Adenovirus-Mediated Extracellular Superoxide Dismutase Gene Therapy Reduces Neointima Formation in Balloon-Denuded Rabbit Aorta

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Background—Restenosis is a frequent problem after invasive treatment of atherosclerotic vessels and is associated with intimal hyperplasia, which is primarily a result of proliferation and migration of smooth muscle cells, leading to the formation of neointima. Because there is no effective conventional medication for restenosis, gene therapy is a potential new treatment to prevent neointima formation.

Methods and Results—In the present study, we analyzed the effects of adenovirus-mediated extracellular superoxide dismutase (EC-SOD) gene transfer (3 × 10^9 pfu/kg AdEC-SOD versus AdLacZ control virus) on neointima formation in balloon-denuded rabbit aortas. Local catheter-mediated gene transfer to the arterial wall reduced restenosis (P < 0.001) and decreased the number of macrophages in the transduced segment (P < 0.001) 2 weeks and 4 weeks after the gene transfer compared with AdLacZ controls. Transgene expression was detected in the arterial wall by RT-PCR 2 weeks after the procedure, and the production of superoxide anion was reduced after the gene transfer. Recovery of the endothelial layer was enhanced in EC-SOD–transduced rabbits compared with LacZ controls (P < 0.001) 2 weeks after the gene transfer. The therapeutic effect was found to be extended, affecting the gene transfer site and flanking aortic segments from the renal arteries to the bifurcation. However, systemic AdEC-SOD gene transfer to liver did not have any effects on restenosis.

Conclusions—The results suggest that EC-SOD gene transfer reduces restenosis and may be useful for the prevention of intimal hyperplasia after vascular manipulations. (Circulation. 2002;106:1999-2003.)

Key Words: angioplasty ■ restenosis ■ oxygen stress ■ antioxidants ■ endothelium

Restenosis is a vascular response to percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG), and coronary stenting, causing intimal hyperplasia in 20% to 30% of patients.1,2 Balloon angioplasty frequently causes intimal tearing, endothelial cell damage, and exposure of subendothelial tissue to blood components, leading to increased oxidative stress, superoxide anion (O_2^-) production, and decreased vascular superoxide dismutase (SOD) concentration.3,4

Extracellular (EC)-SOD as an antioxidative enzyme may offer a potential tool for vascular gene therapy.5 Approximately 50% of the total SOD activity in human aorta is EC-SOD–derived.5,6 In most of the other tissues, EC-SOD represents only a minor part of the total SOD activity, which suggests that EC-SOD has a significant physiological role in the redox balance of vascular wall.6 EC-SOD has been shown to reduce O_2^-–mediated macromolecular and cellular damage, suggesting that EC-SOD gene transfer may be used to attenuate tissue damage caused by oxygen-derived free radicals.5,9–11 Only a few cardiovascular gene therapy studies with EC-SOD have been published.10–12 However, EC-SOD is potentially more suitable for vascular gene transfer than other SODs, because it is a secreted enzyme13 capable of binding reversibly to heparan sulfate proteoglycans on cell membranes14 and has a remarkably long half-life (20 hours) in the circulation.15

To study the effects of EC-SOD on intimal hyperplasia, adenoviral gene transfer was performed in rabbit aorta after endothelial denudation. EC-SOD significantly reduced neointima formation and macrophage accumulation in arterial wall in rabbits after balloon denudation. According to the present work, EC-SOD may be an efficient therapeutic molecule to prevent intimal hyperplasia after vascular manipulations.
Methods

Animal Experiments
Thirty-six New Zealand White rabbits (Finnish National Experimental Animal Center, Kuopio, Finland) were kept on a 0.25% cholesterol diet for 2 weeks before balloon denudation. Animals were anesthetized by 0.5 mL Hypnorm (Janssen) s.c. and 0.8 mL Dormicum (Roche) i.m. Two-week (EC-SOD, n=10, and LacZ, n=10) and 4-week (EC-SOD, n=4, and LacZ, n=4) time points were studied for local gene transfer and the 2-week time point also for systemic gene transfer (EC-SOD, n=4, and LacZ, n=4). Three days after denudation, local adenosine-mediated gene transfer (3x10^9 pfu/kg) was performed in a 2-cm segment in the middle part of the abdominal aorta with a Dispatch catheter (Boston Scientific), which allows continuous blood flow during transduction. Systemic gene transfer was performed by a similar method without the Dispatch catheter. Serum samples were collected before gene transfer, 3 and 7 days after gene transfer, and at the end of the experiment. Tissue samples were collected to determine the biodistribution of adenosine. Aortic sections were collected at the gene transfer site, 2 cm proximal and 2 cm distal from the gene transfer site. The Kuopio University Experimental Animal Committee approved all animal experiments.

