Common Protective and Diverse Smooth Muscle Cell Effects of AAV-Mediated Angiopoietin-1 and -2 Expression in Rat Cardiac Allograft Vasculopathy

Antti I. Nykänen, Katri Pajusola, Rainer Krebs, Mikko A.I. Keränen, Olivier Raisky, Petri K. Koskinen, Kari Alitalo, Karl B. Lemström

Abstract—Angiopoietin-1 (Ang1) and Ang2 regulate the maintenance of normal vasculature by direct endothelial and indirect smooth muscle cell (SMC) effects. Dysfunction of vascular wall cells is considered central in cardiac allograft vasculopathy (CAV), where inflammation and arterial injury initiate subsequent intimal SMC proliferation. In this study, we investigated the effect of exogenous Ang1 and Ang2 in chronically rejecting rat cardiac allografts by intracoronary adeno-associated virus (AAV)-mediated gene transfer. Bioluminescent imaging of AAV-transfected syngeneic grafts revealed gradual and stable transgene expression in graft cardiomyocytes. In cardiac allografts, both AAV-Ang1 and AAV-Ang2 decreased inflammation and increased antiapoptotic Bcl-2 mRNA and Bcl-2/Bax ratio at 8 weeks. Only AAV-Ang2 decreased the development of CAV, whereas AAV-Ang1 activated arterial SMC and increased PDGF-A mRNA in the allograft. Collectively, our results show that exogenous Ang1 and Ang2 have similar antiinflammatory and antiapoptotic effects in cardiac allografts. Prolonged AAV-mediated Ang1 transgene expression also induced SMC activation, whereas AAV-Ang2 lacked the SMC activating effects and decreased CAV. Our results thus highlight the common protective and diverse SMC effects of Ang1 and Ang2 in cardiac allograft microenvironment and the importance of timing of angiopoietins to achieve therapeutic effects. (Circ Res. 2006;98:1373-1380.)

Key Words: endothelium ■ inflammation ■ muscle, smooth ■ angiogenesis ■ arteriosclerosis ■ transplantation

Side effects of immunosuppressive drugs and concentric neointimal thickening—cardiac allograft vasculopathy (CAV)—are the major limitations to long-term survival of heart transplant recipients.1 Allograft endothelial cell layer is important in regulating alloimmune reactions and the development of pathological arterial remodeling.2,3 In addition to traditional cardiovascular risk factors, perioperative ischemia, host alloimmune reactions, and viral infections may also injure endothelial cells (EC) in transplanted organs.2 Activated allograft endothelium in capillaries and postcapillary venules may directly enhance allorecognition4 and recruit inflammatory cells to allograft parenchyma by expressing adhesion proteins and chemokines5 and possibly by increasing vascular permeability.6,7 EC injury in arteries may also alter the delicate interaction between vascular EC and smooth muscle cells (SMC). This in turn may initiate the proliferative phase of CAV where SMC accumulate to the intima of affected arteries.2,6,7

Angiopoietin (Ang) family is an attractive target for modulating EC functions and is currently composed of four known ligands named Ang1 to -4.8 Angiopoietins regulate vascular development and maintenance and inflammatory reactions through Tie2 receptor,9,10 which is mainly expressed on the surface of EC and certain hematopoietic cells.8,9 Ang1 is a survival factor12–16 for EC and promotes interactions between endothelium and underlying supporting cells.17–19 Ang1 induces EC to express growth factors, such as endothelial-derived heparin binding EGF-like growth factor (HB-EGF)20 and serotonin,21 that recruit pericytes and SMC to form support for newly formed vessels. In addition to its functions on vascular development and maintenance, Ang1 also inhibits inflammation and vascular permeability.18,19 Ang2 has been shown to reduce EC adhesiveness to leukocytes,22 inhibit tissue factor expression,23 and alter cell-cell junctional complexes.24,25

