Gene Transfer of a Broad Spectrum CC-Chemokine Inhibitor Reduces Vein Graft Atherosclerosis in Apolipoprotein E–Knockout Mice

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Background—Accelerated atherosclerosis is a major cause of vein graft failure after bypass surgery. Several CC-chemokines (CC-CKs) mediate monocyte/macrophage recruitment in native atherosclerotic plaques; we hypothesized that CC-CKs may be critical in the development of accelerated atherosclerosis in vein grafts.

Methods and Results—Using in vivo gene transfer, we administered a soluble CC-CK binding protein ("35K") to apolipoprotein E–knockout (ApoE−/−) mice that underwent interposition bypass grafting of the vena cava from isogenic donor mice to the common carotid artery. Two days before operation, a recombinant adenovirus encoding either 35K (Ad35K) or green fluorescent protein (AdGFP; control) was injected into recipient mice via the tail vein. 35K greatly reduced CC-CK activity in mouse plasma. After 14 days, vein graft atherosclerotic lesion area, smooth muscle α-actin–positive neointimal area, and total vessel wall thickness were strikingly reduced by Ad35K gene transfer compared with AdGFP controls. Furthermore, 35K gene transfer dramatically reduced macrophage content by ~90% and cell proliferation by 95%. After 28 days, lesion area and vessel wall thickness remained significantly less in Ad35K mice.

Conclusion—A single intravenous injection of the CC-CK inhibitor 35K significantly reduced atherosclerosis in carotid–caval vein grafts in ApoE−/− mice. This study highlights the importance of the CC-CK class in accelerated atherosclerosis, and its role as a potential target for improving vein graft patency. (Circulation. 2005;112[suppl I]:I-235–I-241.)

Key Words: gene therapy ■ inflammation ■ atherosclerosis ■ veins ■ surgery
injury, and stent injury. In vein grafts, upregulation of MCP-1 gene expression in the neointima led to an increase in monocyte/macrophage recruitment to the vessel wall with subsequent increased intimal hyperplasia. In keeping with the aggressive nature of accelerated atherosclerosis, it is likely that many other CC-CKs are also important in lesion formation.

Therapeutic interventions aimed at modulating the CC-CKs could provide a rational and effective strategy in reducing accelerated atherosclerosis. However, targeting a single CC-CK may be limited by potential redundancy. As many as 9 CK receptors have been identified, many of which can bind numerous ligands with similar affinities.

The Vaccinia virus strain Lister expresses a 35KD all protein (35K), which inactivates a wide range of CC-CKs. We demonstrated recently that recombinant 35K could inhibit CC-CK activity in vitro and in vivo. Accordingly, we sought to evaluate the effects of CC-CK inhibition, using 35K gene transfer, on the development of accelerated atherosclerosis in murine vein grafts.

**Methods**

**Generation of Adenovirus-Expressing 35K**

The recombinant adenovirus encoding the broad spectrum CC-CK binding protein “35K” (Ad35K) was generated as described previously. The viral construct incorporated a C-terminal hemagglutinin (HA) epitope tag to aid in identification. A recombinant adenovirus encoding enhanced green fluorescent protein (AdGFP) was used as a control for viral infection. Viruses were purified using CsCl gradient ultracentrifugation, as described previously.

**Western Immunoblotting**

To detect the presence of 35K protein, diluted mouse plasma was incubated overnight with monoclonal anti-HA agarose-conjugated beads (Sigma). After washing, bound proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and detected using a rat monoclonal anti-HA antibody (Roche).

**Lipid and Lipoprotein Analysis**

Total plasma cholesterol and triglyceride concentrations were measured using enzymatic assay (Roche) on a Cobas Mira Plus automated analyzer (Roche).

