Association of Thrombospondin-1 and Cardiac Allograft Vasculopathy in Human Cardiac Allografts

Xiao-Ming Zhao, MD; Yenya Hu, MD, PhD; Geraldine G. Miller, MD; Richard N. Mitchell, MD, PhD; Peter Libby, MD

Background—Despite the expression of angiogenic growth factors in transplanted hearts, neovessel formation appears scant. We therefore hypothesized that cardiac allografts contain endogenous inhibitors of angiogenesis. In particular, we tested the involvement in cardiac allografts of thrombospondin-1 (TSP-1), a matrix glycoprotein that inhibits angiogenesis and facilitates smooth muscle cell (SMC) proliferation.

Methods and Results—Levels of TSP-1 mRNA in endomyocardial biopsy samples of human cardiac allografts substantially exceeded those in normal hearts. The ratio of TSP to GAPDH mRNA determined with quantitative RT-PCR was 6.54±1.6 in cardiac allografts versus 0.26±0.02 (P=0.001) in normal hearts. Analysis in sequential biopsies revealed a strong association between persistent elevation of TSP-1 in allografts and the development of cardiac allograft vasculopathy (CAV). The CAV score was 2.4±0.8 in patients with persistent TSP-1 elevation compared with 0.2±0.2 in patients without elevation (P=0.001). Immunohistochemistry demonstrated intense expression of TSP-1 in cardiac allografts, predominantly in cardiac myocytes and neointimal SMCs. In vitro experiments demonstrated that T cells expressed TSP-1, acidic fibroblast growth factor, and vascular endothelial cell growth factor on allogeneic stimulation. Cytokines known to be elevated in cardiac allografts (interleukin-1β, interferon-γ, and tumor necrosis factor-α) induced TSP-1 in SMCs but inhibited TSP-1 in endothelial cells.

Conclusions—Persistent elevation of TSP-1 in cardiac allografts correlates with the development of CAV. Allogeneic stimulation induces angiogenic growth factors and TSP-1 in T cells. Cytokines differentially regulate TSP-1 expression in SMCs versus endothelial cells. Increased levels of TSP-1 in human cardiac allografts may alter vascular responses to angiogenic growth factors by inhibiting angiogenesis and promoting SMC proliferation characteristic of CAV.

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Key Words: glycoproteins ■ growth factors ■ transplantation ■ coronary disease ■ cytokines

Cardiac allograft vasculopathy (CAV) poses a major limitation for long-term survival of heart transplant recipients. In this disease, smooth muscle cells (SMCs) accumulate in the intima of both large epicardial and small intramyocardial coronary arteries. In 1989, we originally hypothesized an immunity-driven pathogenic scheme for CAV mediated by cytokines and growth factors, a model now widely substantiated by experimental evidence. Yet, current immunosuppressive regimens have had little effect on the prevention of CAV despite improved control of acute allograft rejection. Provocative results with angiogenic growth factors in conventional coronary artery disease and peripheral vascular disease have prompted interest in the use of those growth factors to promote angiogenesis in cardiac allografts with severe CAV. However, angiogenic growth factors might accelerate CAV in transplanted hearts. The finding that increased expression of endogenous growth factors in transplanted hearts correlates with the development of CAV accentuates this concern. Cardiac allografts exhibit augmented expression of several angiogenic growth factors, including acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) and vascular endothelial cell growth factor (VEGF). Nevertheless, transplanted hearts generally do not develop abundant collateral vessels. We hypothesized that other factors expressed in cardiac allografts may alter vascular responses to those growth factors by inhibiting angiogenesis and promoting SMC proliferation characteristic of CAV.

