Angiopoietin-1 Protects Against the Development of Cardiac Allograft Arteriosclerosis

Antti I. Nykänen, MD; Rainer Krebs, MSc; Anne Saaristo, MD; Päivi Turunen, MSc; Kari Alitalo, MD, PhD; Seppo Ylä-Herttuala, MD, PhD; Petri K. Koskinen, MD, PhD; Karl B. Lemström, MD, PhD

Background—Angiopoietin (Ang)–1 is an angiogenic growth factor that counteracts the permeability and proinflammatory effects of vascular endothelial growth factor and other proinflammatory cytokines. Recently, we demonstrated that vascular endothelial growth factor enhances cardiac allograft arteriosclerosis. Here, we studied the roles of Ang1, its natural antagonist Ang2, and their receptor Tie2 in rat cardiac allograft arteriosclerosis.

Methods and Results—Heterotopic cardiac allografts and syngrafts were transplanted from Dark Agouti (DA) to Wistar-Furth rats and from DA to DA rats, respectively. Immunohistochemistry disclosed that only a few mesenchymal cells expressed Ang1 in normal hearts and syngrafts, whereas no immunoreactivity was found in cardiac allografts undergoing chronic rejection. Ang2 and Tie2 immunoreactivity was induced mainly in capillaries and postcapillary venules in chronic allografts when compared with syngeneic controls, but no immunoreactivity was found in arterial endothelium. Intracoronary perfusion of cardiac allografts with a clinical-grade adenoviral vector encoding human Ang1 (Ad.Ang1) protected against the development of allograft arteriosclerosis. Ad.Ang1 perfusion reduced Ang2 expression in microcirculation, the numbers of graft-infiltrating leukocytes, and the level of immunoactivation and interstitial fibrosis, as well as both the incidence and intensity of intimal lesions. Ad.Ang1 perfusion also increased CD34+ stem cell counts in peripheral blood.

Conclusions—Our findings suggest that the antiinflammatory properties of Ang1 may offer an entirely new therapeutic approach to prevent cardiac allograft arteriosclerosis.

Key Words: angiogenesis ■ endothelium ■ inflammation ■ arteriosclerosis ■ transplantation

vascular-specific growth factors have important roles in prenatal and postnatal vascular development and are carefully regulated to build normal vasculature. These growth factors also regulate inflammation and, in fact, vascular endothelial growth factor (VEGF) was first known for its permeability properties. VEGF also induces endothelial adhesion molecule expression, enhances leukocyte rolling and adhesion, and is a direct chemoattractant to monocytes through VEGF receptor-1. Therefore, it is no surprise that inappropriate induction of VEGF results in immature leaky and hemorrhagic vessels and is associated with several pathological conditions such as diabetic retinopathy, tumor growth, arteriosclerosis.

See p 1237

Angiopoietin (Ang)–1 and Ang2 belong to another vascular-specific growth factor family and are both ligands for Tie2 receptor, expressed on endothelial and certain hematopoietic cells. Ang1 induces Tie2 receptor activation, whereas Ang2 functions as a natural antagonist for Tie2. In an early phase of vessel remodeling such as tissue repair, female reproductive organs, and tumors in adults, Ang2 provides a destabilizing signal for endothelial cells, leading to vessel regression or sprouting depending on the presence of other angiogenic factors. Ang1, on the other hand, stabilizes the immature and leaky vessels and also helps to maintain the integrity of mature vessels by promoting interactions between endothelial cells and underlying supporting cells. Possible mechanisms for the antipermeability and antiinflammatory properties of Ang1 include alteration of cell-cell junctional complexes, reduction of endothelial adhesiveness to leukocytes, and inhibition of tissue factor expression.

We recently demonstrated that VEGF enhances cardiac allograft arteriosclerosis, which is associated with increased influx of macrophages into the graft. As both in vitro and in vivo studies suggest that Ang1 counteracts the permeability and proinflammatory effects of VEGF and other proinflam-
matory cytokines on endothelial cells,15,17 we hypothesized that Ang1 would have favorable effects on cardiac allograft arteriosclerosis. Our findings in cardiac allografts demonstrate that Ang2 and Tie2 are induced in capillaries and postcapillary venules in chronic rejection. Moreover, Ang1 reduces intragraft inflammation and protects against the development of allograft arteriosclerosis.

