosporine A–treated groups to −22% and 18%, respectively (P<.01 for each). Immunohistochemistry revealed increased staining for major histocompatibility complex II, T cells, interleukin-1β, TNF-α, and fibronectin in donor coronary arteries from saline-treated animals when compared with TNFsr- and cyclosporine A–treated animals. Grade 3 myocardial rejection was observed in both saline- and TNFsr-treated groups, but only grade 1 was apparent in the cyclosporine A–treated group.

Conclusions In vivo blockade of TNF-α suppresses the acute development of neointimal formation by selectively reducing the vascular immunoinflammatory reaction and accumulation of fibronectin, whereas cyclosporine A suppresses both the myocardial and the vascular immune reaction. (Circulation. 1994;89:2768–2779.)

Key Words • cytokines • vasculopathy • allograft • transplantation • cholesterol

Graft arteriopathy is a major complication of cardiac transplantation and accounts for approximately 40% of deaths in the first 3 postoperative years.1,2 The pathophysiology is thought to be related to an immunoinflammatory reaction in the vessel wall involving activation of endothelial cells, adherence and transendothelial migration of inflammatory cells, and release of cytokines associated with proliferation and migration of smooth muscle cells into the subendothelium and accumulation of extracellular matrix components.3 It is not known whether humoral-mediated vascular reaction or even early cellular reaction, which can be independent of myocardial rejection, is a prerequisite for the later development of the graft arteriopathy.4,6

In our previous studies, we used piglets after heterotopic cardiac transplantation to investigate the mechanisms underlying the development of graft arteriopathy. We demonstrated in the donor coronary arteries endothelial cells expressing major histocompatibility complex (MHC) II molecules associated with adherence and infiltration of T-cell subsets, granulocytes, and macrophages. There were changes in the extracellular matrix (specifically, increased accumulation of fibronectin), especially in the subendothelium and inner media. This feature was accompanied by increased expression of the cytokine interleukin (IL)-1β, primarily in the endothelium and inner media. We speculated that the increase in fibronectin could be modulated by IL-1β in an autocrine or a paracrine manner7 and that the fibronectin might be playing a dual role in directing transendothelial lymphocyte migration and smooth muscle cell migration into the subendothelium.

By culturing smooth muscle cells from host and donor coronary arteries, we demonstrated a cause-and-effect relation between increased IL-1β and fibronectin. We showed that smooth muscle cells cultured from donor coronary arteries produce more fibronectin and IL-1β than those from host arteries and that neutralizing antibodies to IL-1β and the IL-1 receptor antagonist reduced fibronectin synthesis in the donor cells to the level of host cells.8 Further studies have indicated that there also is increased synthesis of fibronectin by donor endothelial cells that is regulated by high levels of endogenous IL-1β.9 We therefore speculated that both paracrine and autocrine mechanisms upregulate synthesis of fibronectin in the donor coronary artery and that fibronectin plays a key role in the development of intimal proliferation associated with graft arteriopathy.

Received November 4, 1993; revision accepted February 26, 1994.

From the Division of Cardiovascular Research, The Hospital for Sick Children, and the Departments of Pediatrics, Pathology (N.C., S.M., M.R.), and Surgery (S.S.), University of Toronto, Canada.

Correspondence to Marlene Rabinovitch, MD, The Hospital for Sick Children, Division of Cardiovascular Research, 555 University Ave, Toronto, Ontario, Canada M5G 1X8.
A number of agents have been tested experimentally for their ability to block the development of graft arteriopathy after cardiac transplantation. For example, in rabbits, the use of angiopeptin, a somatostatin analogue, reduced intimal lesions by approximately 30% to 50%.10 This same group of investigators demonstrated similar beneficial effects using estradiol.11 Recently, the use of antibodies to block the expression of adhesion molecules in the grafted endothelium was reported to reduce by 45% the number of vascular lesions in rabbits.12 Although anticytokine therapy, with either antibodies13 or soluble receptors, has been used to study allograft rejection,14 the efficacy in controlling graft arteriopathy remains unexplored.

Tumor necrosis factor (TNF)-α mediates intragraft T-cell immune responses in the rejection process.15 Increased expression of TNF-α in a rat cardiac allograft was described,14 and increased circulating levels of this cytokine were related to rejection in liver transplantation.16 The use of anti-TNF antibodies in combination with cyclosporine A prolonged rat cardiac allograft survival.13 Although the involvement of TNF-α in the pathogenesis of the graft arteriopathy has not specifically addressed, it is likely to play an important role given its multiple biological activities related to endothelial activation18; increased expression of adhesion molecules19; induction of other cytokines, especially IL-1β; and angiogenesis.20 Although Mauviel and collaborators21 showed that TNF-α downregulated fibronectin synthesis in human fibroblasts, Varani and colleagues22 demonstrated upregulation of fibronectin in normal melanocytes and malignant melanoma cells by both TNF-α and interferon-γ. Moreover, recent in vitro studies in our laboratory indicate that TNF-α may initiate a cytokine cascade whereby it induces IL-8 and increases fibronectin synthesis in piglet coronary artery smooth muscle cells.23

The introduction of cyclosporine A for immunosuppressive therapy after cardiac transplantation has had a major impact in prolonging patient survival. However, despite control of myocardial rejection, the appearance of graft arteriopathy lesions after transplantation does not appear to be reduced by the use of cyclosporine A. On the contrary, a trend toward more vascular lesions in patients treated with cyclosporine A was observed.24 The possible mechanisms involved in mediation of the vascular lesions by cyclosporine A remain speculative, but it is thought that cyclosporine A–induced high plasma lipoproteins and systemic hypertension may play a role.25,26 In a rabbit model of carotid artery endothelial denudation, Ferns and coworkers27 have more specifically addressed this issue and showed that in cyclosporine A–treated rabbits, there was increased intimal thickening, mainly as a result of smooth muscle cell vacuolization and accumulation of foam cells.