Thrombospondin-1 (TSP-1) is a 190-kDa multifunctional matrix glycoprotein that modulates many important vascular cell functions. It is present in large amounts in platelet α-granules and is expressed by endothelial cells (ECs) and SMCs in a highly regulated manner. Its expression rapidly and dramatically increases after vascular injury or exposure of the cells to platelet-derived growth factor (PDGF) or bFGF. Atheroma and balloon-injured vessels contain...
abundant TSP-1. Antibodies to TSP-1 reduce neointima formation in balloon-injured rat carotid arteries, suggesting an important functional role. In regard to angiogenesis, TSP-1 potently inhibits angiogenesis in vivo and blocks microvascular EC proliferation by angiogenic factors. TSP-1 also enhances SMC proliferation and migration in response to FGFs and PDGF, growth factors overexpressed in human cardiac allografts. Thus, we tested the hypothesis that the elevated expression of TSP-1 in human cardiac allografts associates with the development of CAV. We further tested the hypothesis that allogeneic stimulation and inflammatory cytokines regulate TSP-1 expression by ECs and SMCs.

Methods

Myocardial Biopsies of Cardiac Allografts and Normal Hearts

One hundred thirty-three endomyocardial biopsy samples from 11 cardiac allografts and 15 biopsy samples from 15 normal hearts were analyzed for expression of TSP-1, CD36, and CD47 mRNA with quantitative RT-PCR. Myocardium from pretransplantation normal donor hearts was obtained from the right ventricle immediately after organ excision. Allograft myocardial biopsies were obtained at routine follow-up biopsy after transplantation or when clinically indicated. At each cardiac catheterization, 5 biopsy samples were obtained for histology to monitor allograft rejection, and 1 was obtained and frozen for RNA extraction.

Annual coronary angiography was used to assess CAV. Coronary angiograms were reviewed and compared with baseline angiograms independently by 2 cardiologists who were unaware of the results of studies on TSP-1. CAV was assessed according to the criteria established by Gao et al, including the presence of focal stenoses, distal tapering or pruning, and loss or tertiary vessels, and were assigned a numerical rating for severity as absent (0), mild (1), moderate (2), or severe (3).

Isolation of RNA From Myocardial Biopsy Samples and RT-PCR

Total RNA was isolated from myocardial biopsies using RNAzol B (Tel-Test) and was used as template for cDNA synthesis. Primers used in PCR are listed in the Table. Quantitative RT-PCR was performed with labeled dCTP to generate radioactively labeled PCR products. PCR products were run on 2% agarose gel, dried, and exposed to a PhosphorImager (Molecular Dynamics) for quantitation. Standard curves within the exponential range of amplification for each gene were generated with known amounts of cDNA template. The concentration of cDNA in each sample was calculated from the standards run at the same time.

RNA from myocardial biopsy samples could not be quantified because of the small size of the samples. Therefore, the amount of cDNA for each gene was normalized to the amount of cDNA for GAPDH, a constitutively expressed gene, in each sample. The ratio between each gene of interest and GAPDH is used for comparison.

Immunohistochemistry

Twenty specimens were obtained from 3 explanted cardiac allografts during autopsy. Normal hearts were obtained from patients who died of noncardiac diseases. Specimens were fixed in 10% formalin and embedded in paraffin for processing. After deparaffinization, slides were incubated sequentially in proteinase K/PBS (5 mg/mL; Boehringer Mannheim) for 20 minutes, 0.3% H2O2/PBS for 20 minutes, 5% horse serum/PBS for 1 hour, and first antibodies in 5% horse serum/PBS for 2 hours. The first antibodies used were goat anti-human TSP-1 (Neo Markers), goat anti-human VEGF (Santa Cruz), and mouse anti-human CD31 (DAKO). Goat or mouse IgG (Santa Cruz) was used as first antibody in negative controls. After incubation and washing in PBS, slides were incubated with biotinylated secondary antibodies (horse anti-goat or mouse; Vector) and developed with use of a Vectastain ABC kit (Vector) and a DAB substrate kit (Vector).

Cell Cultures

Human SMCs were isolated from saphenous veins and grown in Medium 199 (BioWhittaker) supplemented with 10% FCS. These experiments used 80% to 90% confluent cells from passages 2 to 3. To induce quiescent SMCs, cells were cultured in IT medium for 24 hours. To study the regulation of TSP-1 expression in SMCs, cells were treated with recombiant human interleukin (IL)-1β (10 ng/mL; R&D Systems), interferon (IFN)-γ (10 ng/mL; R&D Systems), or tumor necrosis factor (TNF)-α (5 ng/mL; R&D Systems), for 4, 8, 12, or 24 hours.

