Vascular Endothelial Growth Factor Enhances Cardiac Allograft Arteriosclerosis

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Background—Cardiac allograft arteriosclerosis is a complex process of alloimmune response, chronic inflammation, and smooth muscle cell proliferation that includes cross talk between cytokines and growth factors.

Methods and Results—Our results in rat cardiac allografts established alloimmune response as an alternative stimulus capable of inducing vascular endothelial growth factor (VEGF) mRNA and protein expression in cardiomyocytes and graft-infiltrating mononuclear inflammatory cells, which suggests that these cells may function as a source of VEGF to the cells of coronary arteries. Linear regression analysis of these allografts with different stages of arteriosclerotic lesions revealed a strong correlation between intragraft VEGF protein expression and the development of intimal thickening, whereas blockade of signaling downstream of VEGF receptor significantly reduced arteriosclerotic lesions. In addition, in cholesterol-fed rabbits, intracoronary perfusion of cardiac allografts with a clinical-grade adenoviral vector that encoded mouse VEGF164 enhanced the formation of arteriosclerotic lesions, possibly secondary to increased intragraft influx of macrophages and neovascularization in the intimal lesions.

Conclusions—Our findings suggest a positive regulatory role between VEGF and coronary arteriosclerotic lesion formation in the allograft cytokine microenvironment. (Circulation. 2002;105:2524-2530.)

Key Words: angiogenesis ■ muscle, smooth ■ arteriosclerosis ■ transplantation ■ rejection

vascular endothelial growth factor (VEGF) has significant roles in angiogenesis, tumor growth, vascular development, and atherosclerosis. VEGF induces migration and proliferation of endothelial cells, increases vascular permeability and extravasation of plasma macromolecules, and stimulates production of the thrombogenic protein tissue factor in endothelial cells and monocytes. It is also a chemoattractant to monocytes. VEGF does not induce proliferation of smooth muscle cells (SMCs) but is a chemoattractant to them and facilitates their migration by matrix metalloproteinase production. Preclinical and clinical therapeutic approaches show that VEGF promotes neovascularization in ischemic myocardial and peripheral vascular limb disease and that it improves endothelium-dependent vasorelaxation. Furthermore, studies with both recombinant protein and arterial gene transfer demonstrate that VEGF enhances reendothelialization and thereby reduces intimal thickening in arterial restenosis and after stent implantation.

In the context of cardiac allograft arteriosclerosis, there are several mechanisms whereby VEGF may modulate migratory and proliferative responses of SMCs. As in restenosis, overexpression of VEGF may prevent intimal thickening by enhancing the regeneration and functional recovery of vascular endothelium damaged by ischemia-reperfusion injury and the alloimmune response. VEGF has many properties that may actually enhance arteriosclerotic lesions, such as chemoattractancy to SMCs and macrophages. The aim of the present study was to investigate how VEGF modulates cardiac transplant arteriosclerosis.

Methods

Experimental Design

To investigate the kinetics of VEGF expression during acute and chronic rejection, we transplanted heterotopic cardiac allografts between fully major histocompatibility complex-mismatched strains from DA (AG-B4, RT1a) to WF (AG-B2, RT1v) rats (Harlan, Horst, Netherlands). In the acute rejection model, we removed the grafts at 5 days. We achieved development of cardiac allograft arteriosclerosis at the 2-month time point using different doses of cyclosporine A (1.0, 1.5, or 2.0 mg ⋅ kg⁻¹ ⋅ d⁻¹ SC; Novartis). To investigate the effect of blockade of VEGF receptor (VEGFR) activation on cardiac allograft arteriosclerosis, recipients received by mouth either vehicle
or PTK 787, a protein tyrosine kinase inhibitor, 50 mg · kg\(^{-1}\) · d\(^{-1}\) twice a day (Novartis Pharma).\(^{16}\) Cyclosporine A was given 2.0 mg · kg\(^{-1}\) · d\(^{-1}\) SC for the first week, followed by 1.0 mg · kg\(^{-1}\) · d\(^{-1}\) SC as background immunosuppression for the study period of 60 days.

