Elevated Serum Levels of the CXCR3 Chemokine ITAC Are Associated With the Development of Transplant Coronary Artery Disease

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**Background**—Human and animal studies of acute allograft rejection have implicated CCR5 and CXCR3 chemokines as causative factors. However, these chemokines have not been assessed in transplant coronary artery disease (TCAD).

**Methods and Results**—Serum levels of chemokines were measured by ELISA. Levels of ITAC/CXCL11 were found to be elevated in patients with severe TCAD compared with long-term survivors of transplantation without TCAD and with healthy volunteers who had not undergone transplantation (1.476 ± 0.274 ng/mL, 0.926 ± 0.466 ng/mL, and 0.741 ± 0.321 ng/mL, respectively; P < 0.05 for all comparisons to TCAD group). Immunohistochemical localization confirmed the presence of CXCR3+ mononuclear cells within lesions and the presence of the ligand, ITAC/CXCL11, on the surface of endothelial cells associated with TCAD.

**Conclusions**—Elevated peripheral blood levels of the CXCR3 chemokine ITAC/CXCL11 are associated with severe TCAD and may serve as a marker for patients at increased risk for the development of this disease. Immunohistochemical localization of the CXCR3 chemokine ligand ITAC/CXCL11 on the endothelial surface of TCAD lesions with underlying infiltration of inflammatory mononuclear cells expressing CXCR3 suggests a causative role for this chemokine in the development of TCAD. The present study is one of the first to demonstrate a role for ITAC/CXCL11 in this disease. (*Circulation. 2003;107:1958-1961.*)

**Key Words:** surgery ■ transplantation ■ cardiomyopathy ■ rejection ■ coronary disease ■

Orthotopic heart transplantation remains the only viable option for many patients suffering from end-stage ischemic and nonischemic cardiomyopathies.1–3 Transplant coronary artery disease (TCAD) accounts for the great majority of late graft loss.1–3 This disease entity affects up to 70% to 80% of patients if examined by intravascular ultrasound by year 2 and will be seen angiographically in up to 50% of patients.3

Chemokines have been linked to the development of acute rejection episodes and TCAD in animal studies and some human studies.4–9 The CCR5 chemokine ligands RANTES/CCL5, Mip-1α/CCL3, and Mip-1β/CCL4 and the CXCR3 chemokine ligands ITAC/CXCL11, MIG/CXCL9, and IP-10/CXCL10 are found in proinflammatory states and are chemotactant for monocytes and Th1 lymphocytes.10,11,12,14 Increased levels of IP-10/CXCL10 were found in a murine model of TCAD.9 In humans, MIG/CXCL9 and IP-10/CXCL10 have been associated with the development of acute rejection episodes.15–17 It is our hypothesis that the CCR5 and CXCR3 chemokine ligands are involved in the pathogenesis of TCAD.

**Methods**

**Patient Population**

Patients who had undergone transplantation and had severe 2- to 3-vessel TCAD (defined as ≥2 vessels with >50% angiographic stenosis; n = 15) and long-term, disease-free survivors of transplantation (defined as angiographic absence of disease at least 8 years after transplantation; n = 15) were enrolled during annual right and left heart catheterization (Table). Ideally, intravascular ultrasound should be performed on all patients; however, this was not practical. At enrollment, 10 mL of whole blood was taken from the femoral artery, placed in sodium heparin tubes on ice, and spun down at 4°C. The plasma was stored at −80°C until analysis. The Office for the Protection of Research Subjects and Medical Institutional Review Board approved the protocol, and all subjects gave written, informed consent.

**ELISA**

CCR5 and CXCR3 chemokine ligands were quantified by using a modification of the double-ligand method, as previously described, with a sensitivity of 50 pg/mL or higher.18 The polyclonal antibodies used were anti-human RANTES/CCL5 (R&D Systems, Minneapolis, Minn), anti-human Mip-1α/CCL3, and anti-human Mip-1β/CCL4 (RMS Laboratory) for the CCR5 ligands, and anti-human MIG/CXCL9
Immunohistochemistry
Paraffin blocks of coronary arteries were obtained from transplanted hearts at the time of death or retransplantation for TCAD. Paraffin-embedded tuberculosis granulomas from lung specimens were used as a positive control for immunolocalization of MIG/CXCL9, IP-10/CXCL10, and ITAC/CXCL11. Slides were initially incubated at 60°C and then immersed in sequential baths of xylene, 100%, 95%, and 70% ethanol. The slides were subsequently placed in 3% H2O2 in methanol and then washed with PBS. Antigen retrieval was performed with the use of a citrated buffer. Normal horse serum (3%) or normal goat serum (3%) was used for the blocking agent for CXCR3, MIG/CXCL9, IP-10/CXCL10, and ITAC/CXCL11. The tissue was subsequently incubated at room temperature with mouse anti-CXCR3 antibody (Pharmingen, San Diego, Calif), mouse anti-MIG/CXCL9 (R&D Systems), goat anti-IP-10/CXCL10, and rabbit anti-ITAC/CXCL11 antibody (PeproTech, Inc, Rocky Hill, NJ). The secondary antibody (Vector Laboratory, Inc, Burlingame, Calif) was made up in 1% of the corresponding blocking serum (normal horse serum or normal goat serum). For staining, horseradish peroxidase-bound avidin-biotin was applied, and the slides were developed with DAB solution (Vector Laboratory, Inc). The slides were examined and interpreted by our cardiac pathologist (M.C.F.).

