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*Circulation* 2000;102;2262-2268
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

# Intravascular Adenovirus-Mediated VEGF-C Gene Transfer Reduces Neointima Formation in Balloon-Denuded Rabbit Aorta

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***Background***—Gene transfer to the vessel wall may provide new possibilities for the treatment of vascular disorders, such as postangioplasty restenosis. In this study, we analyzed the effects of adenovirus-mediated vascular endothelial growth factor (VEGF)-C gene transfer on neointima formation after endothelial denudation in rabbits. For comparison, a second group was treated with VEGF-A adenovirus and a third group with *lacZ* adenovirus. Clinical-grade adenoviruses were used for the study.

***Methods and Results***—Aortas of cholesterol-fed New Zealand White rabbits were balloon-denuded, and gene transfer was performed 3 days later. Animals were euthanized 2 and 4 weeks after the gene transfer, and intima/media ratio (I/M), histology, and cell proliferation were analyzed. Two weeks after the gene transfer, I/M in the *lacZ*-transfected control group was 0.57±0.04. VEGF-C gene transfer reduced I/M to 0.38±0.02 ($P<0.05$ versus *lacZ* group). I/M in VEGF-A–treated animals was 0.49±0.17 ($P$=NS). The tendency that both VEGF groups had smaller I/M persisted at the 4-week time point, when the *lacZ* group had an I/M of 0.73±0.16, the VEGF-C group 0.44±0.14, and the VEGF-A group 0.63±0.21 ($P$=NS). Expression of VEGF receptors 1, 2, and 3 was detected in the vessel wall by immunocytochemistry and in situ hybridization. As an additional control, the effect of adenovirus on cell proliferation was analyzed by performing gene transfer to intact aorta without endothelial denudation. No differences were seen in smooth muscle cell proliferation or I/M between *lacZ* adenovirus and 0.9% saline–treated animals.

***Conclusions***—Adenovirus-mediated VEGF-C gene transfer may be useful for the treatment of postangioplasty restenosis and vessel wall thickening after vascular manipulations. **(*Circulation*. 2000;102:2262-2268.)**

**Key Words:** viruses ■ genes ■ restenosis ■ growth substances

Local gene transfer to vascular wall offers a promising alternative for the treatment of restenosis after PTCA and coronary stenting. Restenosis is a frequent complication after PTCA, leading to the obstruction of dilated arteries in 20% to 30% of patients within 6 months after the procedure.[1] One of the key elements in the pathogenesis of restenosis is damage to the endothelium. Dysfunctional or absent endothelium also predisposes arteries to various other pathological conditions, such as thrombosis and spasm. Therefore, strategies to protect endothelium or enhance endothelial regrowth have received increased attention. Vascular gene transfer could be used as a treatment to improve endothelial dysfunction in vivo.[2]

Vascular endothelial growth factors (VEGF) are a family of angiogenic growth factors that stimulate endothelial cell proliferation, increase endothelial permeability, and act as endothelial "survival factors" in retinal vessels.[3–5] In addition to direct angiogenic effects, some VEGFs also induce nitric oxide and prostacyclin release from vascular endothelium.[6,7] Five VEGF-A isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) are generated by alternative splicing from a single VEGF gene[3,8] and are distinguished by their heparan sulfate–binding properties. Most of these splice variants bind to 2 tyrosine kinase receptors, VEGFR-1 and VEGFR-2, which are expressed almost exclusively on the endothelial cells.[3,4] VEGF-C is another member of the VEGF family and is proteolytically processed to the active form.[9,10] VEGF-C binds to VEGFR-2 and VEGFR-3 and has been shown to stimulate both angiogenesis and formation of lymphatic vessels.[9,10] Other members of the VEGF family have also been characterized: VEGF-B[11] binds to VEGFR-1,

Received April 13, 2000; revision received June 1, 2000; accepted June 8, 2000.

