Objective—This study aims to determine the effects of periadventitial vascular endothelial growth factor (VEGF) gene transfer on neointima formation and macrophage accumulation induced by collar placement around the carotid artery in hypercholesterolemic rabbits.

Methods and Results—Collar placement around the carotid artery in cholesterol-fed rabbits induced intimal thickening with increased neointimal macrophage content. Liposome-mediated VEGF gene transfer, confirmed by transgene-specific RT-PCR, caused a marked inhibition of both intimal thickening and macrophage accumulation compared with a lacZ control gene. VEGF gene transfer was not accompanied by a significant increase in adventitial neovascularization. Collaring of carotid arteries in hypercholesterolemic rabbits also upregulated endothelial VCAM-1 expression. Inhibition of neointimal macrophage infiltration in VEGF-transduced, collared arteries was associated with decreased endothelial VCAM-1.

Conclusions—VEGF gene transfer inhibits collar-induced intimal thickening, macrophage accumulation, and VCAM-1 expression in cholesterol-fed rabbits. These findings support the concept that low-level VEGF expression can exert arterioprotective effects in the presence of high blood cholesterol. (Arterioscler Thromb Vasc Biol. 2004;24:1074-1080.)

Key Words: VCAM ■ vascular smooth muscle ■ endothelium ■ atherosclerosis ■ collar

Intimal thickening caused by accumulation of vascular smooth muscle cells (VSMC) and macrophages is a central feature of early atherosclerotic lesions and a frequent cause of failure of arterial bypass grafting.1–3 Whereas most models of neointimal VSMC accumulation involve injury to the endothelium and medial smooth muscle, such as balloon catheter endothelial denudation, neointimal lesions rich in macrophages are experimentally induced by hypercholesterolemia in animals maintained on cholesterol-rich diets. Neointimal thickening can also be induced by periadventitial placement of an inert silicone collar around the rabbit carotid artery.4 In the collar model, neointimal thickening is increased by collar placement over a period of up to 14 days without major damage to the endothelium or medial VSMC,5 and the endothelium remains essentially intact and continuous throughout the study period.6,7 Neointimal thickening is caused predominantly by the accumulation of VSMC and, in rabbits on a normocholesterolemic diet, involvement of macrophages, T lymphocytes, or other cell types is minimal. However, collar placement in rabbits maintained on a high-cholesterol diet results in a striking neointimal accumulation of macrophages in the collared carotid artery.6 Several mechanisms have been proposed to explain collar-induced neointima formation,8 but none has yet been firmly estab-
this concept, liposome-mediated VEGF gene transfer was found to inhibit collar-induced formation of a VSMC-rich neointima. In this study, we tested whether local VEGF gene delivery could reduce intimal thickening and macrophage involvement in the collared carotid arteries of hypercholesterolemic rabbits. The results show that liposome-mediated VEGF gene transfer markedly reduced neointima formation in this model and caused a striking decrease in neointimal macrophage accumulation. Cell adhesion molecules were strongly upregulated in collared arteries by hypercholesterolemia, but a decrease in endothelial VCAM-1 expression was associated with decreased neointima formation in VEGF-transduced arteries. The finding that VEGF gene transfer is able to inhibit formation of macrophage-rich neointimal lesions in hypercholesterolemic rabbits supports the concept that VEGF can exert local arterioprotective effects, which may be therapeutically valuable for the treatment of vascular proliferative disease.

Methods

Plasmid Preparation

The pCMV-VEGF164 and pCMV-LacZ plasmids (gifts of Professor Seppo Yla-Herttuala, Gene Vector Laboratory, Al Virtanen Institute, Kuopio, Finland), were produced using Qiagen Mega Columns (Qiagen, Calif) and were free of any microbiological or endotoxin contamination.

Collar Placement and Gene Transfer

All experiments were conducted in accordance with the Animal Care and Ethics Guidelines of University College London, UK. Twenty-six New Zealand White male rabbits (2.5 to 3.2 kg) were fed a normal diet supplemented with 1.5% cholesterol for 1 week before collar placement and throughout the experiment. In parallel, 22 rabbits were maintained on normal diet without added cholesterol. Placement of a biologically inert, silastic collar (Ark Therapeutics Ltd) around the right carotid artery was performed in anesthetized rabbits as described. The contralateral carotid artery was shammed-operated by surgical dissection from surrounding tissues and exposure to a similar degree of stretch. The wounds were sutured and animals were allowed to regain consciousness. Five days later, the collared arteries were exposed and 200 μL of lipofectamine (Invitrogen) solution in Ringers lactate buffer, containing 25 μg of either LacZ (n=22) or VEGF164 (n=22) plasmids, were placed within the space between collar and artery using a pipette, and the wound was sutured. Nine days after gene transfer, serum samples were taken for assay of total cholesterol (Boehringer-Mannheim), animals were euthanized and collared and contralateral control arteries excised. Arteries were flushed with ice-cold saline and divided into 2 segments. The proximal 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 distal part was immersed fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) pH 7.4, for 30 minutes, rinsed in PBS for 15 minutes, embedded in OCT compound (Miles) and stored at −80°C, or snap-frozen in liquid nitrogen and stored at −80°C for total RNA extraction.