The functional role of Ang2 is more complex, as it has both agonistic and antagonistic properties.8,26 Ang2 binds to Tie2 and acts as a competing Ang1 antagonist but can also phosphorylate Tie2.27 Ang2 may activate different downstream Tie2 signaling cascades and thus have different effector functions as Ang1.8 Correspondingly, Ang2 elicits similar antiapoptotic effects on EC than Ang1,11,27 but it lacks the...
effects on SMC recruitment.\textsuperscript{20,28} Ang2 regulates inflammation in a context-dependent manner. Ang2 induces vascular leakage and neutrophil chemotaxis but may also decrease leukocyte migration and cellular infiltration to tissues during ongoing inflammation or after proinflammatory cytokine stimulation.\textsuperscript{29,30} Interestingly, both Ang1 and Ang2 have integrin-mediated effects that promote cell attachment and cardiomyocyte survival and do not involve Tie2 receptor.\textsuperscript{31,32}

We previously found that Ang1 transgene expression during the early phase of CAV with adenovirus vectors has antiinflammatory effects and protects against the development arteriosclerotic changes in cardiac allografts.\textsuperscript{33} Here we used adeno-associated-virus (AAV) vectors to induce prolonged Ang1 or Ang2 transgene expression during the proliferative phase of CAV. We found that both Tie2 ligands induced similar antiinflammatory and antiapoptotic effects in cardiac allografts. Interestingly, only Ang2 effectively decreased CAV, whereas prolonged Ang1 exposure led to SMC activation in the allografts.

Materials and Methods

Heterotopic Heart Transplantation

Specific pathogen-free inbred male Dark Agouti (DA, RT1\textsuperscript{av1}) and Wistar Furth (WF, RT1\textsuperscript{b}) rats (Harlan, Horst, The Netherlands) of 2 to 3 months of age were used. Permission for animal experimentation was obtained from the State Provincial Office of Southern Finland. The rats received care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by National Academy Press (ISBN 0-309-05377-3, revised 1996). Heterotopic cardiac transplantations were performed between DA rats (syngrafts) or between fully major histocompatibility complex–mismatched DA and WF strains (allografts) using isoflurane (Isofluran Baxter, Deerfield, Ill) anesthesia.

Intralipid (Fresenius Kabi, Bad Homburg, Germany) subcutaneously diluted cyclosporine A (CsA, Novartis, Basel, Switzerland) was administered intravenously after the transplantation or, in case the beat of the allograft fell to 30 per minute, determined by daily palpation.

Cardiac allografts were perfused with AAV encoding either enhanced green fluorescence protein (AAV-Luc) or human Ang1, Ang2, or human Ang1 plus human Ang2. To investigate the kinetics of AAV-mediated transgene expression in cardiac transplants, syngrafts were perfused with AAV encoding either enhanced green fluorescence protein (AAV-Luc) or human Ang1, Ang2, or human Ang1 plus human Ang2. Allografts were harvested 8 weeks after the transplantation or, in case the beat of the allograft fell to <30 per minute, determined by daily palpation.

Production of AAV-Ang1 and AAV-Ang2

Human Ang-1 and Ang-2 cDNAs were blunt-end cloned into the MuI site of pshb-CMV-WPRE.\textsuperscript{36} 293T cells were cotransfected with a recombinant rAAV vector plasmid, the AAV packaging plasmid pAAV/Ad-rep (ACG), and the adenovirus helper plasmid pBS-E2A-VA-EA.\textsuperscript{36} Sixteen hours later, the medium was replaced by fresh complete growth medium. The cells were collected 48 hours after transfection, and rAAVs were released by 3 freeze-thaw cycles in liquid nitrogen. rAAV was purified by an iodixanol-gradient ultracentrifugation and heparin-Sepharose high-performance liquid chromatography.

Histology

CAV was determined by 2 independent observers in a blinded manner from paraffin-paraffin–fixed paraffin sections stained with hematoxylin–eosin and resorcin–fuchsin for internal elastic lamina using computer-assisted image processing (NIH Image version 1.62; http://rsb.info.nih.gov/nih-image) and measuring the area surrounded by the internal elastic lamina and vessel lumen. Arterial occlusion percentage was determined as the ratio of neointimal area and internal elastic lamina area. Additionally, arterial occlusion grade was determined using the Billingham criteria\textsuperscript{34,37} that may be more sensitive for moderate arterial changes detected in the present study: 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, some foam or vacuolated endothelial cells may be present; grade 2: <50% occlusion of the lumen; grade 3, >50% but <100% occlusion of lumen; and grade 4, 100% vessel occlusion of lumen.