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and VEGF-D[12] binds to VEGFR-2 and VEGFR-3. VEGFR-3 differs from other VEGF receptors by its characteristic expression and effects in lymphatic vessels.[10,13] The newest member of the growing VEGF family is the virus-encoded VEGF-E, which has functional characteristics similar to those of VEGF-A but binds only to VEGFR-2.[14,15]

A single dose of recombinant VEGF-A protein in the bloodstream or locally has the capacity to accelerate reendothelialization in balloon-injured rat carotid arteries.[16] Recombinant VEGF-C has also been shown to induce angiogenesis in vivo.[17] Injection of VEGF-A plasmid in ischemic rabbit hindlimbs and adventitial surface of rabbit carotid arteries has been shown to improve the status of the treated vessels.[6,18] Beneficial effects of VEGF-A gene transfer in human peripheral arteries and ischemic myocardium have also been reported.[19–21] Even though intravascular gene transfer efficiency in human atherosclerotic arteries is low,[22] secreted products, such as VEGF, can be used for therapeutic gene transfer trials using novel infusion-perfusion catheters, designed primarily for intravascular drug infusions.[23]

In this study, we analyzed the effects of adenovirus-mediated VEGF-C and VEGF-A gene transfers on neointima formation in rabbits. Because both VEGFs share one receptor (VEGFR-2) but differ with respect to the other receptor, it has remained unclear whether VEGF-C and VEGF-A might have overlapping but distinct effects in the vessel wall. It was found that VEGF-C gene transfer reduced intimal thickening in balloon-denuded rabbit aorta. VEGF-C may be useful for the treatment of restenosis after vascular manipulations.

## Methods

### Adenovirus Constructs

Adenovirus containing the complete human prepro–VEGF-C open-reading frame[9] was constructed as follows: cytomegalovirus (CMV) promoter was excised from the pcDNA3.1 vector (Invitrogen), and a full-length human VEGF-C cDNA containing the 1997-bp sequence was excised from the previously constructed VEGF-C pREP7 expression vector.[9] A human growth hormone polyadenylation signal was excised from an $\alpha$-MHC gene promoter construct (a gift from Dr Jeffrey Robbins). The CMV promoter, VEGF-C cDNA, and the polyadenylation signal fragments were ligated into a pCRII vector (Invitrogen). The transcriptional unit was cloned into pAdenogal vector.[22] This construct was then used to generate recombinant adenovirus. VEGF-A (murine $VEGF_{164}$)[24] and nucleus-targeted *lacZ* adenoviruses were constructed in a way similar to that previously described.[22] Replication-deficient E1-E3–deleted clinical GMP–grade adenoviruses were produced in 293T cells.[22,25] Adenoviruses were analyzed to be free from helper viruses, lipopolysaccharide, and bacteriological contaminants.[22,25]

### VEGF-C and VEGF-A Secretion by Transfected Cells

Secretion of VEGF-C and VEGF-A was tested in rabbit aortic smooth muscle cells (RAASMCs).[26] *LacZ* transfection was used as a control. Cells were incubated for 30 minutes in serum-free medium containing recombinant VEGF-C, VEGF-A, or *lacZ* adenovirus at a multiplicity of infection of 1000. Conditioned medium was analyzed by Western blotting with the following monoclonal antibodies: VEGF-A, clone sc-7269 corresponding to amino acids 1 to 140; and VEGF-C, clone sc-1881 raised against a peptide at the carboxy-terminus of the VEGF-C precursor (Santa Cruz Biotechnology).

### Endothelial Cell Tube Formation Assay

The ability of conditioned medium from SMCs transfected with adenoviruses coding for VEGF-A, VEGF-C, and lacZ to induce endothelial cell (EA hy926) tube formation was analyzed in Matrigel.[27] Tube formation was measured by counting the number of connected cells in 10 randomly selected fields per well and dividing that number by the total number of cells in the current field.

### Animal Experiments

Sixty New Zealand White rabbits were divided into 2 major groups, the first having a 0.25% cholesterol diet for 2 weeks and balloon denudation before gene transfer and the second having only the gene transfer. Gene transfer was performed in the first group of rabbits 3 days after the denudation, and the animals were euthanized 2 or 4 weeks after the gene transfer. The number of rabbits in each study group (*lacZ*, VEGF-C, and VEGF-A) at both time points was 6. The whole aorta was denuded twice with a 4.0F arterial embolectomy catheter (Sorin Biomedical). Three days later, the gene transfer was performed with a 3.0F channeled-balloon local drug delivery catheter (Boston Scientific). Under fluoroscopic control, the catheter was positioned caudal to the left renal artery in a segment free of side branches. A virus titer of $1.15\times10^{10}$ pfu was used in the final volume of 2 mL in 0.9% saline, and the gene transfer was performed at 6 atm pressure for 10 minutes (0.2 mL/min). In the second study group, the rabbits had only the gene transfer without a cholesterol diet or balloon denudation, and they were euthanized 2 or 4 weeks after the gene transfer. There were 3 rabbits in each study group (0.9% saline, *lacZ*, VEGF-C, and VEGF-A). All studies were approved by the Experimental Animal Committee of the University of Kuopio.