In the present study, we used cholesterol-fed rabbits after heterotopic cardiac transplantation following a protocol that produces coronary intimal lesions in a short experimental period28 and neutralized TNF-α with a human recombinant TNF-α–soluble receptor (TNFRs) to investigate whether this might prevent or reduce the development of vascular lesions. We also studied a group of cyclosporine A–treated animals to assess whether the expected cyclosporine A–induced reduction of myocardial rejection was associated with modulation of the vascular lesions. In the TNFRs-treated animals, despite severe myocardial rejection, we observed a marked reduction in the number of vessels with intimal proliferation compared with the control group. Moreover, the degree of intimal proliferation in the affected vessels was less severe. Immunohistochemistry for T cells, MHC II antigens, IL-1β, TNF-α, and fibronectin revealed that the mechanism of reduction in intimal proliferation by TNF-α blockade was associated with suppression of the immunoinflammatory response, the accumulation of fibronectin, or both. In the cyclosporine A–treated group, only mild myocardial rejection was observed, and this was accompanied by a reduction in the vascular lesions and associated immunoinflammatory-related features. Thus, it appears that the vascular lesions observed in this early time frame may be initiated by the rejection process, but their selective downregulation by TNFRs reflects a more independent pathophysiological evolution.

Methods

Experimental Animal Preparation

New Zealand White female rabbits (Charles River Laboratories) weighing between 3.5 and 4.0 kg underwent heterotopic cardiac transplantation following an experimental protocol previously described28 and approved by the Animal Care Committee of The Hospital for Sick Children, Toronto. The animals were not selected to favor an HLA mismatch, as the host was Pasteurella free and the donor was an outbred animal. Both host and donor rabbits were fed Purina 5321 0.5% cholesterol diet (Research Diets Inc) starting 4 days before transplantation, and the recipient was continued on the diet until completion of the experimental period. The anesthesia consisted of face-mask induction with halothane followed by 0.3 mg atropine IM plus 3 mg/kg acepromazine IM. During the surgical procedure, the animals were ventilated with a final concentration of 1.5% halothane in a mixture of 50% nitrous oxide and 50% oxygen.

A vertical incision was performed in the anterior aspect of the neck of the recipient rabbit, and the left common carotid artery and the ipsilateral external jugular vein were isolated. A thoracotomy was performed in the donor rabbit, and the heart was quickly removed and immersed in cold Ringer’s solution. The cardiac allograft was placed in the neck by anastomosing the donor aorta end-to-side to the host carotid artery and the donor pulmonary artery end-to-side to the host external jugular vein.29 The vena cava and pulmonary veins were ligated so that the blood from the host common carotid artery perfused the donor coronary arteries and the myocardium, and the donor venous blood from the pulmonary artery returned to the host venous circulation via the external jugular vein. The period of ischemia to which the donor hearts were subjected varied from 20 to 25 minutes. After the surgery, the animals received penicillin-G (0.5 g IM) and standard postoperative care in compliance with guidelines formulated by the Canadian Council of Animal Care.

Beginning on the day of transplantation, animals were randomized and treated with either 1 mL of saline solution SC daily (control group, n = 5), with TNFRs (a gift from Dr C. Jacobs, Immunex Corp, Seattle, Wash; 2.5 mg/kg [1 mL] SC; n = 6), or with cyclosporine A (Sandimmune, Sandoz; 10 mg/kg SC on alternate days; n = 5). Cyclosporine levels were monitored and were in the therapeutic range, ie, 218 to 332 ng/mL. No other immunosuppression therapy was administered. The grafts were monitored daily by palpation. Based on pilot studies and the work of others using this model,28 we decided to terminate the experimental period at 10 days, a point at which coronary lesions could be expected. In two animals—one from the saline-treated group and one from the TNFRs-
treated group—it was, however, necessary to terminate the experimental period at 9 days because of the absence of a palpable heartbeat in the graft.

**Preparation of the Hearts**

After the animals were killed by a lethal dose of euthanol (480 mg IV), host and donor hearts were removed and perfused with saline through the aorta followed by light fixation by perfusion with 2% paraformaldehyde (Sigma Chemical Co). Because of previous descriptions indicating that cardiac graft arteriopathy in the rabbit is equally distributed throughout the coronary circulation,10 the hearts were then sectioned transversely from base to apex. Different sections of the hearts were then saved in 10% Formalin for light microscopy or immediately frozen in O.T.C Compound Tissue Tek (Miles Inc) for specific immunohistochemistry studies.