Immunohistochemistry and Immunofluorescence

Cryostat sections were stained using peroxidase ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, Calif), and the reaction was revealed by 3-amin-9-ethylcarbazole (AEC) (Vector Laboratories).\textsuperscript{34} Immunofluorescent staining was performed from cryostat sections using a sequential approach with Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Molecular Probes, Carlsbad, Calif) fluorescent secondary antibodies and DAPI nuclear staining (VECTASHIELD Mounting Medium with DAPI; Vector Laboratories). Antibodies and dilutions used were CD4 (5\textsubscript{ug}/mL, 22021D), CD8 (5\textsubscript{ug}/mL, 22071D), ED1 (5\textsubscript{ug}/mL, 22451D) from BD Pharmingen (San Diego, Calif); EGFP (1:1000, ab290) from AbCam (Cambridge, UK); human high-molecular-weight melanoma-associated antigen (HMW-MAA) to detect activated SMC\textsuperscript{38} (1:25, SB02–626/Mab 225.28), a kind gift from Prof Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY); Ki67 (1:2000, NCL-Ki67p) from Novocastra Laboratories (Newcastle, UK); and ICAM-1 (10\textsubscript{ug}/mL, MMS-141P) from BAbCO (Richmond, Calif), and ICAM-1 (10\textsubscript{ug}/mL, clone 1A29) from Seikagaku (Tokyo, Japan). All analyses were performed blinded by 2 independent observers. Inflammatory cells, Ki67\textsuperscript* capillaries, and ICAM-1\textsuperscript* capillaries in allograft parenchyma were counted from 4 random fields from each quadrant of the section with x40 magnification and are given as the mean number of positive cells per millimeter squared. Ki67\textsuperscript* and HMW-MAA\textsuperscript* arteries were counted from whole allograft cross-sections and are given as the mean number of positive vessels per cm\textsuperscript{2}.

In Situ Apoptosis Detection

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) was performed in cryostat sections with CardioTACS In Situ Apoptosis Detection kit (TA5353, R&D Systems, Minneapolis, Minn) according to the instructions of the manufacturer. TUNEL–positive-staining capillaries were counted from 4 random fields from each quadrant of the section with x40 magnification and are given as mean number of positive cells per millimeter squared.
RNA Isolation and Reverse Transcription

Total RNA was extracted using TRIzol (Invitrogen) method and RNeasy Mini Kit (Qiagen, Hilden, Germany) (n=5 per group). Reverse transcription of mRNA was performed from 100 ng of total RNA in a final volume of 20 µL, using 200 U of M-MLV reverse transcriptase (Sigma Aldrich, St Louis, Mo), with 20 U of recombinant RNasin ribonuclease inhibitor (Promega, Madison, Wis), 0.5 mmol/L dNTPs (Sigma), and 2.5 µmol/L random nonamers (Sigma). After RT, 40 µL of nuclease-free water was added to each cDNA and 3 µL of each sample were used in each subsequent PCR reaction.

Proof of AAV-Mediated Transgene Expression

For proof of AAV-mediated transgene expression, a construct specific forward primer (5'-GGATGTT GCCCTTTACTTC-TAGG-3') and backward primers specific for Ang1 (5'- CCTATGTGA GTCAGAATGGCAGCG-3') and Ang2 (5'- TGCA CACGAT TCAGAC CGTA-3')9 were used. PCR reactions were performed using HotStarTaq Master Mix Kit (Qiagen). PCR programs started with enzyme activation at 95°C for 15 minutes, followed by 35 cycles of amplification (for Ang1: denaturation at 94°C for 30 seconds, annealing at 63°C for 10 seconds, extension at 72°C for 8 seconds; for Ang2: denaturation at 94°C for 30 seconds, annealing at 55°C for 10 seconds, extension at 72°C for 16 seconds) and a final extension step at 72°C for 10 minutes. One microliter of each PCR product was taken for repetition of the respective run. Five microliters of the final product were used for agarose gel electrophoresis. For negative controls, each primer set was used on cDNA samples of the respective other transgenic group.