**Chemotaxis Assay**

We quantified specific CCR5-mediated cell migration and primary ApoE−/− aortic SMC migration as described previously. For CCR5 migration, 293 cells were grown to 50% confluence in DMEM plus 10% (v/v) FCS, followed by transfection with plasmids encoding CCR5 and enhanced GFP. Cells were incubated at 37°C overnight on 8-µm porous membranes and allowed to migrate toward mouse plasma placed in the chamber below the membrane. In primary SMC experiments, culture medium (10 µL) known to contain high levels of 35K protein was added to ApoE−/− mouse plasma. SMCs were cultured as described previously. Cell migration was quantified from fluorescent microscopic images of cells on the underside of the membrane. Experimental samples were analyzed in duplicate and the mean of 3 separate images quantified for each membrane.

**Gene Transfer and Vein Graft Procedure**

All animal procedures were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Vein grafts were constructed in 16- to 20-week-old C57BL/6J ApoE−/− mice (Jackson Laboratories). Mice received water and standard chow diet ad libitum. A total of 1×10^11 viral particles of Ad35K or AdGFP in 300 µL was injected into the vein graft recipient via the tail vein 2 days before surgery in accordance with the findings of dose response studies done previously. Vein graft surgery was performed as described previously. Briefly, mice were anesthetized using a combination of Hypnorm (25 mg/kg; Bayer) and Hypnovel (25 mg/kg; Roche) administered subcutaneously. The right common carotid artery was isolated and mobilized from the thoracic inlet to the bifurcation, divided at its midpoint, and cuffs placed over the ends. The artery was inverted over the cuffs and ligated with 8-0 silk sutures. The supradiaphragmatic vena cava from an isogenic donor mouse was harvested and grafted as an interposition graft by sleeving the vein over the 2 ends of the carotid artery and ligating them with 8-0 silk sutures. Vigorous pulsation in the conduit vessel confirmed successful engraftment.

**Tissue Preparation, Histology, and Lesion Quantification**

Grafts were harvested at 14 (n=6 per treatment group) or 28 days (n=8 per treatment group) after surgery and perfusion fixed in situ with 4% phosphate-buffered paraformaldehyde. Grafts were excised, fixed in paraformaldehyde overnight, dehydrated in graded ethanol, and paraffin embedded. Before embedding, each graft was divided at its midpoint to provide sections from the body of the graft, avoiding the cuff anastomoses. Grafts were sectioned for 150 µm from the midpoint, collecting 5-µm sections. Three representative sections, separated by 50 µm, were stained with Masson/Goldner stain for analysis.

**Immunohistochemical Staining**

Immunohistochemistry was performed as described previously. Briefly, paraffin-embedded vein graft sections were stained for SMC α-actin using a mouse monoclonal anti-α-actin antibody (Sigma). Cell proliferation was identified using Ki67 rat anti-mouse monoclonal antibody (DakoCytomation), and monocyte/macrophages were visualized using MAC-3 immunostaining (Pharmingen).

**Statistical Analysis**

Data are presented as mean±SEM. Data were compared using the Mann–Whitney U test for nonparametric data. A value of P<0.05 was considered statistically significant.

**Results**

**35K Is Expressed in ApoE−/− Mice After Adenoviral Gene Transfer**

Efficient gene transfer after tail vein injection of AdGFP was confirmed by widespread GFP fluorescence in hepatocytes 14 days after surgery. As expected for an E1-deleted adenoviral vector, transgene expression was no longer evident by 28 days (data not shown). However, immunoblotting of mouse plasma from animals receiving Ad35K revealed an HA-tagged 35KDal protein at 14 days and 28 days after surgery, suggesting some persistence of circulating 35K protein beyond the duration of transgene expression (Figure 1). No HA-tagged proteins could be detected in plasma from control mice administered AdGFP. There were no significant differences in plasma total or high-density lipoprotein cholesterol concentrations between the groups (data not shown).
Plasma and Inhibits SMC Migration