**Grading of Rejection**

Tissue specimens from the donor hearts were also fixed in 10% Formalin and subsequently stained with hematoxylin and cosin for histological grading of rejection according to Billingham’s criteria: grade 0, absence of rejection; grade 1, mild rejection (scant perivascular and endocardial lymphocytic infiltration); grade 2, moderate rejection (prominent interstitial and perivascular lymphocytic infiltration with early focal myocytolysis); and grade 3, severe rejection (interstitial hemorhage with lymphocytic and polymorphonuclear leuocyte infiltration and prominent myocytolysis). The sections were graded by one of the senior pathologists (Dr G.J. Wilson) at The Hospital for Sick Children without knowledge of whether the donor hearts came from untreated, TNFsr-treated, or cyclosporine A–treated animals.

**Qualitative Assessment of Fat Infiltration in Host and Donor Hearts**

To assess whether there were differences in the amount of fat infiltrating both host and donor hearts as well as the presence of fatty arterial lesions, we performed standard oil Red O staining on frozen heart sections from all animals in the saline-, TNFsr-, and cyclosporine A–treated groups.

**Quantitative Assessment of Coronary Arteries by Light Microscopy**

Three different tissue sections from both host and donor hearts from saline-, TNFsr-, and cyclosporine A–treated rabbits were fixed in 10% Formalin, embedded in paraffin, and stained by the Movat pentachrome method. Light microscopic morphometric analysis was carried out using a Zeiss microscope attached to a computer-generated video analysis system (New Vision Percepts). In each section, the diameter of each vessel was measured, and the coronary arteries were categorized as small (diameter, ≤100 μm), medium (diameter, >100≤500 μm), or large (diameter, >500 μm). For each heart, the total number of vessels in each size category with intimal proliferation was expressed as a percent of total vessel number. The severity of intimal thickening was quantitatively assessed in each affected vessel according to a method previously described.8,9 The areas encompassed by the outer medial layer (ML), the internal elastic lamina (IEL), and the lumen were measured, and the area of intimal thickening (IT) related to the vessel area was calculated by the formula: IT = (IEL – lumen area)/(ML – lumen area) × 100. In the host hearts, a total of 643 coronary arteries in the saline-treated group, 729 in the TNFsr-treated group, and 246 in the cyclosporine A–treated group were analyzed. In the donor hearts, a total of 566 coronary arteries in the saline-treated group, 721 in the TNFsr-treated group, and 240 in the cyclosporine A–treated group were analyzed.

**Immunohistochemistry Studies**

In all immunohistochemistry analyses, we compared coronary arteries from host and donor hearts; from saline control, TNFsr, and cyclosporine A groups; in the different size ranges; and with and without intimal proliferation. The relative abundance of each specific antigen studied in the sections examined was graded semiquantitatively as minimal (+), little (++), moderately abundant (+++), or very abundant (++++) by three of the authors independently (N.C., S.M., and M.R.). The final scoring was based on the average of the individual gradings. There was complete agreement in the grading of more than 80% of the cases.

**Characterization of Inflammatory Cells**

To determine the presence of an immune reaction and inflammatory cells adherent to the coronary artery endothelium and within the vessel wall, we performed immunoperoxidase staining using monoclonal antibodies to rabbit MHC II antigens and rabbit T cells (both IgG2 subtypes and ascitcs fluid were gifts from Dr Peter Libby, Brigham and Women’s Hospital, Boston, Mass). Frozen sections were air-dried for 2 hours, fixed in acetone for 20 minutes, and rinsed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by immersing the sections in PBS plus 1% hydrogen peroxide (BDH) for 30 minutes. After a nonspecific blocking step using 5% normal goat serum (Jackson ImmunoResearch Laboratories, Inc), we applied the antibodies to the sections for 1 hour at a 1:10 dilution at room temperature. The sections were then rinsed, incubated with goat anti-mouse peroxidase-conjugated secondary antibody (Bio-Rad) for 45 minutes, and developed with 3,3′-diaminobenzidine (DAB) (Sigma) for 10 minutes. Control sections were treated with PBS only and/or with normal mouse isotypic IgG2 (Dako Corp).

**Assessment of Interleukin-1β**

To determine whether there was a qualitative difference in the localization or amount of IL-1β in host and donor coronary arteries in the different groups studied, we performed immunoperoxidase staining using an affinity purified polyclonal goat anti-rabbit IgG IL-1β antibody (starting concentration, 1 mg/mL; Cytokine Science). Frozen sections were air-dried for 2 hours followed by permeabilization for 30 minutes with Triton X 100 (BDH) in 1% hydrogen peroxide in PBS to block endogenous peroxidase activity. After a nonspecific blocking step with normal swine serum (Jackson), the primary antibody anti-IL-1β was applied to the sections at a 1:100 dilution for 1 hour at room temperature. The sections were rinsed in PBS, incubated with swine anti-goat peroxidase-conjugated antibody (Tago Inc) at a 1:50 dilution for 45 minutes, and rinsed in PBS. The sections were then developed with DAB. Control sections were treated with normal goat serum (Dako) and/or PBS.