Real-Time PCR

External standards were used to generate a standard curve for each gene of interest. The templates of these standards consisted of PCR fragments generated with the same primers as used in real-time PCR. The DNA concentrations were determined by spectrophotometry (Eppendorf, Hamburg, Germany), followed by calculation of the PCR fragment concentrations. Ten-fold serial dilutions were made starting from 10^7 PCR fragments. The number of copies of the gene of interest was extrapolated from the corresponding standard curve using LightCycler software (Roche, Basel, Switzerland).

Real-time PCR reactions were performed in a LightCycler using LightCycler FastStart DNA MasterPLUS SYBR Green I mix (Roche), primer concentrations of 0.4 µmol/L, and 3 µL of cDNA sample in a reaction volume of 10 µL. A typical protocol included a 10-minute denaturation step at 95°C followed by 35 cycles with a 95°C denaturation step for 10 seconds, annealing at 59°C for 10 seconds, and extension at 72°C, depending on the length of the product (1 second for 25 bp). Measurement of the PCR product was performed at the end of each extension period. Amplification specificity was checked using melting curve analysis. Results are given in relation to β-actin molecules.

The following primers for rat PDGF A chain (GenBank accession no. BC061731), PDGF-B chain (accession no. Z14117), HB-EGF (accession no. L05489), Bcl-2 (accession no. L14680), Bax (acces-

Figure 1. AAV-mediated transgene expression in rat heart transplants. Coronary arteries of cardiac syngrafts were perfused with AAV-luciferase and the transgene expression was revealed by bioluminescent imaging (A and B). Transgene expression in the syngraft (arrows) increased gradually, and there was also a transient expression in the liver (arrowheads) at 2 and 3 weeks (A). At 8 weeks, the transgene expression was diffuse in cross-sections of syngrafts, whereas no luciferase activity was detected in the recipient heart (B). Intramyocardial AAV-Luc injection to nontransplanted hearts resulted in local luciferase activity that remained stable for the entire study period of 1 year (C). In AAV-EGFP-perfused allografts at 8 weeks, EGFP immunoreactivity was detected through the allograft cross-section (D) and was localized to cardiomyocytes along allograft capillaries (E and F). Omitting the primary antibody did not show any specific immunoreactivity (E, inset). RT-PCR with vector-specific forward primers confirmed AAV-mediated Ang1 and Ang2 transcription in the corresponding groups (G). SR indicates square root (B). Scale bars=100 mm. EGFP in green, RECA-1 endothelial marker in red, and DAPI nuclear staining in blue (F).
sion no. NM_017059), HO-1 (accession no. NM_012580), MCP-1 (accession no. M57441), and /H9252-actin (accession no. V01217) were used: PDGF-A forward 5'H11032-GACAAACCTGAGAGCCCATG-3'H11032, reverse 5'H11032-ATGTCACACGCCACGTACAT-3'H11032; PDGF-B forward 5'H11032-AGGTGTTCCAGATCTCGC-3'H11032, reverse 5'H11032-GTCACTGTGGCCT-TCTTG-3'H11032; HB-EGF forward 5'H11032-TCTGTCTTCTTGTCATCGTG-3'H11032, reverse 5'H11032-ACTTGCTCCTGCTTTCTTCT-3'H11032; and /H9252-actin forward 5'H11032-AAGTCCCTCACCCTCCCAAAAG-3'H11032, reverse 5'H11032-AAGCAATGCTGTCACCTTCCC-3'H11032; Bcl-2 forward 5'H11032-CTGTGGATGACTGAGTACCTGAAC-3'H11032, reverse 5'H11032-AGAGACAGCCAGGAGA-AATCAAAC-3'H11032; Bax forward 5'H11032-TCATGAAGACAGGGGCC-TTTT-3'H11032, reverse 5'H11032-CAATCATCCTCTGCAGCTCCA-3'H11032; HO-1 forward 5'H11032-GGGTGACAGAAGAGGCTAAGACC-3'H11032, reverse 5'H11032-AGATTCTCCCCTGCAGAGAGAAG-3'H11032; MCP-1 forward 5'H11032-TCACCTCCACGACCCGAGGAAG-3'H11032, reverse 5'H11032-CTGACCTCCACCTGACCCGAGGAAG-3'H11032; Bax forward 5'H11032-TCATGAAGACAGGGGCC-TTTT-3'H11032, reverse 5'H11032-CAATCATCCTCTGCAGCTCCA-3'H11032; HO-1 forward 5'H11032-GGGTGACAGAAGAGGCTAAGACC-3'H11032, reverse 5'H11032-AGATTCTCCCCTGCAGAGAGAAG-3'H11032; MCP-1 forward 5'H11032-TCACCTCCACGACCCGAGGAAG-3'H11032, reverse 5'H11032-CTGACCTCCACCTGACCCGAGGAAG-3'H11032.