Statistical Analysis
Statistical analysis was carried out with the use of StatView. Nonparametric analysis was used to analyze the data. For comparison of the 3 groups, Kruskal-Wallis analysis was used, and for comparisons between groups, Mann-Whitney analysis was performed.

ELISA
The CCR5 chemokine ligands were not associated with TCAD and were not studied further (Figure 1A). Of the CXCR3 chemokine ligands studied, only ITAC/CXCL11 was found to be elevated in patients with TCAD compared with control subjects (Control) and survivors without TCAD (No TCAD) (1.476±0.274 ng/mL, 0.741±0.321 ng/mL, and 0.926±0.466 ng/mL, respectively; \( P = 0.043 \) compared with Control, \( ** P = 0.034 \) compared with No TCAD).

Immunohistochemistry
Immunolocalization of MIG/CXCL9, IP-10/CXCL10, ITAC/CXCL11, and CXCR3 was performed. Paraffin blocks of coronary arteries obtained at the time of death or retransplantation for TCAD were examined. Staining with anti-CXCR3 antibodies demonstrated the presence of CXCR3+ mononuclear cells within the lesions of TCAD (Figure 2A). Staining with anti-ITAC/CXCL11 antibodies showed intense staining along the endothelium of TCAD lesions (Figure 2B). In contrast, staining with anti-MIG/CXCL9 or anti-IP-10/CXCL10 was absent in the TCAD specimens.

Discussion
TCAD, a form of chronic rejection or chronic inflammation, is characterized by intimal smooth muscle proliferation and
TCAD is seen in native coronary arteries, TCAD is a concentric, accelerated atherosclerosis. However, unlike atherosclerosis seen in native coronary arteries, TCAD is a concentric, diffuse disease involving the distal coronary arteries and small branches. Mortality rate after the initial diagnosis of TCAD is >40% after 2 years. It is important, therefore, to prevent TCAD and prolong the life of the original transplant.

Histologically, mononuclear cell inflammation, fibrocellular intimal hyperplasia, and endothelial damage characterize TCAD. Chemokines have been implicated in acute and chronic solid organ rejection, and the CCR5 and CXCR3 chemokine ligands have been most closely associated with cardiac allograft rejection. In a recent human study in which endomyocardial biopsies were used, elevated levels of ITAC/CXCL11 and IP-10/CXCL10 mRNA were found to be associated with TCAD, and elevated levels of IP-10/CXCL10 and MIG/CXCL9 mRNA were found during episodes of acute rejection. We demonstrate elevated plasma protein levels of the CXCR3 chemokine ligand, ITAC/CXCL11, in patients with severe TCAD. Immunohistochemical studies localized ITAC/CXCL11 to the endothelial surface of TCAD lesions and demonstrated the presence of CXCR3 T-lymphocytes and macrophages. In contrast, we did not find positive immunostaining for either MIG/CXCL9 or IP-10/CXCL10. This is the first study demonstrating elevated serum levels and arterial immunohistochemical evidence of ITAC/CXCL11 involvement in TCAD.

To our knowledge, this is the first study of peripheral blood used to implicate chemokines in the development of TCAD. Several chemokines that have been implicated in animal models of TCAD—namely RANTES/CCL5, IP-10/CXCL10, and MIG/CXCL9—were not found to correlate with the presence of TCAD in our study. Most of the studies looking at the CC and CXC chemokines in TCAD have been in animal models of transplant disease. It is unknown if human pathology follows a similar time course or mechanism as the animal models. Also, our study was based on peripheral blood levels, and levels of chemokines other than ITAC/CXCL11 may be diluted to nondetectable levels or may be bound to circulating red blood cells. It is conceivable that direct sampling of the coronary sinus would provide a more accurate profile of chemokine secretion. Future studies with this sampling technique will help elucidate this possibility.

Conclusions
This study, to our knowledge, is the first demonstrating a correlation between circulating chemokine levels and development of TCAD in humans. Of the chemokines studied, only ITAC/CXCL11, a CXCR3 ligand, was associated with TCAD by elevated serum levels and immunohistochemical localization. ITAC/CXCL11 may have a causative role in the pathogenesis of this disease and may provide a way to prospectively identify patients at risk for the development of TCAD. Further prospective trials will be needed to confirm this hypothesis.

Acknowledgments
This project is funded in part by a 2001-2002 American College of Cardiology Foundation/Merck Fellowship Award (Dr Kao). Additional funding was provided by National Institutes of Health grants P01 HL67665, HL04493, and HL66027.

References


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*Circulation*. 2003;107:1958-1961; originally published online April 14, 2003; doi: 10.1161/01.CIR.0000069270.16498.75
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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