To understand how VEGF regulates cardiac allograft arteriosclerosis, we used adenovirus-mediated mouse VEGF\(_{164}\), gene transfer in heterotopic cardiac allografts transplanted from Dutch-Belted to New Zealand White rabbits (Harlan, Bicester, UK). We chose to use rabbits to validate the results in another model in which recipients were treated with low-dose cyclosporine A (3.0 mg · kg\(^{-1}\) · d\(^{-1}\) SC; Novartis) and a 0.5% cholesterol diet (Altromin Int). After the donor heart was harvested, coronary arteries were perfused through the aorta with 1 × 10\(^{10}\) plaque-forming units of adenoviral vector encoding either mouse VEGF\(_{164}\) or β-gal under control of the cytomegalovirus (CMV) promoter containing 10\(^{-7}\) mol/L acetycholinc to permeabilize the endothelium. The heart, with its main arteries cross-clamped, was placed in ice-cold PBS for 30 minutes and thereafter was anastomosed to the right carotid artery and the inferior jugular vein of the recipient.

**Adenoviral Constructs**

Mouse VEGF\(_{164}\) and nucleus-targeted lacZ adenoviruses were produced as described previously.\(^{17}\) Briefly, cDNAs were subcloned into pAdCMV plasmid, constructed by subcloning the human CMV immediate early promoter, the multiple cloning site, and the bovine growth hormone polyA signal from pcDNA3 plasmid (Invitrogen, Groningen, Netherlands) into a pAdBIII vector. Replication-deficient E1-E3–deleted clinical GMP-grade adenoviruses were produced in 293T cells. Adenoviruses were analyzed to be free of replication-competent viruses, lipopolysaccharide, mycoplasma, and other microbiological contaminants. The functionality of adenoviruses has been demonstrated in our recent study.\(^{17}\)

**Histology**

Transplant arteriosclerosis was scored as followed from sections stained with Mayer’s hematoxylin-eosin and resorcin fuchsin for internal elastic lamina: grade 0, normal artery with intact internal elastic lamina; grade 1, <10% occlusion of lumen by arterial intimal thickening and proliferation, disruption of internal elastic lamina, and possibly the presence of some foam or vacuolated endothelial cells; grade 2, <50% but >10% occlusion of lumen; grade 3, >50% but <100% occlusion of lumen; and grade 4, 100% occlusion of lumen.\(^{18}\) The slides were examined by 2 independent observers in a blinded review. In the rabbit model, the arteries were divided into 3 categories according to their diameter: large (>120 μm), medium (40 to 120 μm), and small (<40 μm).

**Immunohistochemistry**

Cryostat sections were stained by the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories), and the reaction was revealed by 3-amin-9-ethylcarbazole (AEC, Vectastain). The following antibodies were used: rabbit anti-mouse, -rat, and -human VEGF (0.5 μg/mL; sc-152, Santa Cruz); mouse anti-β-galactosidase (Monosan, AM Uden); rabbit anti-mouse VEGF\(_{164}\) (2 μg/mL; sc-1856, Santa Cruz); mouse anti-rabbit CD4+ (5 μg/mL; PS5400 mol/L, Biodesign International); and CD8+ (5 μg/mL; PS5402 mol/L, Biodesign) T cells and RAM11+ macrophages (0.3 μg/mL; M0633, Dako). Intimal neocapillary formation was examined by an antibody against CD31 (3.3 μg/mL; M0823, Dako). Specificity controls were performed with the same immunoglobulin concentration of species- and isotype-matched antibodies; for VEGF staining, this involved the use of a working dilution of polyclonal antibody after overnight incubation with a 20-fold molar excess of peptide antigen. The intensity of VEGF immunoreactivity was scored from 0 to 3 as follows: 0, no visible staining; 1, few cells with faint staining; 2, moderate intensity with multifocal staining; and 3, intense diffuse staining of the cells analyzed. Positive staining for inflammatory cell markers was quantified as total number of cells per cardiac cross section.