Human ECs were isolated from human saphenous veins and grown on gelatin-coated plates in Medium 199 (BioWhittaker), supplemented with 10% FCS. To induce quiescence in ECs, cells were cultured in Medium 199 supplemented with 0.5% BSA for 24 hours. Quiescent cells were treated with cytokines as described earlier.

Mixed Lymphocyte Cultures

Peripheral blood mononuclear cells were isolated from normal healthy donors with lymphocyte separation medium (ICN). Stimulator cells were prepared with irradiation (45 Gy). Responder cells (5×10^5/well) were cultured with irradiated stimulator cells (5×10^5/ well) in 6-well plates at 37°C in a 5% CO2 humidified incubator. After 5 days of coculture, cells were collected and T cells were isolated with a T-cell enrichment column (R&D Systems). Purified T cells typically had 85% to 90% CD3-positive cells on flow cytometry (data not shown). Those cells were used for RNA extraction (RT-PCR analysis) or cytospinning (immunohistochemistry).

CTC Preparation

T cells before and after allostimulation were isolated with T-cell enrichment column (R&D Systems) and cytacentrifuged onto slides. The slides were fixed in acetone for 5 minutes, dried, and stained with goat anti-aFGF (Santa Cruz), goat anti-IFG (Santa Cruz), goat anti-VEGF (Santa Cruz), or goat anti-TSP-1 (Neo Markers). After being washed in PBS, slides were incubated with biotinylated horse anti-goat antibody and developed with a Vectastain ABC kit (Vector) and a DAB substrate kit (Vector).

Northern Blot Analysis

The regulation of TSP-1 mRNA expression in SMCs was studied with Northern blot analysis. mRNA was extracted from SMCs with RNAzol B. Total RNA (20 μg) from each sample was denatured and run on formaldehyde agarose gels. The RNA was transferred to nylon membrane and UV cross-linked. The probe used in Northern blot analysis was the antisense oligonucleotide for TSP-1. A probe for GAPDH was used as positive control to ensure equal loading of total RNA in each sample. The probes were labeled with α-^32P-dCTP with terminal deoxynucleotidyl transferase (GIBCO BRL).

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**Table: Primers Used in RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>TSP-1</td>
<td>5'-AGA ATG TCC TGC GTG CTG TT-3'</td>
<td>5'-TTT CTT GCA GCC TTT GGT CT-3'</td>
</tr>
<tr>
<td>CD36</td>
<td>5'-AAA TGT AAC CCA GGA CGC TG-3'</td>
<td>5'-GGC TG1 GAT GGA AGA ACA AA-3'</td>
</tr>
<tr>
<td>CD47</td>
<td>5'-CCA CTG TCC CCA CTG ACT TT-3'</td>
<td>5'-GGC GAC GAA GGA GGT TA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ATC GAG CTC ATC CCA TCA CCA TCT TCC AGG-3'</td>
<td>5'-ACA TCT AGA GGC ATC ATC AGG CCA CAG TTT CCC-3'</td>
</tr>
</tbody>
</table>
Western Blot Analysis

Cells were washed with cold PBS once, scraped from the plastic plates, and resuspended in 1 mL of cold PBS. After centrifugation at 9.5 g for 1 minute, cells were solubilized in 100 μL of lysis buffer (1% Triton X-100, 50 mmol/L HEPES, 10 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mg/mL aprotinin, 1 μg/mL leupeptin, and 2 mmol/L PMSF). Supernatants were collected after centrifugation at 12.5 g for 15 minutes at 4°C. Protein concentration was determined with BCA protein assay reagent (Bio-Rad). For Western blot analysis, 50 μg of proteins was separated by 7.5% SDS-PAGE and transferred to a nylon membrane. After blocking with 5% serum/PBS, the membrane was subsequently incubated with monoclonal anti-human TSP-1 antibody (1:1000 dilution; Neo Marker) and peroxidase-conjugated goat anti-mouse antibody (1:1000; Vector). The signals were detected by ECL Western blot analysis system (GIBCO BRL).