**Assessment of TNF-α**

To determine whether there was a qualitative difference in the expression of TNF-α in the coronary arteries, we performed immunoperoxidase staining using a monoclonal antibody to rabbit TNF-α (IgG2 subtype in ascites fluid; a kind gift from Dr Hideo Naruchi, University of Tokyo, Japan). Frozen sections were air-dried for 2 hours, fixed in acetone for 20 minutes, and rinsed with PBS. Endogenous peroxidase activity was blocked by immersing the sections in PBS plus 1% hydrogen peroxide for 30 minutes. After a nonspecific blocking step using 5% normal goat serum, the antibodies were applied to the sections at a 1:100 dilution overnight at 4°C. The sections were then rinsed, incubated with goat anti-mouse peroxidase-conjugated secondary antibody for 45 minutes, and developed with DAB for 10 minutes. Control sections were treated with PBS only and/or with normal mouse isotypic IgG2.
Assessment of Fibronectin

Immunoperoxidase staining was performed using deparaffinized sections. The specimens were immersed for 30 minutes in 1% hydrogen peroxide solution in methanol to block endogenous peroxidase activity and then submitted to enzyme digestion with 0.1% pronase solution (Boehringer-Mannheim) in Tris buffer (Sigma) for 30 minutes at 37°C. After a nonspecific blocking step with 5% normal goat serum, the sections were incubated overnight with a monoclonal IgG anti-human cellular fibronectin antibody (ascites fluid; UBI) at a 1:50 dilution at 4°C. Then the sections were rinsed and incubated for 45 minutes at a 1:50 dilution of goat anti-mouse peroxidase-conjugated antibody at room temperature, washed in PBS, and developed with DAB. Control sections were treated with PBS only and/or with normal mouse isotypic IgG.

Statistical Analysis

The data were expressed as mean±SEM. For comparisons between host and donor coronary arteries from the saline-, TNFsr-, and cyclosporine A–treated groups, related both to the number of vessels with intimal thickening and to the severity of the lesions in affected vessels, one-way ANOVA with post-hoc paired subgroup testing performed by Newman-Keuls analysis was performed, taking into account P value correction for multiple comparisons. The differences were considered statistically significant at P<.05.

Results

Assessment of Myocardial Rejection

Analysis of hematoxylin and eosin sections of the rabbit cardiac allografts revealed a similar degree of severe rejection in both the saline- and TNFsr-treated groups with extensive lymphocytic infiltration, myocyte necrosis, and hemorrhage (Fig 1B and 1C). In the cyclosporine A–treated group, however, only mild rejection was observed, with scant lymphocytic infiltration and mostly a normal-appearing myocardium, similar to the host heart (A). Magnification ×100.

Assessment of Infiltration of Fat

There was minimal fat accumulation observed, as judged by oil Red O staining, in both the myocardium and the vessels from the host saline-, TNFsr-, and cyclosporine A–treated hearts. In the donor hearts of the cyclosporine A–treated group, however, the staining for oil Red O in the myocardium was slightly increased compared with that observed in the saline and the TNFsr groups, but the staining was negative in the coronary arteries. There were, however, more animals in which oil Red O staining in the coronary arteries was positive in the TNFsr group compared with the saline group (three of six versus one of five).

Light Microscopic Morphometric Assessment of the Coronary Arteries

We observed in the host hearts in the TNFsr- and saline-treated groups a similar small proportion of coronary arteries with intimal proliferation (16±3.4% and 13±1.6%, respectively), but there was a significant
increase in the number of vessels affected in the cyclosporine A–treated group (30±3.2%, \*P < .01, versus saline- and TNFsr-treated groups). In the donor hearts of the saline group, however, 68±4.3% of the vessels had lesions, whereas in the TNFsr and cyclosporine A groups, there was a significant reduction in the number of vessels with intimal proliferation to 35±7.1% and 32±0.84%, respectively (\(P < .01\) for both versus saline) (Figs 2 and 3). The proportion of affected vessels was similar in the small (≤ 100-μm diameter) and medium-size (> 100≤500-μm diameter) vessels, but the larger vessels appeared to be relatively spared.

The severity of the intimal thickening in the affected vessels was also assessed in each group. We observed overall a similar degree of intimal thickening, as judged as percent of vessel area, in the coronary arteries from the host hearts in the saline-, TNFsr-, and cyclosporine A–treated groups (21±1.59%, 23±1.73%, and 22±4.4%, respectively). There was, however, a significant increase in the severity of intimal thickening in the small vessels (< 100 μm) in the cyclosporine A group compared with the saline group (\(P < .05\)). In the coronary arteries from the donor hearts in the saline group, the degree of intimal thickening, judged as percent of vessel area, was almost twofold greater, ie, 38±2.43%, whereas it was only 22±1.08% in the TNFsr group and 18.4±1.63% in the cyclosporine A group (\(P < .01\) for both versus saline), values that were comparable to those observed in the host coronary arteries. The reduction in severity of intimal thickening was seen in small (≤ 100-μm diameter) and medium-size (> 100≤500-μm diameter) vessels in each group but was especially evident in the medium-size vessels of the cyclosporine A group compared with the saline group (\(P < .01\)) (Fig 4).