**Methods**

**Experimental Design**

To investigate the kinetics of Ang1, Ang2, and Tie2 expression during acute and chronic rejection, we transplanted heterotopic cardiac allografts between fully major histocompatibility complex (MHC)–mismatched strains from Dark Agouti (DA) (AG-B4, RT1\(^b\)) to Wistar-Furth (WF) (AG-B2, RT1\(^b\)) rats (Harlan, Horst, the Netherlands).19 Syngeneic controls were done from DA to DA rats.

In the acute rejection model, no immunosuppression was given and grafts were harvested 5 days after the operation. In the chronic rejection model, recipients received cyclosporin A (Novartis) 2.0 mg/kg per day SC for the first week and 1.0 mg/kg per day thereafter to prevent acute rejection and to achieve the development of cardiac allograft arteriosclerosis. Grafts were harvested 8 weeks after the transplantation or if the function of the graft fell to <30 beats per minute, which was determined by daily palpation.

To determine how Ang1 regulates the development of cardiac allograft arteriosclerosis, the chronic rejection model was used. Coronary arteries of the donor hearts were perfused through the aorta with 2×10\(^{5}\) plaque-forming units of adenoviral vector encoding either human Ang1 (Ad.Ang1) or β-galactosidase (Ad.lacZ) under the control of cytomegalovirus promoter with 10\(^{-2}\) mol/L acetylcholine to permeabilize the endothelium. After perfusion, the heart with its main arteries cross-clamped was placed in ice-cold PBS for 30 minutes and was then anestomosed to the abdominal aorta and inferior vena cava of the recipient.

**Construction and Functionality of the Adenoviruses**

Full-length human Ang1 cDNA was fused to a sequence encoding the carboxyl terminus of Myc epitope, and adenoviruses were produced using an Adeno-X Expression System kit (BD Biosciences Clontech). Nuclear-targeted lacZ adenoviruses were produced as described.20 All E1- to E3-deleted GMP-grade adenoviruses were analyzed to be free of replication-competent viruses, lipopolysaccharide, mycoplasma, and other microbiological contaminants. To determine the in vitro functionality of Ad.Ang1, 293EBNA cells were transfected with Ad.lacZ or Ad.Ang1. The cells were metabolically labeled and subjected to immunoprecipitation with Myc-specific antibody (BAbCO [CRP, Inc]), to detect recombinant Ang1, or to binding assay with soluble Tie2-immunoglobulin fusion protein (R&D Systems). The bound proteins were precipitated with protein G Sepharose, separated in 10% SDS-PAGE, and analyzed by autoradiography. Adenoviral transgene expression in vivo was determined by X-gal staining from cardiac allograft and recipient liver and spleen at days 2, 7, and 14 after transplantation of Ad.lacZ-perfused allograft (n=1 to 2 per time point). Cryosections were fixed in 0.5% glutaraldehyde in PBS at room temperature for 10 minutes and incubated in a staining solution of (in mmol/L) 20 potassium ferricyanide, 20 potassium ferrocyanide, and 2 MgCl\(_2\), and 1 mg/mL X-gal in PBS at 37°C overnight.

**Histology**

Transplant arteriosclerosis was determined in a blinded manner from paraformaldehyde-fixed paraffin sections stained with hematoxylin-eosin and Resorcin fuchsins for internal elastic lamina using computer-assisted image processing (NIH Image version 1.62, NIH; http://rsb.info.nih.gov/nih-image/) and measuring the area surrounded by the internal elastic lamina and vessel lumen. Occlusion percentage was determined as a ratio of neointimal area and internal elastic lamina area. Graft interstitial fibrosis was determined from sections stained with Masson’s trichrome and scored semiquantitatively (0 to 3) as follows: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; and 3, intense interstitial fibrosis.