### Histology

Three hours before death, animals were injected with 50 mg IV of bromodeoxyuridine (BrdU) dissolved in 40% ethanol. After death, the transfected segment was removed, flushed gently with saline, and divided into 5 equal parts. The proximal part was snap-frozen in liquid nitrogen and stored at −70°C. The next part was immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 hours, rinsed in 15% sucrose (pH 7.4) overnight, and embedded in paraffin. The medial part was fixed in 4% paraformaldehyde/PBS (pH 7.4) for 10 minutes, rinsed in PBS, embedded in OCT compound (Miles), and stored at −70°C. The fourth part was fixed in 70% ethanol overnight and embedded in paraffin. The distal part was stained for $\beta$-galactosidase activity in X-Gal staining solution at +37°C for 16 hours, followed by fixation similar to that for the second part. Paraffin sections were used for detection of SMCs (HHF35, DAKO, dilution 1:50), macrophages (RAM-11, DAKO, 1:50), endothelium (CD31, DAKO, 1:50), T cells (MCA 805, DAKO, 1:100), and VEGF receptors (VEGFR-1 clone sc-316, 1:50; VEGFR-2 clone sc-6251, 1:500; and VEGFR-3 clone sc-637, 1:300; Santa Cruz Biotechnology). Controls for immunostainings included incubations with class- and species-matched immunoglobulins and incubations in which primary antibodies were omitted. Evaluation of the gene transfer efficiency was done with X-Gal staining of OCT-embedded tissue sections.[6] Detection of BrdU-positive cells was done according to the manufacturer's instructions. Morphometry was done with Image-Pro Plus software with an Olympus AX70 microscope (Olympus Optical). Measurements were done from randomly selected multiple sections independently by 2 observers (M.O.H., M.L.) without knowledge of the origin of the sections. Means of the 2 measurements are reported.

### Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from transfected aortic segments with Trizol reagent (Gibco-BRL), and 2 $\mu$g of RNA was used for cDNA synthesis. Primers were designed to distinguish between endogenous and transduced genes by selection of the 5′ primers from the CMV promoter and the 3′ primers from the coding regions. For *lacZ*, amplification primers were 5′-TTGGAGGCCTAGGCTTTTGC-3′