Data Analysis

Differences in levels of TSP-1, CD36, and CD47 mRNA in transplanted hearts with and without severe CAV as well as in normal hearts were analyzed by unpaired Student’s t test. A value of P<0.05 was considered statistically significant.

Results

Neovascularization in Coronary Arteries With Severe CAV

Levels of angiogenic growth factors increase substantially in human cardiac allografts compared with normal hearts, yet angiographic studies demonstrate little or no collateral formation. We evaluated the extent of neovascularization in explanted human cardiac allografts with antibody to CD31, a marker for endothelial cells. Lesions of severe CAV showed a distinct and unusual pattern of microvascularization (Figure 1). Microvessels were abundant in the abluminal (outer) layers of the neointima but were sparse in the luminal (inner) layer of the thickened intima. Microvascular density in the parenchyma in cardiac allografts resembled that in normal human hearts (data not shown).

Human Cardiac Allografts Express TSP-1, TSP-1 Receptors, and VEGF

The lack of parenchymal neovascularization in cardiac allografts in the presence of high levels of angiogenic growth factors prompted the hypothesis that cardiac allografts expressed inhibitors of angiogenesis. We therefore investigated the expression of TSP-1, an angiogenesis inhibitor, in 133 human cardiac allograft biopsy samples. Levels of TSP-1 mRNA in cardiac allografts substantially exceeded those in normal hearts. The mean TSP-1–to-GAPDH ratio was 6.54±1.61 in allografts (133 biopsy samples from 11 allografts) versus 0.26±0.21 in normal hearts (15 endomyocardial specimens from 15 normal hearts, P<0.001). Figure 2 shows a representative RT-PCR gel for 5 allograft and 4 normal hearts samples.

To test whether elevated TSP-1 mRNA correlates with the development of CAV, we analyzed TSP-1 mRNA levels in sequential biopsy samples from 11 heart transplant recipients who had 2-year posttransplantation coronary angiography. Biopsy samples were obtained from each patient at routine follow-up biopsy or when clinically indicated. On average, 10 to 17 biopsy samples were obtained from each patient during the 2-year period.

TSP-1 mRNA expression generally followed 2 distinct patterns. Five patients demonstrated persistent elevation of TSP-1 mRNA in sequential biopsies, after a rise in TSP-1 mRNA levels immediately after transplantation (Figure 3A). Six other patients demonstrated an initial elevation of TSP-1 mRNA after transplantation, followed by return to normal levels (Figure 3A). Patients with persistent elevation of TSP-1 developed severe CAV as determined with coronary angiography.
angiography. Their mean CAV score was 2.4 ± 0.8 compared with 0.2 ± 0.2 for patients with only transient TSP-1 elevation (P < 0.001) (Figure 3A). The mean TSP-1-to-GAPDH ratio (over 2 years) was 14-fold higher in patients with CAV than in those without CAV (12.43 ± 6.09 versus 0.87 ± 0.25, P < 0.0003) (Figure 3B).

The expression of 2 known TSP-1 receptors also increased but to a lesser extent. The CD36-to-GAPDH ratio was 0.78 ± 0.08 in cardiac allografts versus 0.53 ± 0.04 in normal hearts (P = 0.01). The CD47-to-GAPDH ratio was 0.93 ± 0.06 versus 0.77 ± 0.10 (P = 0.05), respectively. The CD36 and CD47 levels did not correlate with the severity of CAV.