Weeks after surgery (Figure 3). However, Ad35K gene transfer of a soluble broad spectrum CC-CK inhibitor, 35K, in a mouse vein graft model of accelerated atherosclerosis. First, we observed that 35K gene transfer significantly

days and 54% (P=0.02) at 28 days (Figure 5). Immunohistochemistry for macrophage content revealed that vein grafts from Ad35K mice had a marked 87% (P=0.008) reduction in MAC-3 staining compared with AdGFP controls at 14 days (Figure 6). However, this difference was not attributable to macrophage staining in the neointima but rather to a striking reduction in macrophages in the remaining vessel wall. In contrast to the differences observed 14 days after surgery, there were no significant differences in macrophage content in either the neointima or the remaining vessel wall at 28 days. (Figure 6)

Discussion

In this study, we report the use of adenoviral-mediated gene transfer of a soluble broad spectrum CC-CK inhibitor, 35K,
reduced CCR5-mediated chemotactic activity and SMC migration after bypass surgery. Second, adenoviral gene transfer of 35K significantly reduced total vessel wall area and thickening 14 days after surgery, with sustained benefit 28 days after surgery. Finally, Ad35K administration dramatically reduced macrophage recruitment, cell proliferation, and neointimal SMC accumulation in vein grafts.

In previous work, we demonstrated that adenoviral delivery of the potent and broad spectrum CC-CK binding protein 35K can reduce native atherosclerosis in the aortic roots of ApoE−/− mice fed a high-fat diet. However, lesion formation in high-fat–fed ApoE−/− mice is driven by severe hypercholesterolemia and is characterized by lipid deposition and foam cell formation rather than by vascular injury, which is characterized by high SMC content and inflammatory cell infiltrate. In the present study, we demonstrate that broad-spectrum CC-CK blockade greatly reduces accelerated atherosclerosis in vein grafts.

In murine vein grafts, α-actin positive SMCs are predominant within lesions 2 weeks after surgery. Ad35K dramat-
ically reduced α-actin–positive cell content 14 days after surgery, with a sustained reduction 28 days after surgery. Until recently, it has been believed that SMCs in atherosclerosis are derived from the media of injured arteries in response to platelet-derived growth factor from platelet aggregates and damaged endothelium. This theory has been challenged by recent findings demonstrating other sources of SMCs, such as adventitial myofibroblasts, bone marrow–derived cells, circulating progenitor cells, and hematopoietic stem cells. Interestingly, CC-CKs are produced in abundance by bone marrow stromal cells in response to inflammatory stimuli and have been implicated in the proliferation and chemotaxis of hematopoietic progenitor cells. It is possible that Ad35K reduced α-actin–positive SMCs by its effects on bone marrow and hematopoietic progenitor cells during the initial response to vascular injury. Alternatively, the beneficial effect could be attributed to a direct antiproliferative effect within the vessel wall. We found a marked 95% reduction in cell proliferation within the α-actin–positive area at 14 days after surgery and a 54% reduction at 28 days (Figure 5). Double-labeling studies in ApoE−/− murine vein grafts have shown that 85% of proliferating cells are α-actin positive and <5% are monocyte/macrophages. Reports also suggest that vascular SMCs respond directly to CKs. For example, human vascular SMCs possess functional CCR5 and MCP-1 receptors. CK receptor-8 has also been shown to mediate vascular SMC chemotaxis and proliferation. In our study, migration of ApoE−/− primary SMCs to plasma was significantly reduced in the presence of 35K (Figure 2B). Thus, broad-spectrum CC-CK blockade by Ad35K may also reduce α-actin–positive area by directly inhibiting SMC function.