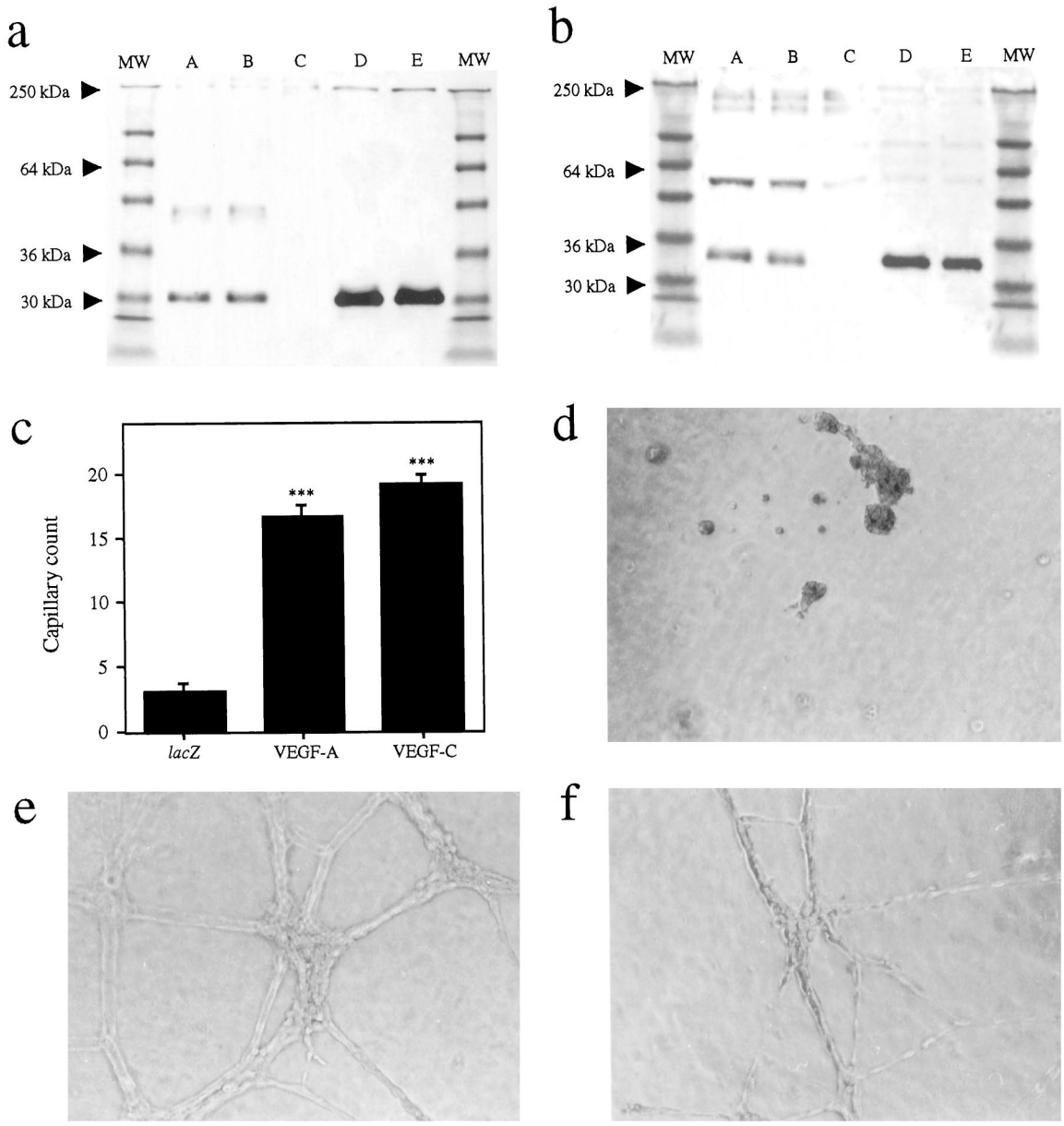


**Figure 1.** Expression of VEGF-C and VEGF-A proteins in vitro and tube formation assay. a, Western blot detection of VEGF-A protein from conditioned medium produced by RAASMCs transfected with recombinant VEGF-A adenovirus. From left: Molecular weight marker (MW), nonreduced VEGF-A proteins (A and B), medium from *lacZ*-transfected cells (C), reduced VEGF-A proteins (D and E), and MW. b, Western blot detection of VEGF-C protein from conditioned medium produced by RAASMCs transfected with recombinant VEGF-C adenovirus. From left: MW, nonreduced VEGF-C proteins (A and B), medium from *lacZ*-transfected cells (C), reduced VEGF-C proteins (D and E), and MW. c through f, Endothelial cell tube forming assay: c, capillary counts in endothelial cell cultures treated with medium from SMCs transfected with adenoviruses encoding for VEGF-A, VEGF-C, or *lacZ*. ***$P<0.001$. d, Endothelial cells treated with medium from *lacZ* adenovirus–transfected SMCs. e, Endothelial cells treated with medium from VEGF-A adenovirus–transfected SMCs. f, Endothelial cells treated with medium from VEGF-C adenovirus–transfected SMCs.

and 5′-ATACTGTCGTCGTCCCCTCA-3′. Five microliters of the first polymerase chain reaction (PCR) product was used for the second PCR with primers 5′-GGTAGAAGACCCCAAGGACTTT-3′ and 5′-CGCCATTCGCCATTCAG-3′. For VEGF-C amplification, primers were 5′-CTGCTTACTGGCTTATCG-3′ and 5′-CCTGTTCTCTGTTATGTTGC-3′. Five microliters of the first PCR product was used for the second PCR with primers 5′-TCTCCAAAAAGCTACACCG-3′ and 5′-CAAGTGCATGGTGGAAGG-3′. For VEGF-A amplification, primers were 5′-TCGATCCATGAACTTTCTGC-3′ and 5′-TTCGTTTAACTCAAGCTGCC-3′. Five microliters of the first PCR product was used for the second PCR with primers 5′-GACCCTGGCTTTACTGCTG-3′ and 5′-GGAACATTTACACGTCTGCG-3′.