**Immunohistochemistry**

Cryostat sections were stained with a peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories), and the reaction was revealed by 3-amino-9-ethylocarbazole (Vectastain).19 Antibodies and dilutions used were as follows: Ang1 (10 μg/mL, RDI-ANGIOPO1Xabr) from Research Diagnostics; Ang2 (2 μg/mL, sc-7017), Tie2 (2 μg/mL, sc-9026), and CD34 (2 μg/mL, sc-7324) from Santa Cruz Biotechnology; CD4 (5 μg/mL, 22021D), CD8 (5 μg/mL, 22071D), ED1 (5 μg/mL, 22451D), and interleukin (IL)–2Rα (5 μg/mL, 22090D) from Pharmingen; RECA-1 (1:10, MCA970) from Serotec; leukocyte common antigen (LCA, 1:100, clone OX10) and major histocompatibility complex (MHC) class II (1:100, clone OX6) from Sera Laboratary; vascular cell adhesion molecule (VCAM)–1 (10 μg/mL, MMS-141P) from BAbCO (CRP, Inc); and ICAM-1 (10 μg/mL, clone 1A29) from Sekigaku. Negative controls were performed without the primary antibody, and for Ang2 staining, primary antibody at 0.5 μg/mL dilution was incubated for 2 hours in room temperature with 50-fold weight excess of Ang2 peptide antigen (sc-7017P, Santa Cruz Biotechnology) before staining. Ang1 is given as mean number of immunoreactive cells and Ang2 and Tie2 as mean number of positive capillaries and postcapillary venules per cross section. Inflammatory cells from graft parenchyma were counted from four random fields from each quadrant of the section with ×40 magnification and given as mean number of positive cells per mm\(^2\).

**Peripheral Blood Analysis**

Peripheral blood was taken from tail vein to EDTA tube 1, 2, 4, 6, and 8 weeks after the transplantation. Blood samples from nonoperated WF rats were used as controls. Leukocyte count was determined with Coulter T890 (Beckman Coulter) cell counter. Immunofluorescence staining was performed by using a lyse-and-wash procedure. Peripheral blood (100 μL) was incubated with the FITC-conjugated antibody for 15 minutes at room temperature after which the erythrocytes were lysed with Cellkit C-04 (Cellset) and washed twice with PBS. Cells (1×10\(^6\) cells) were analyzed with a FACScan (Becton Dickinson) flow cytometer. Antibodies used were CD34-FITC (sc-7324, Santa Cruz Biotechnology), ED1-FITC (MCA341F, Serotec), and IgG1-FITC (X0927, Dako) as negative isotype control. Absolute circulating cell numbers were determined by multiplying

**Figure 1.** Kinetics of Ang1 (A), Ang2 (B), and Tie2 (C) immunoreactivity in acute and chronic rejection of rat cardiac allografts. Ang1 is given as number of positive cells per cross section, whereas Ang2 and Tie2 are given as number of positive capillaries and postcapillary venules per cross section. Data are mean±SEM, by Student t test. N values are the following: normal heart, 5; acute syngraft, 5; acute allograft, 5; chronic syngraft, 6; and chronic allograft, 11.
the leukocyte count by the percentage of positive cells determined by fluorescence-activated cell sorting.

Statistics
All data are mean±SEM and analyzed by parametric Student t test or nonparametric Mann-Whitney U test (StatView 4.1 program; Abacus Concepts, Inc). Graft survival between groups was analyzed by log-rank test (Medstat, Astra Group A/S). Peripheral blood cell counts were analyzed by ANOVA for repeated measures over time. P<0.05 was regarded as statistically significant.

Results

Ang2 and Tie2 Immunoreactivity Is Induced in Capillaries and Postcapillary Venules of Cardiac Allografts

Only a few Ang1-immunoreactive cells were detected in normal heart, acute syngrafts and allografts, and chronic syngrafts, but no Ang1+ cells were detected in chronic allografts (P<0.001; Figure 1A). Ang1+ cells were round or oval in shape, were located in cardiac mesenchyme (Figure 2A), and did not show any immunoreactivity for antibodies against endothelial cells (RECA-1), leukocytes (LCA), or stem cells (CD34) on serial sections (data not shown).

Ang2 immunoreactivity was detected in normal hearts, syngrafts, and allografts mainly in endothelial cells of capillaries and postcapillary venules (Figure 2B and 2C). The number and immunoreactivity of Ang2+ capillaries and postcapillary venules were increased in chronic allografts (P<0.01; Figures 1B, 2B, and 2C) compared with respective syngeneic controls. In chronic allografts, Ang2 was expressed also in some cardiomyocytes (Figure 2D) and inflammatory cells (Figure 2E), but no Ang2 expression was detected in arterial endothelium (Figure 2E, arrow). In chronic allografts with severe arteriosclerotic changes, intimal neocapillaries showed low Ang2 immunoreactivity (Figure 2F).