**In Situ Hybridization**

Human VEGF antisense and sense RNA probes were generated from linearized pGEM3Zf(+) plasmid (Promega), which contained a fragment corresponding to nucleotides 57 to 639 of human VEGF cDNA. Radiolabeled RNA was synthesized with T7 polymerase and [\(^{35}\)S]UTP (Amersham). In situ hybridization was performed as described previously.\(^{19}\) Paraffin sections were treated in prehybridization solutions and hybridized overnight at 52°C. After high-stringency washes for 60 minutes at 65°C, the sections were dehydrated, air-dried, dipped into NTB-2 emulsion (Eastman Kodak), and exposed at 4°C for 4 to 6 weeks. The sections were developed in D19 (Eastman Kodak), fixed in sodium fixative (Eastman Kodak), and counterstained in hematoxylin (Shandon).

The intensity of VEGF mRNA expression was scored from 0 to 3 as follows: 0, no visible expression; 1, few cells with faint expression; 2, moderate intensity with multifocal expression; and 3, intense diffuse VEGF mRNA expression of the cells analyzed.

**Statistical Analysis**

All data are mean ± SEM and were analyzed by Mann-Whitney U test (StatView 512 program; Brain Power Inc). Linear regression analysis was applied to evaluate a possible relation of intragraft VEGF immunoreactivity to intimal thickening. \(P<0.05\) was regarded as statistically significant.

**Results**

**Kinetcs of VEGF in Acute and Chronic Rejection**

In situ hybridization disclosed that VEGF mRNA is expressed at a minimal level by cardiomyocytes in nontransplanted rat hearts (Figure 1a). In syngeneic grafts, VEGF mRNA expression was mild in cardiomyocytes (Figure 1c). During acute rejection, VEGF mRNA expression was moderate in cardiomyocytes but was saliently expressed in infiltrates of mononuclear inflammatory cells (Figure 1e). In long-term surviving cardiac allografts with mild intimal thickening and preserved myocardium, moderate VEGF mRNA signals were localized to cardiomyocytes and, to some extent, to graft-invading mononuclear inflammatory cells (not shown). In chronically rejecting cardiac allografts with severe arteriosclerotic changes, moderate VEGF mRNA expression was localized to cardiomyocytes of vital myocardium (Figure 1g) and was particularly strong in cardiomyocytes adjacent to infarcted areas. Mild to moderate VEGF mRNA expression was localized to intiisitital and periadicular mononuclear inflammatory cells (Figure 1i). We did not observe any specific signal of VEGF mRNA expression in vascular structures in any specimen (Figure 1i).

Immunohistochemical examination localized VEGF protein in cardiomyocytes and media cells of arteries in nontransplanted hearts (Figure 1b) and in syngeneic grafts (Figure 1d). During acute rejection, VEGF immunoreactivity was moderate in cardiomyocytes, but strong expression was localized to inagraft infiltrates of interstitial mononuclear inflammatory cells (Figure 1f). In long-term surviving cardiac allografts with mild arteriosclerotic changes, VEGF protein expression was mild in cardiomyocytes, in interstitial mononuclear inflammatory infiltrates, and in the media, intima, and endothelium of arteries (not shown). In cardiac allografts with severe arteriosclerotic changes, VEGF immunoreactivity was moderate in vital myocardium (Figure 1h) but was particularly strong in cardiomyocytes adjacent to infarcted areas. VEGF protein expression was moderate in intisitital mononuclear inflammatory infiltrates and...
in the media, intima, and endothelium of arteries (Figure 1j). Linear regression analysis revealed a clear correlation between intimal thickening and VEGF immunoreactivity in intimal cells, interstitial inflammatory cells, arterial endothelial cells, cardiomyocytes, postcapillary venules/capillaries, and media cells of arteries, in that order (Figure 2).