Adenovirus Production
The EC-SOD expression cassette containing elongation factor 1α promoter was cloned into an adenosine vector (AdBglIII). LacZ adenosine was used as a control. Clinical-grade adenoviruses were produced in 293 cells and analyzed to be free of microbiological contaminants, mycoplasma, endotoxin, and replication-competent viruses. The expression of transduced \( ec-sod \) mRNA in various tissues was determined by reverse transcription–polymerase chain reaction (RT-PCR) analysis using an avian enhanced RT-PCR kit (Sigma). The method was specific for the transgene and did not amplify endogenous rabbit EC-SOD mRNA. The reaction conditions for reverse transcription were as follows: denaturation at 80°C for 10 minutes, random primer annealing at 25°C for 5 minutes, and first-strand synthesis at 60°C for 50 minutes. Of the first-strand reaction product, 15 μL was used for PCR, and 2 μL of the first PCR product was used for the second PCR reaction. The primers 5′-TGATGTGGGGCGACCG-3′ and 5′-GGATGTGTGCAAAGTG ACCAGGC-3′ were used for the first PCR and the inner 3′-end primer 5′-GTGAGCGCCTGCCAGATCTC-3′ for the nested PCR. The reaction conditions for both PCRs were as follows: denaturation at 96°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 30 cycles. The initial denaturation was 5 minutes at 96°C, and the final extension was 10 minutes at 72°C.

Histological Analysis
A transduced segment of the aorta was removed, flushed gently with saline, and divided into 3 equal parts to be used for histological paraffin sections, cryo sections, and a snap-frozen sample stored at −70°C. Paraffin sections were immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 hours, rinsed overnight in 15% sucrose (pH 7.4), and embedded in paraffin. Cryo sections were fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 10 minutes, rinsed in PBS, embedded in OCT compound (Miles Scientific, Elkhart), and stored at −70°C. Gene transfer efficiency was determined by X-gal staining for 6 hours at +37°C. Neointima formation was measured after hematoxylin-eosin (HE) staining using Image-pro Plus software with an Olympus AX70 microscope (Olympus Optical). In situ superoxide anion production was determined by the dehydrodriildium (DHE) labeling method. Briefly, fresh-frozen sections (15 μm) of aortas were incubated with DHE (Molecular Probes) for 5 minutes, rinsed, mounted, and analyzed with a fluorescent microscope.

The following antibodies were used to study the effect of gene transfer: CD31 (endothelium, dilution 1:50, DAKO), RAM 11 (macrophages, dilution 1:200, DAKO), HRP35 (smooth muscle cells [SMCs], dilution 1:50, DAKO), p67phox (NADPH oxidase, dilution 1:100, Transduction Laboratories), inducible nitric oxide synthase (dilution 1:25, Transduction Laboratories), vascular endothelial growth factors (VEGF)-A (dilution 1:100, Santa Cruz), VEGF-C (dilution 1:100, Santa Cruz), and nuclear factor-κB (dilution 1:50, Transduction Laboratories). An avidin-biotin–horseradish peroxidase system was used for signal detection (Vector Elite Kit). Apoptosis was detected with an ApopTag kit (Intergen) according to the manufacturer's instructions.

Autopsy Analysis and Clinical Chemistry
Autopsy analysis was done at the National Veterinary and Food Research Institute, Kuopio Department, Finland, by Dr Vet. P. Syrjala. Basic clinical chemistry analyses (CRP, ASAT, ALAT, APOS, creatinine, cholesterol, triglycerides) were analyzed at Kuopio University Hospital Central Laboratory with an autoanalyzer (Kone Instruments).

Statistical Analysis
Statistical analysis was done by ANOVA and modified t test.

Results
The effect of adenosine-mediated gene transfer of EC-SOD on intimal thickening was evaluated in a rabbit restenosis model after both local and systemic adenosine-mediated gene transfer. New Zealand White rabbits were kept on a 0.25% cholesterol diet for 2 weeks before balloon catheter injury of aortic endothelium. Cholesterol feeding increased plasma total cholesterol levels from 2 to 2.5 mmol/L to 7 to 10 mmol/L (ie, closer to human values) but did not yet induce any foam cells or fatty streak formation.

Expression of LacZ and EC-SOD
Biodistribution of adenoviruses was determined by X-gal staining from the LacZ group and by RT-PCR from the EC-SOD group. In both the local and systemic gene transfer groups, X-gal staining was frequently seen in spleen, lung, liver, and vessel wall (data not shown). RT-PCR analysis for \( ec-sod \) expression showed a pattern of tissue distribution similar to that of X-gal staining for LacZ expression (Figure 1a). Total plasma SOD activity, which was measured before the gene transfer and 3, 7, and 14 days after the gene transfer (Figure 1b), was shown to be significantly (P<0.01) lower 3 days after the gene transfer in the control rabbits than in the EC-SOD group, indicating that adenosine-mediated EC-SOD gene transfer attenuated the reduction in the total plasma SOD activity. In subsequent experiments, we determined that a similar reduction in total plasma SOD activity also occurred after balloon denudation without any adenovi-
rus gene transfer (data not shown). Thus, the reduction in SOD activity is related to the balloon-denudation procedure itself and not to the adenoviral gene transfer.