## In Situ Hybridizations

The localization of VEGF receptors 1 to 3 mRNAs were determined by in situ hybridization using [$^{33}$P]UTP-labeled riboprobes as previously described.[28] For VEGFR-1 in situ hybridization, a probe covering nucleotides 1647 to 2251 (GenBank accession number AF063657) was selected; for VEGFR-2, a probe covering nucleotides 1756 to 2262 (GenBank accession number AF063658) was

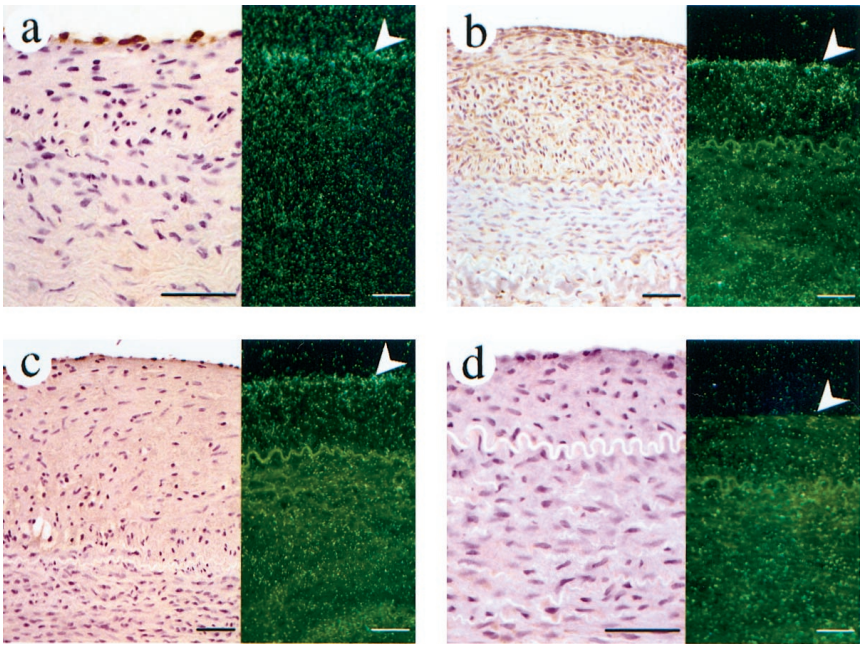


**Figure 2.** Expression of VEGF receptors in *LacZ* adenovirus–transfected rabbit aorta 2 weeks after balloon denudation. [$^{33}$P]UTP-labeled riboprobes were used for in situ hybridizations. a, VEGFR-1 immunostaining (clone sc-316; left) and in situ hybridization (right). b, VEGFR-2 immunostaining (clone sc-6251; left) and in situ hybridization (right). c, VEGFR-3 immunostaining (clone sc-637; left) and in situ hybridization (right). d, Nonimmune control (left) and in situ hybridization sense control (right) for VEGFR-3. Sense controls for VEGFR-1 and VEGFR-2 were also negative (data not shown). Bars=50 $\mu$m. Arrowheads point to endothelium in in situ hybridization figures.

used; and for VEGFR-3, a probe covering nucleotides 1 to 595[29] was selected. Corresponding sense probes were used as controls.

### Statistical Analyses

ANOVA followed by modified *t* test was used to evaluate statistical significances. A value of $P<0.05$ was considered statistically significant. Numerical values for each measurement are shown as mean±SEM.

## Results

### Functionality of VEGF-C and VEGF-A Adenoviruses

Secretion of recombinant VEGF-C and VEGF-A proteins from transfected vascular SMCs (RAASMCs) was tested in vitro. Proteins were detected from cell culture media by Western blotting (Figure 1A and 1B). VEGF-C was proteolytically processed to a correct size (≈34 kDa; Figure 1B). The size of VEGF-A (≈30 kDa; Figure 1B) was somewhat greater than that reported previously (≈23 kDa).[30] The difference may be due to differences in analytical conditions, because the structures of both VEGF vector constructs have been verified by sequencing (data not shown). Both VEGFs induced endothelial tube formation in matrigel in vitro compared with the *lacZ* control group. The number of connected cells in the *lacZ* group (n=10) was 3.1±0.9. VEGF-A (n=10) induced connection to the level of 16.6±3.6 ($P<0.001$) and VEGF-C (n=10) to the level of 19.1±3.6 ($P<0.001$) (Figure 1C through 1F).