**Figure 1.** Kinetics of VEGF mRNA (left) and protein (right) expression in acute and chronic rejection of rat cardiac transplants. a and b, Sections of normal hearts showed faint VEGF mRNA (arrows) and protein expression in cardiomyocytes. c and d, In syngeneic grafts, VEGF mRNA and protein were mildly expressed in cardiomyocytes. e and f, In acute rejection, VEGF mRNA and protein expression was moderate in cardiomyocytes (e, inset) and in graft-infiltrating mononuclear inflammatory cells. g and h, In long-term surviving cardiac allografts with severe arteriosclerotic changes, moderate VEGF mRNA and protein expression was localized to cardiomyocytes. i and j, Note that no VEGF mRNA was localized to arterial wall structures in any specimen, but VEGF protein expression was observed in media, intima, and endothelium of arteries (dotted line indicates internal elastic lamina). Reactions with sense probe did not result in any specific hybridization (g, inset). Incubation of antibody with excess amount of VEGF peptide did not reveal any specific immunoreactivity (h, inset). Hematoxylin counterstaining for immunostaining.

**Blockade of VEGFR Activation Reduces Cardiac Allograft Arteriosclerosis**

The mean number of arteries analyzed in cardiac allograft cross sections was 37±4 (total of 220 arteries from 6 allografts) in allografts of vehicle-treated rats and 31±6 (total of 184 arteries from 6 allografts) in allografts of PTK
787–treated rats. In cardiac allografts of vehicle-treated rats, 88±7% of arteries (Figure 3A) were affected by intimal thickening, and the mean grade of intimal thickening was 1.8±0.3 (Figures 3b and 3c). Treatment with PTK 787 reduced the percentage of affected arteries to 67±5% (P<0.05; Figure 3A) and the mean grade of intimal thickening to 1.0±0.2 (P<0.05; Figures 3b and 3d) compared with vehicle-treated rats.

**VEGF Gene Transfer Enhances Allograft Arteriosclerosis**

In rabbit cardiac allografts, moderate transgene expression persisted for ≥30 days as determined by staining with an antibody against β-galactosidase (Figure 4a) and by X-gal staining (not shown). Expression was robust in endothelial cells of arteries, capillaries, and postcapillary venules and mild in medial cells of arteries and cardiomyocytes. Mild to moderate mouse VEGF164 expression in transfected rabbit allografts was localized to cardiomyocytes (Figure 4b). The mean number of arteries in cardiac cross sections analyzed was 88±7 (total of 616 arteries from 7 allografts) in Ad.lacZ-perfused allografts and 79±7 (total of 472 arteries from 6 allografts) in Ad.VEGF-perfused allografts. Allograft perfusion with Ad.VEGF increased the incidence of arteries with arteriosclerotic lesions from 55±7% to 79±7% (P<0.05; Figure 4e) and the mean grade of intimal thickening from 1.1±0.1 to 1.7±0.1 (P<0.05; Figures 4c, 4d, and 4f) in medium-sized arteries compared with Ad.lacZ-perfused allografts.

**VEGF Overexpression Increases Macrophage Influx and Intimal Vessel Formation**

Immunohistochemical analysis of allograft cross sections showed that perfusion with Ad.VEGF led to an increased number of graft-infiltrating activated macrophages and a decreased number of CD8+ T cells compared with Ad.lacZ-perfused allografts (P<0.05; Figure 5). No difference was observed in the numbers of CD4+ T cells between the 2 groups. Hardly any CD4+ or CD8+ T cells or macrophages were observed in the developing intima. In addition, we
examined advanced arteriosclerotic lesions for the presence of intimal neovascularization, identified by immunohistochemistry using an antibody against the endothelial cell marker CD31. These newly formed intimal capillaries were not observed in arteries with an intimal thickness score <2. The incidence of intimal neocapillaries in advanced arteriosclerotic lesions was increased 29-fold in Ad.VEGF-perfused allografts compared with Ad.lacZ-perfused allografts (Figure 6; P<0.01).