**Histological Analysis**

The gene transfer site and adjacent segments of abdominal aorta from the renal arteries to the bifurcation point were analyzed histologically to determine the effect of local adenoviral EC-SOD gene transfer on restenosis at the 2- or 4-week time points. At 2 weeks, the intima-media ratio in EC-SOD animals (0.09 ± 0.05) was significantly (P < 0.001) reduced compared with LacZ controls (0.32 ± 0.14; Figure 2, a and b and right column). Interestingly, inhibition of the neointima formation was observed not only in the gene transfer site but also in the flanking segments of the abdominal aorta, suggesting a more widespread effect on the prevention of restenosis. The 4-week time point showed an 0.13 ± 0.02 intima-media ratio for the EC-SOD group and 0.54 ± 0.20 for the LacZ control group (P < 0.001) (Figure 3, a and b and right column). Inhibition of the neointima formation was extended outside the gene transfer site as at the 2 week-time point. In additional experiments, we studied whether systemic AdEC-SOD gene transfer given directly into the aortic blood stream reduced restenosis compared with AdLacZ. However, this was not the case, because intima-media ratios at the early time point after systemic AdEC-SOD

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**Figure 1.** a, RT-PCR analysis for ec-sod mRNA 2 weeks after local gene transfer. Lanes 1 to 7, EC-SOD group; lanes 9 to 15, LacZ group. Lanes 1 and 9, gene transfer site; lanes 2 and 10, proximal control of aorta; lanes 3 and 11, heart; lanes 4 and 12, lung; lanes 5 and 13, spleen; lanes 6 and 14, liver; lanes 7 and 15, kidney; lane 8, negative control; lane 16, positive EC-SOD control. In RT-PCR, a strong signal was detected from spleen, liver, and kidney and only a weak signal from aorta and heart. b, Total SOD activity measured from plasma at time points 0 (pre), 3, 7, and 14 days after gene transfer. Top curve, total plasma SOD activity in EC-SOD group; bottom curve, total plasma SOD activity in LacZ group. Plasma total SOD activity decreased 3 days after gene transfer, but the decrease was significantly (P < 0.01) attenuated in EC-SOD group.

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**Figure 2.** Histological analysis of serial sections from abdominal aorta 2 weeks after local gene transfer. a, c, and e, EC-SOD group; and b, d, and f, LacZ group. Arrows indicate location of IEL. In columns, *** indicates significant (P < 0.001) difference. a and b, HE staining; column, neointima formation between EC-SOD and LacZ groups; c and d, endothelium staining (CD-31); column, endothelial recovery percents; e and f, macrophage staining (RAM-11); column, number of macrophages accumulated in vessel wall. Magnification ×200. Bars = 100 μm.

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**Figure 3.** Histological analysis of serial sections from abdominal aorta 4 weeks after local gene transfer. a, c, and e, EC-SOD group; b, d, and f, LacZ group. Arrows indicate location of IEL. In columns, *** indicates significant (P < 0.001) difference. a and b, HE staining; column, neointima formation between EC-SOD and LacZ groups; c and d, endothelium staining (CD-31); column, endothelial recovery percents; e and f, macrophage staining (RAM-11); column, number of macrophages accumulated in vessel wall. Magnification ×200. Bars = 100 μm.
and AdLacZ gene transfers (3×10⁹ pfu/kg) were 0.25±0.13 and 0.25±0.12, respectively.