Statistics
All data are mean±SEM and analyzed by parametric ANOVA with Dunnett’s correction to compare the treatment groups with the control group, nonparametric Kruskall–Wallis with Dunn test to compare the treatment groups with the control group, or log-rank test to compare the graft survival using SPSS for Windows version 11.5.1 (SPSS Inc). P<0.05 was regarded as statistically significant.

Results
AAV-Mediated Gene Transfer Induces Stable Transgene Expression In Rat Heart Transplants
Bioluminescent imaging of AAV-Luc–perfused syngrafts revealed that transgene activity in the syngrafts increased gradually over time (Figure 1A, arrows) and was diffuse in the syngraft cross-sections at 8 weeks (Figure 1B). No luciferase activity was localized in the recipient heart (Figure 1B), whereas a transient transgene expression was detected in the recipient liver at 2 and 3 weeks (Figure 1A, arrowheads). Intramyocardial injection of a nontransplanted rat hearts with AAV-Luc heart produced local transgene expression that remained stable for the entire follow-up period of 1 year (Figure 1C).

EGFP immunoreactivity was detected across the allograft cross-section (Figure 1D) in AAV-EGFP–perfused allografts at 8 weeks. The transgene expression was localized to cardiomyocytes along allograft capillaries (Figure 1E and 1F). In contrast, we did not detect transgene expression in allograft endothelial cells (Figure 1F) or smooth muscle cells (data not shown). AAV-mediated Ang1 and Ang2 gene expression was confirmed in the corresponding AAV-Ang1– and AAV-Ang2–perfused allografts using RT-PCR with vector specific forward primers (Figure 1G).

AAV-Ang2 Reduces Cardiac Allograft Vasculopathy
Allograft coronary arteries were perfused with AAV encoding EGFP (n=11) Ang1 (n=8) or Ang2 (n=7) to study the effect of Ang1 and Ang2 overexpression on CAV. Two AAV-EGFP–perfused allografts were lost, whereas all AAV-Ang1– and AAV-Ang2–perfused allografts survived the study period of 8 weeks (P=NS, analyzed by log rank). The

Figure 2. The effect of AAV-mediated Ang1 and Ang2 gene transfer on rat cardiac allograft vasculopathy. Allograft coronary arteries were perfused with AAV encoding EGFP (n=11), Ang1 (n=8), or Ang2 (n=7). Allograft recipients received cyclosporin A 2.0 mg/kg per day SC for the first week and 1.0 mg/kg per day SC thereafter as immunosuppression. AAV-Ang2 perfusion decreased the incidence of intimal lesions (A), arterial occlusion percentage (B) (P=NS), and Billingham arterial occlusion grade (C). Representative photomicrographs of medium-sized coronary arteries in AAV-EGFP (D), AAV-Ang1 (E), and AAV-Ang2 (F) groups stained with hematoxylin–eosin and resorcin–fuchsin for internal elastic lamina. Data are mean±SEM, by ANOVA with Dunnett’s correction (A and B) or Kruskall–Wallis with Dunnett’s correction (C), comparing the treatment groups with the control group. Scale bars=50 μm.
mean number of arteries in cardiac allograft cross-section per animal analyzed was 37±3 in AAV-EGFP group, 33±2 in AAV-Ang1 group, and 30±4 in AAV-Ang2 group. Perfusion with AAV-Ang2 decreased the incidence (Figure 2A) and intensity (Figure 2B; \( P=\text{NS} \)) of arterial lesions and arterial occlusion grade (Figure 2C), as determined by the Billingham criteria.\(^3\) In contrast, AAV-Ang1 perfusion had no effect on CAV (Figure 2A through 2C).