### Expression of Transfected VEGFs and Their Receptors in the Aortic Wall

Transfection efficiency 2 weeks after the intravascular catheter–mediated gene transfer was 1.1±0.5% as analyzed by the X-Gal staining method for $\beta$-galactosidase activity. The $\beta$-galactosidase activity was also detected at the 4-week time point at the level of 0.3±0.1%. The mRNA expression of transfected genes was verified by reverse transcription–PCR (data not shown). VEGFR-1, VEGFR-2, and VEGFR-3 expression was analyzed by immunostainings and in situ hybridization. We found that the expression of all receptors was localized to endothelium. VEGFR-2 was also expressed in neointimal SMCs (Figure 2).

### Effects of VEGF-C and VEGF-A on Neointima Formation, Cell Proliferation, and Endothelial Regrowth

Balloon denudation of the rabbit aorta results in intimal thickening and SMC proliferation. The first study group was euthanized 2 weeks after the gene transfer. The *lacZ* control group had the highest intima/media ratio (I/M) (0.57±0.04), whereas the VEGF-C (0.38±0.02) and VEGF-A (0.49±0.17) groups showed decreased intimal thickening. The difference in I/M between *lacZ* and VEGF-C groups was significant ($P<0.05$) at the 2-week time point. At the 4-week time point, no significant differences were observed (Figure 3). Hematoxylin-eosin and immunostainings of the transfected arteries are shown in Figure 4: intimal thickening in all arteries was composed predominantly of SMCs. No signs of inflammation or foam cell accumulation were detected, as judged by macrophage and T-cell immunostainings (data not shown).

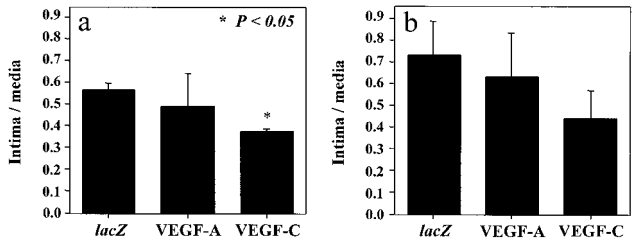


**Figure 3.** I/M in study groups after balloon denudation and gene transfer (mean±SEM). A, I/M 2 weeks after gene transfer. *$P<0.05$. B, I/M 4 weeks after gene transfer. n=6 in each study group.