Because the neointima formation is dependent on the degree of endothelial balloon damage, we determined the integrity of the internal elastic lamina (IEL) from each aortic sample. The measurement showed 4±6% damage to the IEL in both the EC-SOD and LacZ groups at 2 weeks and 5±6% and 2±2% damage at 4 weeks, respectively, indicating a similar degree of damage to the endothelium after the denudation. Aortic sections immunostained with CD31 for endothelial cells showed 86±13% recovery of the endothelium after denudation in the EC-SOD group at 2 weeks, whereas in the LacZ control group, the endothelial recovery was only 21±13% (Figure 2, c and d), showing a significant difference (P<0.001). Immunohistological analyses of factors that could be involved in this effect (endothelial nitric oxide synthase, inducible nitric oxide synthase, VEGF-A, VEGF-C, and nuclear factor-kB) showed no marked differences between the EC-SOD group and the LacZ control group (data not shown). Endothelial recovery of the control samples approached that of the EC-SOD group at 4 weeks, being 88±13% for the EC-SOD group and 81±19% for the LacZ control group (Figure 3, c and d). RAM-11 immunostaining showed a significantly (P<0.001) decreased infiltration of macrophages in neointima in EC-SOD group at both time points (Figures 2, e and f, and 3, e and f). The EC-SOD group showed 10-fold fewer macrophages than LacZ controls 2 weeks after the gene transfer and 20-fold less accumulation 4 weeks after the gene transfer. NADPH oxidase, which is reported to be upregulated in balloon-injured rabbit aortas,4 was detected in the same areas as macrophages (data not shown). SMC α-actin immunostaining indicated that at 2 weeks, intimal thickening was caused primarily by migration and proliferation of SMCs. Apoptosis was remarkably higher in LacZ control vessels than in the EC-SOD group at 2 weeks, but the difference was not present at 4 weeks (data not shown). Superoxide anion production in aorta was determined by DHE assay after the gene transfer. Analysis revealed only scattered fluorescence-positive nuclei in the EC-SOD–treated rabbits (Figure 4a), whereas LacZ-treated control animals showed widespread positive nuclei in the luminal part of the neointima and within the media (Figure 4b). Autopsy and clinical chemistry analysis of the basic laboratory parameters showed no significant differences between the study groups (data not shown).

**Discussion**

SMC proliferation is a common consequence after balloon angioplasty, causing intimal hyperplasia within 6 months after operation.1 In the present study, we have shown that local adenovirus-mediated EC-SOD gene transfer attenuated balloon denudation–related loss of total plasma SOD activity and resulted in a significant inhibition of neointima formation in balloonotted rabbit aortas. Macrophage accumulation in the denuded aorta was also markedly reduced. These findings indicate that oxidative stress may play an important role in the pathogenesis of restenosis22 and are in line with recent clinical results in which both probucol23 and a probucol analogue24 have been reported to reduce restenosis in humans. Restenosis can be effectively inhibited in experimental animals by gene transfer of several different genes, such as VEGFs and nitric oxide synthases.25 However, several of the tested genes do not reduce the infiltration of macrophages. Macrophages are known to secrete many cytokines and growth factors that induce SMC proliferation and participate in neointima formation.26 The reduced infiltration of macrophages suggests an anti-inflammatory role for EC-SOD in addition to its antioxidative and antiapoptotic roles, which we have shown in previous studies.5,9 EC-SOD gene transfer reduced apoptosis at 2 weeks. The exact role for apoptosis in intimal hyperplasia is unknown, but at early stages after balloon denudation, apoptosis has been suggested to stimulate restenosis by provoking the wound-healing process, whereas at later stages, apoptosis may inhibit neointima formation by balancing the number of proliferating cells with the rate of neointimal SMC death.27 The importance of oxidative stress in the pathogenesis of restenosis was recently suggested by results showing that lucigenin reductase activity, which reflects the amount of O₂⁻, is increased shortly after injury to rabbit arterial rings.22 In the present study, our results suggested a reduced production of O₂⁻ in AdEC-SOD–transfected aortas compared with AdLacZ controls, which indicates that at least part of the beneficial effects may be a result of the antioxidative nature of EC-SOD in addition to the effects on macrophage recruitment and endothelial recovery. The therapeutic effect was also extended from the gene transfer site to adjacent abdominal aortic segments. This may be because of the ability of plasma EC-SOD to bind to heparan sulfate proteoglycans on the cell membranes at and near the site of gene transfer, creating a more favorable environment for endothelial cells to grow and spread out, causing the extended effect. In this respect, the remarkably long half-life (20 hours) of the secreted EC-SOD in vivo may be an advantage. To exclude possible systemic effects of AdEC-SOD gene transfer, intraaortic gene transfer was also performed. Even though the biodistribution of the adenoviruses was relatively similar after both local and systemic gene transfers, the antirestenotic effect was not seen after the systemic gene transfer. The results are in line with recent findings that neither a systemic increase in EC-SOD activity in AdEC-SOD–transduced LDL

![Figure 4](image-url) Production of superoxide anion in abdominal aorta as analyzed by DHE method. a, AdEC-SOD–treated rabbits show only scattered fluorescence-positive nuclei; b, AdLacZ control rabbits reveal much stronger fluorescence, predominantly within luminal part of vessel, suggesting presence of higher oxidative stress in AdLacZ control animals. Magnification ×200. Bars=100 μm.
receptor–deficient mice nor the lack of EC-SOD activity in EC-SOD knockout mice affects atherogenesis.

In conclusion, our results suggest that local catheter-mediated delivery of EC-SOD adenoviruses can reduce restenosis in rabbits and may provide a useful tool for the prevention of restenosis after vascular manipulations in humans.

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