![Fig 2. Representative photomicrographs of a normal-appearing host coronary artery (A) and a donor coronary artery with marked intimal thickening (B) from rabbit cardiac allografts from the saline group. Movat pentachrome stain. Magnification x200.](http://circ.ahajournals.org/)

![Fig 3. Bar graph of effect of administration of tumor necrosis factor–soluble receptor (TNFsr) or cyclosporine A (CsA) on the number of vessels with intimal proliferation from host and donor hearts. A decrease in the number of vessels with intimal proliferation is observed in the donor hearts from the TNFsr and CsA groups compared with the saline group (\(P < .01\)). In the host hearts, there is no significant difference related to the use of TNFsr compared with the saline group with respect to the incidence of vessels with graft arteriopathy, but there is an increase in the number of vessels affected in the CsA group compared with the saline and TNFsr groups (\(P < .01\)). In the host hearts, a total number of 643 coronary arteries in the saline-treated group, 729 in the TNFsr-treated group, and 246 in the CsA-treated group were analyzed. In the donor hearts, a total number of 566 coronary arteries in the saline-treated group, 721 in the TNFsr-treated group, and 240 in the CsA-treated group were analyzed.](http://circ.ahajournals.org/)

![Fig 4. Bar graph of effect of administration of tumor necrosis factor–soluble receptor (TNFsr) or cyclosporine A (CsA) on the severity of the arterial lesions as assessed by intimal thickening (percent of total vessel area) related to vessel size in host and donor coronary arteries. A reduction in the degree of intimal thickening, similar to the levels in the host coronary arteries of the saline group, is observed in both small (\(P < .01\)) and medium-size (\(P < .05\)) donor coronary arteries from the TNFsr- and CsA-treated groups compared with the saline group. No significant effect is observed with the use of the TNFsr on the severity of intimal lesions in the host coronary arteries, but there is an increase in the intimal thickening observed in the small host vessels from the CsA group (\(P < .05\)).](http://circ.ahajournals.org/)
Characterization of the Immunoinflammatory Reaction

Immunohistochemical analysis of inflammation revealed negative staining for MHC II molecules or T cells (Fig 5A and 5E) in host heart coronary arteries in all rabbits from saline-, TNFsr-, and cyclosporine A–treated groups and positive staining (+ to +++) for MHC II molecules (Fig 5B) and T cells (+ to ++++) (Fig 5F) in donor coronary arteries in all animals from the saline group (Table). In the TNFsr-treated group, however, positive staining for MHC II antigens was present in only three of six allografts, and positive staining for T cells was present in only two of six animals. In the remaining animals, the expression of these markers in the donor coronary arteries was similar to that observed in the host arteries (Fig 5C and 5G). The expression of these molecules was more consistently downregulated in the cyclosporine A–treated group with minimal staining for MHC II antigens present in only one of five animals (Fig 5D) and for T cells in two of six animals (Fig 5H). In all groups, positive staining for these markers was observed in small and medium-size vessels with and without intimal lesions. The three of six animals from the TNFsr group in which MHC II expression was not present also had no T-cell infiltration, suggesting a coordinate downregulation of these features by the TNFsr. This pattern was also observed in the cyclosporine A group.

Morphological Localization of IL-1β in the Coronary Arteries

In host coronary arteries from the saline-, TNFsr-, and cyclosporine A–treated groups, the intensity of immunoperoxidase staining for IL-1β was negative to minimal (− to ±) (Fig 6A). In donor vessels in four of five animals from the saline group, more intense IL-1β immunostaining was observed (+ in one and ++ in three) (Table), which was predominantly localized to the endothelium and immediate subendothelium (Fig 6B). Occasionally, positive staining was associated with individual cells that appeared to be inflammatory cells. In donor coronary arteries from the TNFsr group, the intensity of the immunostaining was similar to host vessels (− to ±) in three of six animals and only little (+) in the remaining three animals (Fig 6C). In the cyclosporine A group, minimal staining was observed in only one of five animals, with the remainder negative (Fig 6D). Thus, the intensity of immunostaining for
Findings in Coronary Arteries From Cholesterol-Fed Rabbits After Cardiac Transplantation

<table>
<thead>
<tr>
<th>Animal</th>
<th>Group</th>
<th>Myocardial Rejection Grade</th>
<th>MHC II</th>
<th>T Cells</th>
<th>TNF</th>
<th>IL-1β</th>
<th>FN</th>
<th>No. of Vessels With IT (% total)</th>
<th>Severity of IT (% vessel area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>55</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>Saline</td>
<td>3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>63</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>Saline</td>
<td>3</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>77</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>3</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>77</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>Saline</td>
<td>3</td>
<td>++</td>
<td>+++</td>
<td>±</td>
<td>++</td>
<td>++</td>
<td>72</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>TNFsr</td>
<td>3</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>TNFsr</td>
<td>3</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>TNFsr</td>
<td>3</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>44</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>TNFsr</td>
<td>3</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>TNFsr</td>
<td>3</td>
<td>+++</td>
<td>+++</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>67</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>TNFsr</td>
<td>3</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>12</td>
<td>CsA</td>
<td>1</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td>13</td>
<td>CsA</td>
<td>1</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>14</td>
<td>CsA</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>+</td>
<td>31</td>
<td>14</td>
</tr>
<tr>
<td>15</td>
<td>CsA</td>
<td>1</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>16</td>
<td>CsA</td>
<td>1</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>31</td>
<td>24</td>
</tr>
</tbody>
</table>

MHC II indicates major histocompatibility complex II; TNF, tumor necrosis factor; IL-1β, interleukin-1β; FN, fibronectin; IT, intimal thickening; TNFsr, tumor necrosis factor-soluble receptor; and CsA, cyclosporine. - , ± , + , ++ , and +++ indicate negative, minimal, little, moderately abundant, and very abundant, respectively.