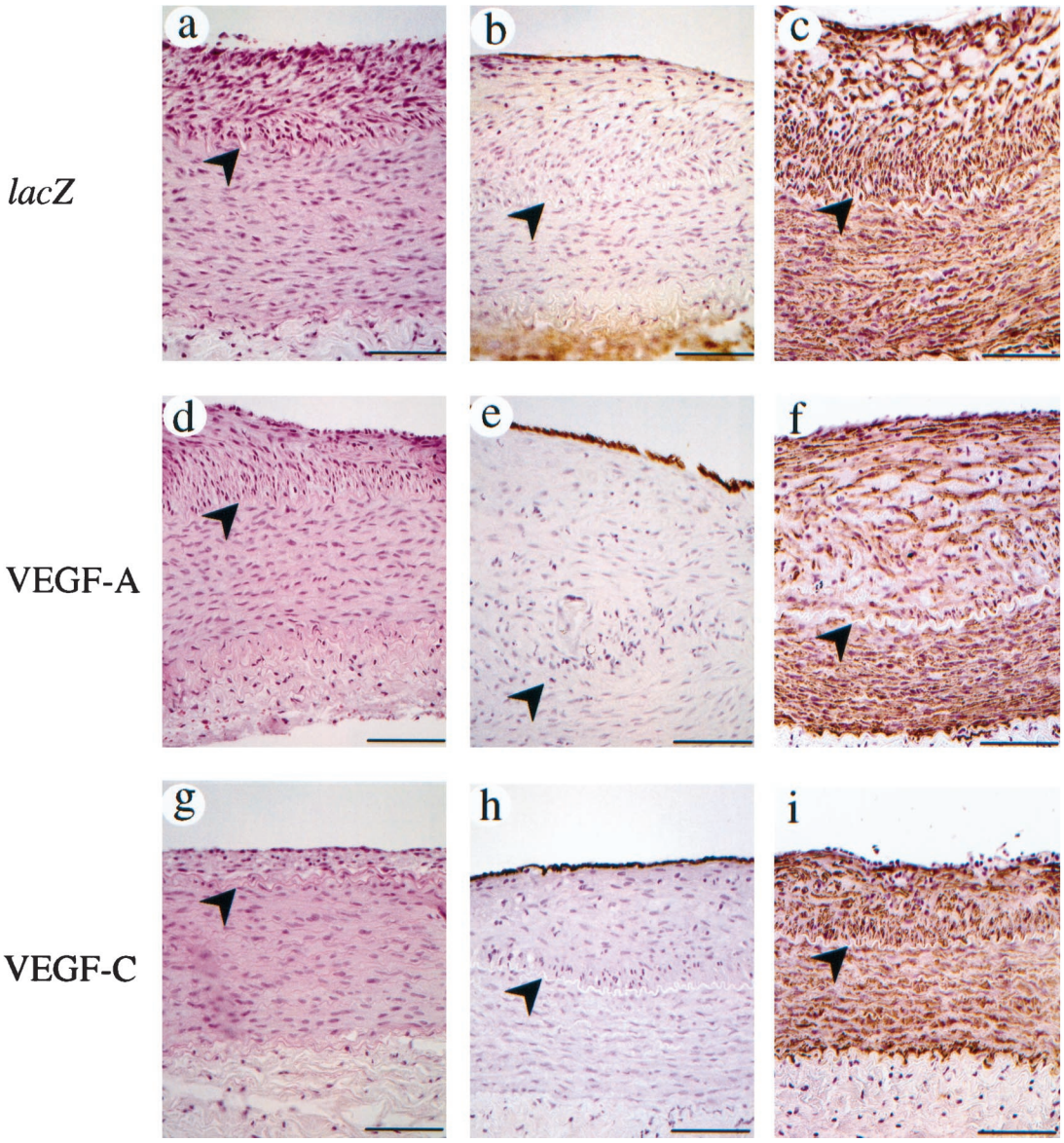


**Figure 4.** Histological characterization of balloon-denuded aortas 2 weeks after gene transfer. a through c, *LacZ* adenovirus–transfected aortas. a, H-E staining. b, Endothelium-specific immunostaining (CD-31). c, $\alpha$-Actin immunostaining (HHF-35). d through f, VEGF-A adenovirus–transfected aortas. d, H-E staining. e, Endothelium-specific immunostaining (CD-31). f, $\alpha$-Actin immunostaining (HHF-35). g through i, VEGF-C adenovirus–transfected aortas. g, H-E staining. h, Endothelium-specific immunostaining (CD-31). i, $\alpha$-Actin immunostaining (HHF-35). Arrowheads denote internal elastic lamina. Bars=100 $\mu$m.

The percentage of proliferating cells was analyzed by BrdU labeling (Table). No significant differences were seen, although we found a tendency that the VEGF-C group had a lower proliferation rate, which is in line with the observation that VEGF-C–transduced arteries had smaller I/M at both time points. The endothelial regrowth was analyzed by measuring the length of intact endothelium from histological sections. No significant differences were found between the study groups (Table).

**Regrowth of the Endothelium, IEL Damage, and Cell Proliferation After Balloon Denudation and Gene Transfer**

| | Intact Endothelium, %±SEM | | IEL Damage, %±SEM | | Proliferating Cells, %±SEM | |
|---|---|---|---|---|---|---|
| | 14 d | 28 d | 14 d | 28 d | 14 d | 28 d |
| *lacZ* | 25±20 | 44±17 | 81±10 | 53±18 | 1.8±0.4 | 0.3±0.1 |
| VEGF-A | 67±26 | 47±21 | 54±7 | 78±11 | 2.2±0.7 | 1.2±0.5 |
| VEGF-C | 6±6 | 73±16 | 77±8 | 67±12 | 1.2±0.0 | 0.3±0.1 |

IEL indicates internal elastic lamina.