RNA Extraction and RT-PCR

Total arterial RNA was extracted using Trizol reagent (Gibco-BRL) and treated with RNase-free DNAse I (Promega) at 37°C for 30 minutes; 1.5 μg total RNA was reverse-transcribed using AMVL reverse-transcriptase and random hexamers according to the manufacturer’s instructions (Perkin Elmer). Polymerase chain reaction (PCR) was performed using Taq polymerase (Boehringer Mannheim) and primers for the pCMV-VEGF164 plasmid. The 3′ primer was derived from the cytomegalovirus promoter region within the expression plasmid, (5′-TCG ATC CAT GAA CTT TCT GC-3′) and the 3′ primer derived from VEGF (5′-TTG TTA CTC AAG CTG CC-3′). The following PCR cycle parameters were used: 4 minutes at 96°C (hot start, enzyme omitted), 35 seconds at 96°C, 40 seconds at 53°C, 90 seconds at 72°C for 39 cycles, and the last extension step was continued for 11 minutes. Products of each reaction were run on a 1.2% agarose gel, alongside 100-kb DNA markers (Promega).

LacZ Staining

Detection of β-galactosidase activity was performed by overnight incubation of whole arterial segments or 6-μm sections in the dark at 37°C in β-gal staining solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L MgCl2, 0.002% nonidet P-40, 0.01% SDS, and adjusted to pH 7.4 with NaH2PO4. Stained vessel segments were briefly rinsed in ice-cold PBS, snap-frozen in liquid N2, and stored at −80°C for sectioning. For thin-section staining, arteries were immersion fixed in 4% paraformaldehyde for 4 hours, then embedded and frozen in OCT compound. Stained sections were counterstained with eosin.

Immunohistochemistry

The following antibodies were used: mouse IgG1 to rabbit VCAM-1 and ICAM-1 (Rb/19 and Rb/39; respectively; gifts from M.I. Cybulsky), each at a 1:100 dilution in frozen sections; macrophage-specific RAM-11 (1:50, Dako); mouse anti-human CD31 (1:500; Dako); VSMC-specific α-actin antibody (1:150; Dako); and anti-VEGF mouse mAb sc-7269 (1:200; Santa Cruz Biotechnology Inc). Primary antibodies were diluted in Tris-buffered saline (TBS), pH 7.2. Staining was performed on deparaffinized or frozen sections. Endogenous peroxidases were blocked with 15-minute incubation in 0.3% H2O2 in methanol. Sections were washed twice with TBS, 10 minutes each. Nonspecific binding was blocked by incubation of tissue with diluted horse serum for 20 minutes. Sections were then incubated at room temperature with primary antibody, followed by 3 washes, 3 minutes each, with TBS containing 1% fetal bovine serum. Secondary antibody from Vectastain Elite ABC Kit (anti-murine IgG; Vector Laboratories) was applied to sections for 30 minutes each. The avidin–biotin complex was applied for 30 minutes and the sections were washed twice with TBS. For visualization, sections were incubated with DAB substrate (Vector) for 5 minutes and counterstained with Gill hematoxylin; 5-μm frozen sections were mounted in OCT compound and fixed for 10 minutes in acetone at −20°C, and then air-dried. Negative controls for all immunostainings were performed by omitting the primary antibody.

Morphometry and Image Analysis

Images of sections at ×5 and ×40 were acquired with a high-resolution color camera (Zeiss microscope; Jenoptik Camera) and analyzed using automated image analysis software (Image J; National Institutes of Health). Intima were defined as the regions between luminal endothelium and the internal elastic lamina. The media was defined as the area between the internal and external elastic laminae. Serial arterial sections were cut at 500-μm intervals, stained with hematoxylin and eosin, and analyzed blindly. The intima/media ratios were averaged and expressed as means±SEM. Neovascularization was quantified by counting the numbers of CD31-positive vessels. CD31 staining was regarded as positive if a single cell or a vessel with a lumen was present and numbers of CD31-positive cells and vessels were expressed per mm2 adventitial area. Total RAM-11 positive macrophages were counted in the intima and expressed per mm2 area. VCAM-1 immunostaining was quantified on high-resolution (1300×1030 pixel) images using OpenLab 3.14 software (Improvement Ltd) and expressed as the number of pixels representing endothelial VCAM-1 immunostaining as a percentage of the total endothelial pixel count.
Statistical Analysis

Differences in serum cholesterol levels and morphometric differences between different treatment groups were evaluated by ANOVA and Bon Ferroni correction (SPSS). \(P < 0.05\) was considered significant.