**AAV-Ang1 and AAV-Ang2 Decrease Allograft Inflammation**

Potential antiinflammatory effects of angiopoietins were investigated by immunohistochemistry. Perfusion with AAV-Ang1 or AAV-Ang2 was associated with reduced numbers of ED1\(^+\) macrophages (Figure 3A), CD4\(^+\) T cells (Figure 3B; \( P=\text{NS} \) for AAV-Ang2) and CD8\(^+\) T cells (Figure 3C) in allograft parenchyma. Perfusion with AAV-Ang1 or AAV-Ang2 was also associated with a decrease in VCAM-1 (Figure 3D; \( P=\text{NS} \)) and ICAM-1 (Figure 3E; \( P=\text{NS} \)) immunoreactivity in allograft capillaries and postcapillary venules.

**AAV-Ang1 Increases Arterial SMC Activation and Proliferation**

As AAV-Ang1 failed to inhibit allograft arteriosclerosis despite its antiinflammatory effects, we investigated the effects of angiopoietins on allograft arterial SMC by antibodies against SMC activation marker HMW-MAA\(^3\) and proliferation marker Ki67. Perfusion with AAV-Ang1 increased the number of arteries with HMW-MAA\(^+\) SMC compared with AAV-Ang2 group (Figure 4A). AAV-Ang1 also increased the number of Ki67\(^+\) arteries compared with AAV-EGFP group (Figure 4B). In contrast, AAV-Ang2 perfusion did not increase SMC activation or proliferation. Investigation of myocardial capillaries showed that AAV-Ang2-perfusion decreased the density of proliferating Ki67\(^+\) capillaries (Figure 4E). Perfusion with AAV-Ang1 and AAV-Ang2 also decreased the density of TUNEL\(^+\) apoptotic capillaries (Figure 4F; \( P=\text{NS} \)).

**AAV-Ang1 Increases Allograft SMC Growth Factors**

Next, we determined the effect of AAV-Ang1 and AAV-Ang2 on expression of potent SMC growth factors by real time RT-PCR. Perfusion with AAV-Ang1 – but not AAV-Ang2 - was associated with a two-fold elevation in allograft mRNA levels of PDGF-A (Figure 5A, \( P<0.05 \)), PDGF-B (Figure 5B; \( P=\text{NS} \)) and HB-EGF (Figure 5C; \( P=\text{NS} \)).

**AAV-Ang1 and AAV-Ang2 Increase Allograft Antiapoptotic Gene Expression**

AAV-Ang1 and AAV-Ang2 was associated with two-fold elevation in mRNA levels of antiapoptotic Bcl-2 (Figure 5D), and Bcl-2/Bax ratio (Figure 5F). AAV-Ang1 did not change HO-1 mRNA whereas lower levels were observed in AAV-Ang2–perfused allografts (Figure 5G; \( P=\text{NS} \)). Perfusion with either AAV-Ang1 or AAV-Ang2 did not change MCP-1 mRNA expression (Figure 5H).

**Discussion**

Our current findings show that AAV vectors may be used to achieve stable transgene expression in cardiac allografts. AAV-mediated Ang1 and Ang2 gene transfer induced similar antiinflammatory and antiapoptotic effects. Interestingly, only Ang2 effectively decreased CAV, whereas prolonged Ang1 exposure increased PDGF-A mRNA expression and SMC activation in the allografts.
Gene therapy is an attractive tool to introduce therapeutic genes into transplanted organs during the perioperative ex vivo time of the allograft. Genes regulating alloimmune reactions, unwanted proliferative responses, or protective genes may be considered for therapeutic purposes. In contrast, adenoviral vectors generally result in rapid but transient transgene expression and may also result in an inflammatory reaction. Thus, AAV vectors may be a better choice than adenovirus vectors if long-lasting transgene expression and low-inflammatory response is required.