These results at the mRNA level supported the hypothesis that interaction between TSP-1 and angiogenic growth factors plays an important role in inhibition of angiogenesis and facilitation of SMC proliferation. The cellular distribution of TSP-1 and angiogenic growth factor proteins in cardiac allografts may provide clues of their roles in CAV. Therefore, we studied the expression of TSP-1 and VEGF in human cardiac allografts with immunohistochemical studies of tissue sections from explanted cardiac allografts and normal hearts. In accordance with the mRNA results, cardiac allografts demonstrated markedly increased TSP-1 proteins in comparison with normal hearts. Cardiac myocytes and intimal SMCs were the predominant sites of TSP-1 expression in cardiac allografts (Figures 4A and 4C). SMCs in the luminal (inner) layer of neointima displayed strong staining for TSP-1, whereas infiltrating cells did not express high levels of TSP-1.
in cardiac allografts (Figure 4B). Consistent with RT-PCR data, the expression of TSP-1 increased early after transplantation. For example, in the heart of a patient who died of a noncardiac cause 3 weeks after transplantation, the coronary arterial media already showed intense TSP-1 expression, predominantly in SMCs (Figure 4D). In contrast, normal hearts showed only scattered TSP-1 expression (Figure 4E).

The cellular distribution of VEGF in cardiac allografts differs from TSP-1, with cardiac myocytes and infiltrating mononuclear inflammatory cells containing the most VEGF in cardiac allografts (Figures 4G and 4H). Intimal SMCs did not express high levels of VEGF (Figure 4F). Normal hearts showed only scattered VEGF in ECs (Figure 4I).

Allogeneic Response Induces TSP-1, aFGF, and VEGF Expression in Lymphocytes

We have proposed that allogenic stimulation of recipient T cells by donor cells contributes to the pathogenesis of CAV. We previously demonstrated expression of aFGF by T-cell clones. Others have reported the expression of VEGF and bFGF in T cells. Here, we investigated the hypothesis that allostimulation modulates the expression of growth factors and TSP-1 in lymphocytes during mixed lymphocyte reaction (MLR).

Naive T cells contained low or undetectable levels of VEGF, aFGF, or TSP-1 transcripts (Figure 5A) but did express bFGF mRNA (data not shown). After coculture with stimulator cells for 5 days, T cells accumulated substantial VEGF, aFGF, and TSP-1 mRNA. The levels of bFGF mRNA in activated T cells resembled those seen in naive T cells. In accordance with mRNA results, allostimulated T cells, but not naive T cells, contained TSP-1, aFGF, and VEGF proteins (Figure 5B).

Cytokines Differentially Regulate TSP-1 Expression in SMCs and ECs

The expression of proinflammatory cytokines increases in human and murine cardiac allografts. We hypothesized that those cytokines modulate TSP-1 expression in SMCs and ECs. Quiescent SMCs expressed TSP-1 mRNA at low levels. The addition of IL-1β greatly increased TSP-1 mRNA expression, being apparent at 4 hours and maximal at 12 hours. TSP-1 mRNA levels remained elevated after 24 hours of IL-1β stimulation (data not shown). IFN-γ stimulation moderately and transiently elevated TSP-1 mRNA, achieving maximal levels at 4 hours. TNF-α produced a delayed and modest increase in TSP-1 expression in SMCs (Figure 6A).

Exposure to IL-1β markedly elevated TSP-1 protein expression (Figure 6B). The time course of TSP-1 protein expression paralleled mRNA expression. The induction depended on the concentrations of IL-1β. Elevation of TSP-1 protein was evident at 0.2 ng/mL IL-1β and maximal at 5 ng/mL. IFN-γ or TNF-α only moderately induced TSP-1 in SMCs.

We next investigated the regulation of TSP-1 expression in ECs. In contrast to SMCs, IL-1β inhibited TSP-1 expression in ECs, with effects apparent at 4 hours and maximal at 12 hours (Figure 6C). IFN-γ or TNF-α did not affect TSP-1 expression in ECs.

Discussion

CAV is the leading cause of late death in heart transplant recipients. The promotion of neovascularization through the administration of angiogenic growth factors might provide a therapeutic approach to this challenging disease. Nevertheless, the levels of endogenous angiogenic growth factors present in transplanted hearts already substantially exceed those in normal hearts. However, angiographically detectable coronary collateral formation in cardiac allografts occurs infrequently if at all. We demonstrate here that although the parenchymal microvascular density appears to be similar in
transplanted and normal hearts, abundant neovascularization occurs in portions of the thickened intima from allografts. Neovascularization in these areas may actually contribute to intimal hyperplasia by providing oxygen, nutrients, infiltrating cells, growth factors, and cytokines. This observation complements the finding of Moulton et al\textsuperscript{24} that neovascularization in atherosclerotic plaques accelerates lesion formation in mice.