It is also likely that the effects of 35K on vein graft atherosclerosis are mediated through direct inhibition of inflammatory cell recruitment. In intercellular adhesion molecule-1–deficient mice, leukocyte recruitment and neointima formation in vein grafts is significantly reduced. Biomechanical injury in vein grafts leads to cell death, expression of adhesion molecules, and release of CKs, all of which promote mononuclear cell recruitment. We observed a striking reduction in monocyte/macrophage infiltration in 35K-treated vein grafts 14 days after surgery but no differences after 28 days, suggesting that CC-CK–dependent inflammatory cell recruitment in the immediate response to injury is critical in modulating neointima formation in vein grafts. Previous studies have shown adherence of inflammatory cells and upregulation of CK receptors within hours after surgery. To ensure sufficient 35K protein was present at the time of surgery, we injected mice 2 days before operation based on studies from our own and other groups showing that E1-deleted adenoviruses under the control of the cytomegalovirus promoter express detectable levels of protein within 8 hours and near-maximal protein production by 24 to 48 hours. Interestingly, we observed marked macrophage infiltration within the adventitia of vein grafts. Indeed, we found no effect of Ad35K on macrophage infiltration into the neointima (defined by α-actin–positive cells). Rather, the marked reduction in macrophage content in Ad35K-treated animals was found within the remaining vessel wall. This finding is in keeping with recent reports suggesting that the vascular adventitia appreciably contributes to atherosclerosis. In accordance with our observations, it is likely that mononuclear cells are infiltrating the vessel wall from the surrounding adventitia, and that 35K can inhibit adventitial and luminal mononuclear cell recruitment. Strategies aimed at
reducing the adventitial response to injury in vein grafts may be clinically relevant, with the opportunity of local delivery at the time of surgery.

Despite the presence of detectable 35K protein in plasma at 28 days (Figure 1), there appeared to be a degree of “catch up” in wall thickness in the Ad35K-treated animals when compared with 14 days. However, when we assessed CCR5-mediated chemotaxis in plasma from Ad35K-treated mice, we observed a significant reduction in cell migration at 14 days but no difference at 28 days. It is possible that a proportion of 35K protein persists in the circulation but is bound to CKs, rendering it biologically inactive. This hypothesis is supported by the absence of green fluorescent cells in the liver 1 month after AdGFP treatment. The host immune response to adenoviral antigens leads to a reduction in transgene expression from 2 weeks to negligible levels at 4 weeks. Future work using viral vectors that provide more prolonged gene expression will allow investigation of the longer-term effects of CC-CK inhibition on vein graft atherosclerosis.

In conclusion, we demonstrate that adenoviral systemic gene transfer via a single intravenous injection of the CC-CK inhibitor 35K inhibited CCR5-mediated chemotaxis and SMC migration in mouse plasma and dramatically reduced vessel wall thickening, cell proliferation, SMC content, and early macrophage recruitment in vein graft accelerated atherosclerosis. These findings highlight the importance of the CC-CKs in accelerated atherosclerosis and raise the prospect of broad spectrum CC-CK blockade as a therapy to improve vein graft patency.

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References


Figure 5. In vivo gene transfer of 35K reduces proliferating cell numbers in the vessel wall of ApoE−/− vein grafts at 14 and 28 days after surgery. Sixteen- to 20-week-old ApoE−/− mice were injected via the tail vein with AdGFP or Ad35K 2 days before carotid–caval vein bypass graft surgery. Ki67 antibody was used to detect proliferating cell content (mm²) in lesions of paraffin-embedded vein graft sections from the midpoint of the graft. The top panels are representative images of Ki67 staining 14 days after surgery and the bottom panel 28 days after surgery for the different treatment groups. *Significant difference between Ad35K and AdGFP control total vessel wall; **significant difference between Ad35K and AdGFP control neointima; P<0.01; n=6 to 8 mice per group. Magnification ×200.

Figure 6. In vivo gene transfer of 35K reduces macrophage numbers in the vessel wall of ApoE−/− vein grafts at 14 but not 28 days after surgery. Sixteen- to 20-week-old ApoE−/− mice were injected via the tail vein with AdGFP or Ad35K 2 days before carotid–caval vein bypass graft surgery. MAC-3 antibody was used to detect total macrophage content (mm²) in lesions of paraffin-embedded vein graft sections from the midpoint of the graft. The top panels are representative images of MAC-3 staining 14 days after surgery and the bottom panel 28 days after surgery for the different treatment groups. *Significant difference between Ad35K and AdGFP control groups; P<0.01; n=6 to 8 mice per group. Magnification ×200.


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