**Discussion**

Our current findings show a positive regulatory role between VEGF and arteriosclerotic lesion formation in cardiac allografts. First, linear regression analysis of rat cardiac allografts demonstrated a strong correlation between intragraft VEGF immunoreactivity and the development of intimal thickening, whereas blockade of signaling downstream of the VEGFR significantly reduced arteriosclerotic lesions. Second, intracoronary perfusion with an adenoviral vector that encoded mouse VEGF164 at the time of transplantation enhanced arteriosclerotic lesions of cardiac allografts in cholesterol-fed rabbits.

Previous studies show that vascular SMCs may produce VEGF in response to hypoxia, cytokines, growth factors, and balloon injury. Therefore, an unexpected finding was that VEGF mRNA expression was absent from the medial layer of arteries examined both during acute rejection and in different stages of arteriosclerotic lesion formation. However, the lack...
VEGF expression was markedly upregulated in cardiomyocytes of areas with vital myocardium and, in particular, in graft-infiltrating mononuclear inflammatory cells. Our findings clearly argue for the role of alloimmune response as an alternative stimulus capable of modulating VEGF expression during allograft arteriosclerosis.

Given the vasculoprotective role of VEGF in ordinary atherosclerosis, intragraft overexpression of VEGF could have prevented intimal thickening by enhancing the regeneration and functional recovery of vascular endothelium damaged by ischemia-reperfusion injury and the alloimmune response. Our findings raise the crucial question, how does VEGF enhance arteriosclerotic lesion formation in cardiac allografts? Although VEGF does not induce SMC proliferation, VEGF could increase SMC migration directly or indirectly by triggering the release of platelet-derived growth factor-BB from adjacent cells. VEGF may also enhance VEGFR receptor-1–mediated infiltration of macrophages and VEGFR-2–mediated migration of endothelial and smooth muscle progenitor cells from bone marrow into the graft, which may contribute to the formation of transplant coronary arteriosclerosis.

Ordinary atherosclerotic lesions in apolipoprotein E–deficient mice and in humans contain intimal vessels, and a recent study on angiogenesis inhibitors demonstrated that intimal neovascularization promotes the progression of atherosclerosis. In the present study, perfusion of cardiac allografts with a clinical-grade adenovirus that encoded mouse VEGF164 increased the incidence of neovascularization in advanced intimal lesions 29-fold compared with allografts perfused with control virus. Thus, the direct or indirect (macrophage mediated) angiogenic features of VEGF could explain, at least in part, the deleterious effects of VEGF overexpression on intimal thickening.

Comparison of syngeneic and allogeneic grafts in the acute rejection model suggests that ischemia-reperfusion injury induces VEGF mRNA expression in cardiomyocytes in syngeneic grafts, whereas an alloimmune response enhances VEGF mRNA expression in cardiomyocytes and in graft-infiltrating mononuclear cells. In long-term surviving cardiac allografts with arteriosclerotic lesions, both myocardial ischemia and local production of proinflammatory cytokines and growth factors by activated macrophages may induce VEGF mRNA expression in cardiomyocytes and in graft-infiltrating mononuclear cells. In the present study, VEGF was strongly induced as a consequence of ischemia due to advanced arteriosclerotic lesions. But importantly, because VEGF expression was markedly upregulated in cardiomyocytes of areas with vital myocardium and, in particular, in graft-infiltrating mononuclear inflammatory cells, our findings clearly argue for the role of alloimmune response as an alternative stimulus capable of modulating VEGF expression during allograft arteriosclerosis.
In conclusion, our observations suggest that VEGF may enhance cardiac allograft arteriosclerosis, possibly in connection with the chemotactic activity of VEGF to SMCs and macrophages, as well as its angiogenic properties, including the induction of intimal capillary formation.

Acknowledgments
This study was supported by grants from the Aarne Koskelo Foundation, the Finnish Foundation for Cardiovascular Research, Helsinki University Central Hospital Research Funds, Sigrid Juselius Foundation, and University of Helsinki, Helsinki, Finland.

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_Circulation_. 2002;105:2524-2530; originally published online May 6, 2002;
doi: 10.1161/01.CIR.0000016821.76177.D2
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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