Thus, rather than promoting potentially beneficial parenchymal angiogenesis in cardiac allografts, elevated angiogenic growth factors may contribute to the development of CAV. A recent study demonstrates that the persistent elevation of aFGF in cardiac allografts correlates with the development of CAV.\textsuperscript{4} Nabel et al\textsuperscript{25} showed that introduction of aFGF into vessel walls substantially augmented intimal neocapillary formation and intimal hyperplasia. VEGF accentuates neointimal thickening at the site of arterial injury but not in normal vessels.\textsuperscript{26} The biological effects of these growth factors (angiogenesis versus vascular intimal hyperplasia) resulted from a complex balance among growth factors, their inhibitors, extracellular matrix, and other factors present in situ. Here, we provide evidence to support the hypothesis that TSP-1 expressed in transplanted hearts alters vascular responses to angiogenic growth factors by inhibiting angiogenesis and accelerating SMC proliferation.

TSP-1 is a multifunctional protein that inhibits EC proliferation, migration, and angiogenesis\textsuperscript{11} but stimulates SMC proliferation and migration.\textsuperscript{18} It also potentiates SMC proliferation in response to other growth factors.\textsuperscript{19} The current study demonstrates significant elevation of TSP-1 in cardiac allografts, with the strongest expression in neointimal SMCs and cardiac myocytes. Although the SMCs from the luminal (inner) layer of the neointima express the highest levels of TSP-1, the greatest VEGF expression occurs in infiltrating inflammatory cells in the abluminal (outer) layers of the intima. Neovascularization colocalizes with VEGF-producing cells and is sparse in areas with intense TSP-1 expression. These data suggest that TSP-1 inhibits angiogenesis in cardiac allografts.

Intense expression of TSP-1 in neointima indicates a role in SMC proliferation. The association between TSP-1 levels and the development of CAV supports this hypothesis. Persistent elevation of TSP-1 in cardiac allografts correlates with the development of CAV, whereas transient early elevation of TSP-1 after transplantation does not seem to influence CAV development.

Our in vitro studies examined the mechanisms that underlie the regulation of TSP-1 and angiogenic growth factors in cardiac allografts. We found that proinflammatory cytokines differentially regulate TSP-1 expression in SMCs and ECs. Cardiac allografts contain abundant IL-1β, TNF-α, and IFN-γ, which may induce TSP-1 production by SMCs in cardiac allografts. We and others found that IL-1β inhibits TSP-1 expression in ECs.\textsuperscript{27} The in vitro data agree with the observation that SMCs, but not ECs, produce TSP-1 in cardiac allografts.

The current study also demonstrates that allogeneic stimulation induces aFGF, VEGF, and TSP-1 expression in T cells. Cardiac allografts contain abundant T cells even in the absence of histologically detectable rejection. Thus, growth factors secreted by T cells may contribute to the persistent elevation of endogenous growth factors present in cardiac allografts. Colocalization of neovessels and VEGF-producing infiltrating cells in the neointima suggests an important role for these cells in the development of CAV. The finding that T-cell activation promotes the production of angiogenic growth factors and inhibitors has implication for many immunity-mediated diseases.

In conclusion, high levels of TSP-1 expressed in cardiac allografts may play a critical role in altering vascular responses to endogenous angiogenic growth factors by inhibiting angiogenesis and promoting neointimal SMC proliferation. Indeed, the deliverance of exogenous angiogenic growth factors to cardiac allografts may accelerate vascular intimal hyperplasia. New approaches to inhibition of TSP-1 expression or block of the biological effects of TSP-1 may offer novel ways to increase angiogenesis and reduce neointimal SMC proliferation in cardiac allografts.

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References


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