IL-1β overall was lower in the donor coronary arteries from the TNFsr group and more so in the cyclosporine A–treated group (Table). In the saline group, negative immunostaining for IL-1β in one animal was associated with reduced MHC II expression, whereas in two of three of the TNFsr-treated animals with increased IL-1 immunostaining, MHC II staining was also intense. This suggests that the suppression of IL-1 by the TNFsr might also be coordinately related to MHC II expression. This is also evident by the overall reduction in the immunoinflammatory reaction observed in the cyclosporine A group associated with less myocardial rejection. The pattern of immunostaining for IL-1β was similar in small and medium-size vessels and was not related to the degree of intimal thickening.

**Morphological Localization of TNF-α in the Coronary Arteries**

In the host coronary arteries from the saline-, TNFsr-, and cyclosporine A–treated groups, there was negative to

---

**Fig 6.** Representative photomicrographs of immunoperoxidase staining for interleukin-1β performed in host (A) and donor coronary arteries from a saline- (B), tumor necrosis factor-soluble receptor (TNFsr) (C), and cyclosporine A (CsA)-treated rabbit (D). Positive staining is observed in the donor coronary artery from the saline-treated rabbit, and decreased intensity in the staining can be appreciated in the coronary arteries from both the TNFsr- and the CsA-treated rabbits. No positive staining is observed in the host coronary artery from a saline-treated rabbit. Magnification ×150.
minimal immunostaining for TNF-α (Fig 7A). In the donor coronary arteries from the saline group, four of five animals had more intense staining for TNF-α (Fig 7B) (Table). In both TNFsr and cyclosporine A groups, however, there was reduction in the expression of TNF-α in five of five and in four of five animals, respectively (Fig 7C and 7D), similar to that observed in the host arteries (Table). Thus, neutralization of TNF-α activity and the use of cyclosporine A were equally effective in reducing the accumulation of TNF-α in the donor coronary arteries.

**Morphological Localization of Fibronectin in the Coronary Arteries**

The intensity of immunostaining for fibronectin was similar in all host coronary arteries from the saline-, TNFsr-, and cyclosporine A–treated groups (± to +) (Fig 8A) (Table). In all donor coronary arteries from the saline group, the intensity of staining was increased (++ to ++++) (Fig 8B). In the TNFsr group (Fig 8C), we observed reduced intensity of the fibronectin staining, which was comparable to that of the host vessels in four of six animals. In the cyclosporine A group, there was a similar reduction in fibronectin staining in all animals (Fig 8D). The intensity of the fibronectin staining observed in the vessels was similar in both small and medium-size vessels but less so in the larger arteries. Also, accumulation of fibronectin in the donor coronary arteries was observed in vessels with and without intimal proliferation (Fig 8B) and in vessels with both concentric and eccentric lesions (Fig 8E through 8H). It was interesting that in the TNFsr group, the three animals with the fewest vessels with intimal thickening (20% to 27%) had both reduced T-cell infiltration and fibronectin accumulation compared with the three other animals, in which a somewhat higher proportion of affected vessels (38% to 67%) was associated with either T-cell infiltration or increased fibronectin accumulation.

**Discussion**

In the present study, we demonstrated that in vivo blockade of TNF-α selectively decreases the incidence and severity of acute coronary artery lesions in rabbits after cardiac transplantation. This was associated with reduced accumulation of fibronectin in the vessel wall and/or decreased vascular immunostaining for MHC II antigens, T cells, and IL-1β, despite grade 3 (severe) rejection in the myocardium. In the cyclosporine A–treated group, a similar reduction in vascular lesions was associated with a decreased immune response, as indicated by grade 1 (mild) rejection in the myocardium. These observations suggest a role for TNF-α more specifically related to the pathogenesis of the allograft intimal thickening and a therapeutic approach, which may interfere with the development of this disease.

After heart transplantation, rabbits fed a cholesterol-rich diet provide a useful experimental model to study events related to the development of graft arteriopathy. This model was first described by Alonso and colleagues in a study using four different experimental groups in which a cholesterol-rich diet was used in two of the groups to accelerate the development of the graft arteriopathy in both the presence and the absence of immunosuppressors. Although we have not measured the concentration of lipids in the rabbit serum, other studies in which the same diet regimen was used have shown a fourfold to fivefold increase in circulating lipid levels. In the present study, we carried out oil Red O staining of heart sections in all animals studied. We observed a tendency toward more myocardial fat infiltration in the cyclosporine A group than in the other two groups and more donor vessels with fat infiltration in the TNFsr group than in the saline and cyclosporine A groups. Thus, we could not relate the presence of fat to the evolution of the vascular lesions. Because high cholesterol levels are not uncommon in patients after cardiac transplantation and are thought to be partially the result of the use of cyclosporine A, hypercholesterolemic experimental models, rather than confounding the study of graft arteriopathy development, may better simulate the clinical setting. It is interesting that a high cholesterol diet per se can induce the expression of adhesion molecules on the rabbit endothelium.