Tie2 immunoreactivity was detected mainly in capillaries and postcapillary venules in normal heart, syngrafts, and allografts (Figure 2H). The number of Tie2+ capillaries was upregulated in acute allografts (P<0.01) and in chronic allografts (P<NS; Figure 1C) compared with respective syngeneic controls. In chronic allografts, Tie2 immunoreactivity was also found in intimal neocapillaries of totally occluded arteries (Figure 2G; same artery as in Figure 2F). These Ang2- and Tie2-immunoreactive intimal neocapillaries were also positive for the endothelial cell marker RECA-1 (Figure 2F, inset).

Functionality of Adenoviruses In Vitro and In Vivo

Medium of Ad.lacZ- or Ad.Ang1-transfected cells was subjected to immunoprecipitation with Myc-specific antibody or soluble Tie2-immunoglobulin fusion protein. Gel electrophoresis concluded that Ad.Ang1-transfected cells produce a 70-kDa recombinant Ang1 polypeptide (Figure 3A), which also binds to Tie2 receptor (Figure 3B). Intracoronary adenoviral perfusion resulted in low to moderate transgene expression in cardiac allografts as well as in the spleen and liver of the recipient, which persisted for at least 7 days as determined by X-gal staining (data not shown).
Ang1 Protects Against Development of Cardiac Allograft Arteriosclerosis

To investigate the effect of Ang1 on cardiac allograft arteriosclerosis, allografts were perfused with an adenoviral vector encoding either lacZ (n=11) or Ang1 (n=9). Seventy-three percent of Ad.lacZ-perfused and 100% of Ad.Ang1-perfused allografts survived 8 weeks (P=NS; Figure 4C). Allograft perfusion with Ad.Ang1 reduced the interstitial fibrosis score from 1.8±0.3 to 0.7±0.3 (P<0.05; Figure 4A, 4B, and 4D) compared with Ad.lacZ-perfused allografts.

The mean number of arteries in cardiac cross section analyzed was 52±5 (total of 570 arteries from 11 allografts) in the Ad.lacZ group and 55±5 (total of 491 arteries from 9 allografts) in the Ad.Ang1 group. Allograft perfusion with Ad.Ang1 reduced the incidence of arteries with arteriosclerotic lesions from 81±7% to 55±6% (P<0.05; Figure 4G), and the mean percentage of vessel occlusion from 48±9% to 20±5% (P<0.05) compared with Ad.lacZ-perfused allografts (Figure 4E, 4F, and 4H).

Ang1 Reduces the Influx of Leukocytes Into the Graft

Perfusion with Ad.Ang1 reduced the numbers of graft-infiltrating LCA+ cells from 850±244 to 254±50 (P<0.05), CD4+ T cells from 433±102 to 169±33 (P<0.05), CD8+ T cells from 122±23 to 83±13 (P=NS), and ED1+ macrophages from 300±79 to 131±38 (P=NS), as well as the level of immunoactivation as determined by the numbers of IL-2R+ cells from 41±15 to 4±1 per mm2 (P<0.05; Figure 5) compared with Ad.lacZ-perfused allografts. Ad.Ang1 perfusion also decreased the numbers of Ang2+ vessels per cross section.
circular ED1+ monocytes, and CD34+ hematopoietic stem cells after transplantation (Figure 6A, 6C, and 6E) compared with nonoperated controls. In recipients with Ad.Ang1-perfused allografts, the levels of CD34+ hematopoietic stem cells increased to >2-fold at 2 weeks compared with the Ad.lacZ group and gradually decreased below the levels of the Ad.lacZ group (Figure 6E). A similar but subtler trend was observed with the peripheral blood leukocyte count and circulating ED1+ monocytes (Figure 6A and 6C).

Discussion
Our current findings show that Ang2 and Tie2 immunoreactivity is induced in capillaries and postcapillary venules of chronically rejecting rat cardiac allografts and that Ang1 protects against the development of allograft arteriosclerosis. Intracoronary perfusion of cardiac allografts with Ad.Ang1 reduced the numbers of graft-infiltrating leukocytes, the levels of immunoactivation and interstitial fibrosis, and both the incidence and intensity of intimal lesions.

Perivascular inflammation and injury to graft coronary arteries plays a pivotal role in the pathogenesis of chronic rejection. Subsequent arterial remodeling, including recruitment of smooth muscle progenitor cells from recipient bone marrow to the injured vessels, results in neointima formation and luminal occlusion of coronary arteries. Early activation of graft microvascular endothelium and upregulation of adhesion proteins, on the other hand, are key steps toward extravasation of inflammatory cells into the graft parenchyma and perivascular area, leading to subsequent vessel injury. Recent studies on angiogenic growth factors show that in addition to mediating endothelial proliferation and construction of new vessels, they also regulate inflammatory responses. VEGF and Ang2 are shown to increase vessel permeability and inflammation, whereas Ang1 may inhibit these effects.