We have previously found that transient adenovirus-mediated Ang1 expression has both antiinflammatory and antiarteriosclerotic effects in cardiac allografts. Interestingly, AAV-mediated Ang1 expression in the current study had antiinflammatory and antiapoptotic effects but failed to inhibit pathological arterial remodeling. This may be explained partly by the delayed transgene expression profile of AAV vectors compared with adenovirus vectors used in our previous study. Antiinflammatory genes, such as Ang1, may be most effective early in the disease progression to efficiently inhibit damage to the allograft and subsequent intimal proliferation. In addition, we found that AAV-mediated Ang1 expression activated allograft arterial SMC, which may also reflect on intimal growth. Previously, Ang1 has been shown to promote SMC migration and proliferation by inducing HB-EGF and serotonin expression from EC. Here, we detected a 2-fold increase in mRNA levels of SMC growth factors PDGF-A, PDGF-B, and HB-EGF in the AAV-Ang1-perfused grafts. Thus, the unwanted SMC mitogenic effects may actually balance the beneficial antiinflammatory properties of AAV-mediated Ang1 transgene expression in cardiac allografts. A similar dual role for Ang1 has been proposed in pulmonary hypertension. Specifically, Ang1 may inhibit lung microvascular apoptosis and thus prevent monocrotaline-induced pulmonary hypertension. On the other hand, Ang1 by itself may also induce SMC hyperplasia and result in progression of pulmonary hypertension.

Previously, both Ang1 and Ang2 have been reported to share antiapoptotic properties. Here we found that both AAV-Ang1 and AAV-Ang2 decreased capillary apoptosis and increased mRNA levels of antiapoptotic Bcl-2 and Bcl-2/Bax ratio in allografts. Bcl-2 may have direct protective effects in cardiac allografts, and, interestingly, adenoviral Ang1 gene transfer activates Bcl-2 pathway in cardiomyocytes through receptor-independent mechanisms. It is thus possible that the protective effects of AAV-Ang1 and AAV-Ang2 in the current study involve Bcl-2 activation and may be both receptor dependent, EC mediated, and also receptor independent, cardiomyocyte mediated.

In addition to the common antiapoptotic effects, both AAV-Ang1 and AAV-Ang2 had antiinflammatory effects in our current study. The antiinflammatory effects of Ang1 are well documented, but Ang2 has been shown to regulate inflammation in a context-dependent manner. Namely, Ang2 by itself promotes vascular leakage and neutrophil migration. On the other hand, Ang2 actually decreases vascular endothelial growth factor (VEGF)-mediated leukocyte migration and inhibits cellular infiltration to tissues during zymosan-induced inflammation. The antiinflammatory effects of Ang2 may thus be restricted to situations in which...
inflammation and inflammatory mediators are already present, such as in cardiac allografts.

Interestingly, in contrast to AAV-Ang1, AAV-Ang2 did not increase expression of SMC growth factors but decreased myocardial capillary proliferation and protected against the development of CAV. The different effects of AAV-Ang1 and AAV-Ang2 on SMC activation in our current study possibly reflect the different effector functions of the 2 Tie2 ligands. Originally, it was believed that Ang2 is a competing antagonist for Ang1, but several recent studies suggest that Ang2 also activates Tie2 receptor. Moreover, Ang2 may have different signaling pathways and thus different effector functions than Ang1. In contrast to Ang1, Ang2 did not induce activation and proliferation of SMC in graft arteries but actually protected against the development of intimal lesions. Therefore, the beneficial effect of AAV-Ang2 on neointimal development observed in our current study may be explained by the antiinflammatory and antiapoptotic effects of prolonged Ang2 exposure, combined with the lack of SMC activation. In addition, the finding that AAV-Ang2 perfusion decreased myocardial capillary proliferation may have an impact on allograft inflammation. Our unpublished findings indicate interplay of inflammation and capillary angiogenesis in cardiac allografts, and a regulatory role for VEGF in the process.

Collectively, our results show that exogenous Ang1 and Ang2 have similar antiinflammatory and antiapoptotic effects in cardiac allografts. Prolonged AAV-mediated Ang1 transgene expression also induced SMC activation, whereas AAV-Ang2 lacked the SMC activating effects, and decreased CAV. Our results thus highlight the common protective and divergent SMC effects of Ang1 and Ang2 in cardiac allograft microenvironment and the importance of timing of angiopoietins to achieve therapeutic effects.

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