This could accentuate the immunoinflammatory reaction likely to be present in the vessel wall associated with the development of graft arteriopathy.
In some of the studies with rabbits, an immunosuppressive therapy regimen is instituted after cardiac transplantation that usually includes cyclosporine A with or without azathioprine and with or without steroids.\textsuperscript{10,31} In the present study, we chose not to use any immunosuppressive agent in the saline control group to allow full development of the immunoinflammatory reaction and the associated cytokine activity. By studying one experimental group treated with cyclosporine A only, which is known to effectively block cellular rejection, we were able to assess the selectivity of TNFsr treatment on the development of intimal thickening. Because of impending myocardial rejection in both the saline and TNFsr groups, we terminated our experimental period at 9 or 10 days. This experimental period is comparable to that reported by Alonso and colleagues\textsuperscript{28} in their experimental groups receiving no immunosuppression.

We have previously described the development of early intimal thickening in piglets receiving a normal diet after cardiac transplantation in the absence and presence of cyclosporine A, albeit in low dosage. Although we examined only epicardial coronary arteries because we used them as sources of cultured cells, intimal thickening in the donor vessels was apparent only at the light microscopic level in 1 of 14 cases. At the electron microscopic level, however, intimal thickening in the donor vessels was evident in all animals and included widening of the subendothelial space with infiltration of inflammatory cells and increased numbers of smooth muscle cells. Immunohistochemistry revealed a local immunoinflammatory reaction with accumulation of fibronectin and IL-1β in the arterial wall.\textsuperscript{7} We considered these findings to be early changes related to the development of graft arteriopathy, and they directed our investigations in the rabbit model.

In the present study, rabbits were used instead of piglets because the scarcity of TNFsr made it unfeasible to administer therapeutic doses to large animals. Comparisons between the two animal models should, however, be made carefully because of the differences in protocols and biological behavior. In the rabbits, approximately 14% of the host coronary arteries had intimal thickening, and the lesions were similar in incidence and severity in untreated and TNFsr-treated groups. Our observations are similar to those of Alonso.
and colleagues, so it is possible that although cholesterol contributes to the development of the lesions in the native vessels, cytokines do not, or at least TNF-α does not, appear to be involved. Interestingly, however, in the host coronary arteries of the cyclosporine A–treated animals, there was a significant increase in the number of vessels with intimal thickening, which suggests, as others have indicated, that cyclosporine A may have independent adverse effects on the vessel wall. It is also possible that the cyclosporine A effect might be related to a high cholesterol level. Although cholesterol levels were not measured, there was more myocardial fat infiltration in this group.

In the rabbit donor hearts from the saline-treated group, we observed the development of neointimal formation in almost 70% of the small and medium-size vessels, and the severity of intimal thickening was approximately 40% of the vessel area. The larger arteries appeared to be relatively spared, which is in keeping with the observations by Eich et al and which, in our study, could be attributed to the relative short experimental period. In the donor hearts from TNFsr-treated animals, we observed a 50% reduction in the number of the vessels with neointimal formation and a similar reduction in the severity of the lesions, as judged by the intimal thickness. In contrast to the observations by Eich and collaborators, which showed a reduction of lesions in both native and transplanted hearts in rabbits treated with dehydroepiandrosterone, the protective effect of TNFsr demonstrated in the present study was selectively observed in the vessels from the donor hearts. Others have shown that the use of estradiol reduces by 60% the severity of the graft arteriopathy lesions in rabbit cardiac allografts, which was accompanied by a protective effect in the native vessels. The use of angiopeptin inhibited intimal hyperplasia in graft arteriopathy lesions in rabbits by approximately 50% and was associated with decreased smooth muscle cell thymidine incorporation. Although these studies report beneficial effects of different agents that appear to act through relatively nonspecific antiproliferative mechanisms, our data suggest that by neutralizing TNF-α activity, a more specific pathophysiological mechanism is selectively blocked.

In the present study, the positive identification of MHC II molecules and T cells in rabbit donor coronary arteries was similar to our findings in piglets. Although these changes were consistent in all animals in the saline-treated group, considerable variability was observed among the TNFsr-treated animals, suggesting that the immunoinflammatory process was not equally suppressed in all cases. Similar to our findings in piglets, there was increased immunostaining for IL-1β in rabbit donor coronary arteries. The fact that it was not seen in all rabbits in the saline group may indicate that its expression is transient. The consistent reduction in IL-1β immunostaining in the donor coronary arteries in the TNFsr-treated animals suggests that its expression was suppressed. In the cyclosporine A group, there was consistent reduction in the expression of MHC II, T cells, and IL-1β, indicating a generalized immunosuppressive effect.