Our previous observations in rat cardiac allografts transplanted between fully MHC-mismatched strains with cyclosporine A immunosuppression show characteristic features of chronic rejection. These include interstitial and perivascular inflammation, a low-level immune activation, interstitial fibrosis, and accumulation of smooth muscle cells into the neointima, whereas mononuclear cells are seldom detected in the intima. Our recent study on allograft arteriosclerosis revealed a clear correlation between intimal thickening and VEGF immunoreactivity, whereas blocking signaling downstream of VEGF receptor activation reduced allograft arteriosclerosis. In addition, intracoronary Ad.VEGF perfusion enhanced allograft arteriosclerosis associated with an increased number of graft-infiltrating macrophages.

Our present results show that alloimmune responses also upregulate Ang2 and Tie2 expression in cardiac allografts, whereas Ang1 expression is decreased. Ang2 immunoreactivity was induced in graft microvessels—where inflammatory cells enter the graft—and also in cardiomyocytes. Although Ang2 immunoreactivity was limited to endothelium of capillaries and postcapillary venules, Ang2 coexpression with Tie2 in neointimal neocapillaries of severely occluded arteries might also have a direct role in neointima formation. Interestingly, our present study shows that Ang1 has a...
protective effect against the development of cardiac allograft arteriosclerosis. As adenovirally mediated gene expression generally lasts 1 to 2 weeks, as was also seen in our study, and intracoronary Ad.Ang1 perfusion was associated with reduction in graft inflammation, Ad.Ang1 probably protected against the early infiltration of inflammatory cells and subsequent parenchymal and vascular damage. Ang1 can exert its antiinflammatory and antipermeability properties through Tie2 activation, but a recent report suggests that angiopoietins also have receptor-independent effects on cell adhesion, which are probably mediated through integrins.27 Therefore, Ad.Ang1 perfusion might also have stabilized arterial endothelium, even though Ang2 and Tie2 expression was only detected in microcirculation.

We detected only a few Ang1-immunoreactive cells in normal rat adult heart, whereas none were detected in chronic allografts. The phenotype and function of these cells remain unclear, as staining of serial sections did not show any immunoreactivity for endothelial cell, leukocyte, or hematopoietic stem cell marker (data not shown). Previous studies state that Ang1 is expressed in pericytes surrounding developing vessels in embryos, but Takakura et al28 also describe an Ang1-expressing round mesenchymal hematopoietic cell type not adhering to vessels.

As recent reports underline the involvement of stem cells in neointima formation after arterial damage,23,24 it is interesting that both VEGF and Ang1 can also mobilize hematopoietic and endothelial progenitor cells from bone marrow,29 which may contribute to vessel remodeling. In our study, Ad.Ang1 perfusion resulted in an increased mobilization of hematopoietic stem cells to peripheral blood. However, further studies are needed to clarify how stem cells migrate to damaged vessels and whether local differentiation signals direct them to participate in endothelial repair or pathological vessel remodeling.

In conclusion, this study provides direct in vivo evidence of the important roles of angiopoietins in cardiac allograft arteriosclerosis. Moreover, our study suggests that the anti-
inflammatory properties of Ang1 may offer entirely new therapeutic approaches to prevent manifestations of chronic rejection in cardiac allografts.

Acknowledgments

This study was supported by grants from Helsinki University Central Hospital Research Funds, the Sigrid Juselius Foundation, Finnish Life and Pension Insurance Companies, the Research and Science Foundation of Farmos, The Finnish Medical Foundation, and the Aarne Koskelo Foundation. We thank M. Sandberg, RN; M. Schoutz, RN; and E. Wasenius, RN, for their excellent technical assistance.

References

Angiopoietin-1 Protects Against the Development of Cardiac Allograft Arteriosclerosis
Antti I. Nykänen, Rainer Krebs, Anne Saaristo, Päivi Turunen, Kari Alitalo, Seppo Ylä-Herttuala, Petri K. Koskinen and Karl B. Lemström

Circulation. 2003;107:1308-1314; originally published online February 17, 2003; doi: 10.1161/01.CIR.0000054623.35669.3F
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

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/107/9/1308

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/