## Effect of Adenoviral Gene Transfer on Intact Aorta Without Balloon Denudation

We tested the potential of clinical-grade adenovirus to cause damage to the vessel wall and neointima formation by doing high-titer adenovirus gene transfer to intact abdominal aorta of New Zealand White rabbits. Control rabbits were treated in the same way with 0.9% saline. The positioning of the gene transfer catheter caused some internal elastic lamina damage and moderate induction of neointima formation after the

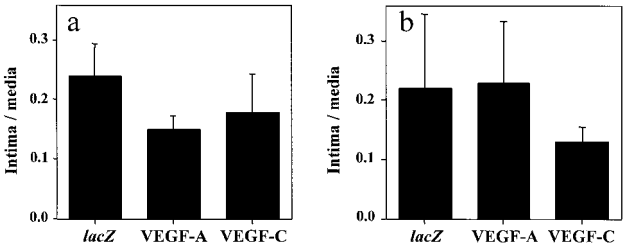


**Figure 5.** I/M in study groups after gene transfer without balloon denudation (mean±SEM). a, I/M 2 weeks after gene transfer. b, I/M 4 weeks after gene transfer. n=3 in each study group.

procedure. At the 2-week time point, the I/M in the *lacZ* group was 0.24±0.06; in the saline control group, 0.28±0.05 (data not shown); in the VEGF-C group, 0.18±0.07; and in the VEGF-A group, 0.15±0.03 ($P$=NS). At the 4-week time point, the *lacZ* group had an I/M of 0.22±0.13, the VEGF-A group 0.23±0.11, and the VEGF-C group 0.13±0.03 ($P$=NS) (Figure 5).

## Discussion

Restenosis has remained a major clinical problem after coronary and peripheral artery angioplasty, coronary bypass operations, and other types of vascular surgery (eg, grafted veins and prostheses).[1,2] The present study shows a beneficial therapeutic effect of intravascular adenovirus-mediated VEGF-C gene transfer on the vessel wall after balloon injury. This is also the first study to compare VEGF-C and VEGF-A adenovirus-mediated gene transfer for the prevention of neointima formation. Although different receptor-binding profiles of VEGF-C and VEGF-A might have led to different biological effects in the vessel wall, the findings support the hypothesis that beneficial effects are mediated primarily through the VEGFR-2 receptor.[31] By using in situ hybridization and immunocytochemistry, we show that VEGF receptors 1, 2, and 3 or highly homologous proteins are expressed in rabbit vessel wall. Our finding that VEGFR-2 is also expressed in intimal SMCs is in line with recently published data.[32] It is likely that the effects of VEGFs are at least partially due to the enhanced production of NO and prostacyclin.[6,33] In addition to endothelial cell proliferation and inhibition of neointima formation, VEGFs may also lead to therapeutic angiogenesis in ischemic tissues. Thus, VEGFs are potential candidates for vascular gene therapy of ischemic atherosclerotic diseases.[2]

Gene transfer was used instead of recombinant protein administration because a single gene transfer can produce a therapeutic effect for several days, whereas the half-life of recombinant VEGF protein in circulation is only a few minutes. Administration of recombinant VEGF protein has also recently been shown to be ineffective in humans.[34] For several atherosclerotic complications, such as postangioplasty restenosis, probably only a temporary expression of the transgene is needed to obtain a therapeutic effect. Despite the low gene transfer efficiency in human arteries with advanced atherosclerotic lesions,[22] the therapeutic effect can be achieved by use of genes encoding secreted gene products, such as VEGFs.[2] It is likely that gene transfer efficiency is limited by internal elastic lamina and calcified atherosclerotic lesions. However, the dissection lines caused by the angioplasty balloon have been shown to allow transfection of the deeper layers of artery.[22]

Problems related to the use of adenoviral vectors include immunological and inflammatory reactions.[35] Immunological reactions may be at least partly explained by the fact that high-titer adenovirus induces expression of NF-$\kappa$B[36] and activates a cytotoxic T lymphocyte response.[37] However, no major immunological reactions were seen in the analyzed arteries. Some of the problems related to the use of adenoviruses may be related to impurities or replication-competent viruses in the virus lots. In this study, human clinical-grade viruses were used, which, together with the short exposure time in the transfected arteries, may explain the absence of severe inflammatory reactions. However, immunostimulatory properties of adenoviruses may limit the use of very high titer viruses or repeated gene transfers.

It is concluded that VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit arteries. Thus, VEGF-C is a potential candidate for gene therapy of postangioplasty restenosis.

## Acknowledgments

This study was supported by grants from the Finnish Academy, Kuopio University Hospital (EVO grant 5130), the Ludwig Institute for Cancer Research, and the European Union (BMH4 CT-95-0329). The authors thank Mervi Nieminen, Eila Pelkonen, Anne Martikainen, Aila Erkinheimo, and Maiju Jääskeläinen for technical assistance; Marja Poikolainen for preparing the manuscript; and Boston Scientific for catheters.

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