In the donor coronary arteries from the saline-treated group, a consistent increase in fibronectin accumulation was observed; this is also in keeping with our findings in the piglets after heterotopic cardiac transplantation. This feature was present in the rabbit donor coronary arteries with and without neointimal formation. In the TNFsr-treated animals, the variability in intensity of immunostaining for coronary artery fibronectin indicates that its expression was not always downregulated. It is interesting that the best results observed with TNFsr in terms of suppression of intimal proliferation were associated with a reduction in both fibronectin expression and the immunoinflammatory reaction. Although we speculate, based on observations in vitro, that TNF induces an increase in donor coronary artery fibronectin through IL-1β, this is difficult to address in the present study, given the variability in immunostaining. In the cyclosporine A group, the immunostaining for fibronectin was consistently reduced compared with the saline group, suggesting that there is a relation between the immune-mediated response and the appearance of fibronectin in the vessel wall.

The increased concentration of fibronectin in the subendothelial area and inner media suggests different roles for fibronectin in the development of the graft arteriopathy. Specifically, this preferential localization of fibronectin found in "prelesion" vessels may function as a gradient to promote smooth muscle cell migration to the subendothelium. In fact, enhanced cellular migration associated with a fibronectin gradient has been demonstrated in cardiovascular development and in studies from our laboratory in the ductus arteriosus. Shekhonin and colleagues also described early fibronectin accumulation in atherosclerotic rabbit aorta associated with changes from a contractile to a synthetic smooth muscle cell phenotype. An additional role for the increase of fibronectin may be related to its interactions with inflammatory cells. The binding of very late antigens (VLA)-4 and VLA-5 on the cell surface of lymphocytes to the CS1 and RGDS motifs of the fibronectin molecule, respectively, may suggest that fibronectin is involved in adhesion of inflammatory cells to the endothelium and subsequent trafficking into the subendothelium. Based on the observations in the present study, fibronectin may be only one of a number of factors that influence T-cell infiltration. We speculate that it might also contribute to their continued presence in the subendothelium and perhaps their state of activation and potential contribution to the progression of the graft arteriopathy.

The presence of TNF-α in cardiac allografts is well documented. The experimental use of antibodies to TNF-α improved cardiac allograft survival associated with reduced infiltration of inflammatory cells, expression of intragraft TNF, and TNF-α circulating levels. Ours is the first study to show that this cytokine does play a role in the development of the cardiac allograft coronary artery neointimal formation. Although we have not measured circulating levels of TNF-α, we showed that the use of TNFsr decreased the local concentration of this cytokine in the vessel wall, which also occurred in the cyclosporine A–treated group, suggesting a paracrine/autocrine loop. It could be speculated that some of the variability in the responses of individual animals may be related to the effectiveness with which TNF-α is suppressed, but it is also likely that other factors and mechanisms contribute to neointimal formation.
In summary, we used rabbits fed a cholesterol-rich diet after cardiac transplantation to demonstrate the development of acute neointimal formation associated with an immunoinflammatory reaction and accumulation of fibronectin and to show the beneficial effect of in vivo neutralization of TNF-α in reducing both the incidence and the severity of the coronary lesions. We also showed that this effect appeared to be selective to the vessel wall because there was no modulation of myocardial rejection, unlike in the cyclosporine A-treated group, in which both rejection and vascular lesions were markedly reduced, at least during this time frame. The lesions observed in the present study are likely the result of a combination of factors that could include cholesterol-mediated injury, vascular rejection and its humoral component, and a cellular immunoinflammatory response associated with a cytokine-associated increase in fibronectin. In the present study, the TNFsr effect appeared to be related to both decreased accumulation of fibronectin and less severe inflammatory reaction and suggests that TNF activity could be modulating the development of coronary intimal lesions by more than one mechanism.

Because the late allograft vasculopathy appears to occur despite immunosuppression with cyclosporine A both clinically and experimentally, we speculate that even a mild immune-mediated cellular response, especially if recurrent, may be sufficient to trigger cytokine induction of matrix accumulation and vascular changes associated with the later progressive development of this disease. If so, our study suggests that this process may be amenable to cytokine blockade with agents such as TNFsr.

Acknowledgments

N.C. is supported by a fellowship from Coordenadoria de Aperfeiçoamento para o Ensino Superior (CAPES, Brazil), S.M. is a Clinical Scientist at The Hospital for Sick Children and is supported by Conselho Nacional de Pesquisa (CNPQ, Brazil), S.S. is supported by the Duncan Gordon Fund from The Hospital for Sick Children Foundation, and M.R. is a Career Investigator of the Heart and Stroke Foundation of Ontario. This work was supported by Medical Research Council of Canada grant MT8546. The authors are grateful to Claire Couber for technical assistance during the cardiac transplants and to Pat Peugay and Lily Morikawa for assistance with tissue processing for histology. We thank Mike Starr for preparation of the color photomicrograph plates and Joan Jowlabar and Susy Taylor for secretarial assistance.

References


40. Shingu M, Nobunaga M, Ezaki I, Yoshioka K. Recombinant human IL-1β and TNF-α stimulate production of IL-1α and IL-1β by vascular smooth muscle cells and IL-1α by vascular endothelial cells. Life Sci. 1991;49:241-246.


In vivo blockade of tumor necrosis factor-alpha in cholesterol-fed rabbits after cardiac transplant inhibits acute coronary artery neointimal formation.

N Clausell, S Molossi, S Sett and M Rabinovitch

Circulation. 1994;89:2768-2779
doi: 10.1161/01.CIR.89.6.2768

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/89/6/2768

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/