Results

Effect of VEGF\textsubscript{164} Gene Delivery on Intimal Thickening in Normocholesterolemic Rabbits

Previous findings showed that periadventitial liposome-mediated VEGF\textsubscript{164} gene transfer into collared arteries significantly reduced collar-induced neointima formation.\textsuperscript{9} To reproduce these findings, we initially performed periadventitial VEGF\textsubscript{164} gene transfer into carotid arteries 5 days after positioning of the collar in rabbits maintained on a normal low-cholesterol diet. Intimal thickness and cellular composition in arterial sections was then measured another 9 days after gene transfer. Consistent with previously published work, liposome-mediated VEGF\textsubscript{164} gene transfer caused a significant decrease in collar-induced intimal thickening as compared with a control lacZ gene (Figure 1).

Cellular Composition of Collar-Induced Neointima in Hypercholesterolemic Rabbits

To examine whether VEGF gene delivery could affect neointimal macrophage accumulation, collar placement was performed in cholesterol-fed rabbits. Collar-induced neointimal lesions in rabbits on a normal diet were composed largely of VSMC, as shown by immunostaining with a VSMC-specific \(\alpha\)-actin antibody, with no detectable macrophage involvement (Figure 2). In a parallel set of rabbits fed a 1.5% cholesterol diet, collar placement induced formation of neointimas composed of VSMC and abundant Ram 11-stained macrophages (Figure 2). Blood cholesterol increased to 12 to 15 and 24 to 29 mmol/L, respectively, after 7 and 21 days on the high-cholesterol diet, most of which was present as low-density lipoprotein cholesterol (Figure 2). Immunostaining of endothelial cells with CD31 antibody indicated that neither collaring nor hypercholesterolemia caused significant discontinuities in the endothelium, which remained essentially intact (Figure 2), in agreement with previous findings.\textsuperscript{6,7} Furthermore, microscopic analysis of histological sections from collared arteries revealed no disruption of either the internal or the external elastic lamina and no detectable damage to the tunica media.

Periadventitial VEGF\textsubscript{164} Gene Delivery in Collared Hypercholesterolemic Rabbits

The effect of VEGF\textsubscript{164} gene delivery on collar-induced intimal thickening and macrophage accumulation in cholesterol-fed rabbits was determined next. Expression of the VEGF\textsubscript{164} transgene was verified in transduced carotid arteries by reverse-transcriptase polymerase chain reaction (RT-PCR). An expected amplicon of 547 bp corresponding to the VEGF\textsubscript{164} transgene and vector was detected only in collared
arteries transduced with the VEGF<sub>164</sub> vector (Figure 3A). Immunostaining of sections of VEGF-transduced arteries with anti-VEGF antibody showed the expression of VEGF in the adventitia, media, and intima including endothelium (Figure 3B). Previous studies demonstrated that liposome-mediated gene transfer in the collared rabbit carotid artery results in a low efficiency of transfection (<0.05%).<sup>21</sup>

Staining for β-galactosidase in arteries transduced with the control lacZ gene revealed a small number of strongly stained cells in the adventitia, consistent with a similarly low efficiency of gene transfer and in agreement with previous findings (results not shown).<sup>9,21</sup> LacZ gene transfer caused no significant change in intimal thickening or neointimal macrophage accumulation relative to saline-treated nontransfected arteries (results not shown).

As shown in Figure 4A, VEGF<sub>164</sub> gene transfer for 9 days caused a marked, highly significant decrease in collar-induced intimal thickening in cholesterol-fed rabbits (P<0.001 for VEGF versus lacZ). Immunostaining with Ram11 showed that VEGF also caused a striking inhibition of macrophage accumulation in the neointima of collared arteries (Figure 4B). Compared with lacZ-transduced arteries, periadventitial VEGF gene delivery significantly decreased the total number of neointimal Ram11-positive cells (Figure 4C) and the neointimal density of Ram11-positive cells (Figure 4D). VEGF also decreased the neointimal density of Ram11-positive cells compared with saline nontransfected collared arteries (224±66 versus 384±54 for VEGF and saline, respectively; P<0.05).

**Neovascularization in VEGF-Transduced Collared Arteries**

Because angiogenesis is a major biological effect of VEGF in vivo, it was examined whether VEGF gene transfer increased neovascularization in collared carotid arteries. Immunostaining of CD31 revealed the presence of new vessels specifically in the adventitia of VEGF-transduced and lacZ-transduced arteries. Quantification of the adventitial density of CD31-positive vessels indicated that neovascularization was increased in VEGF-transduced arteries compared with lacZ transfectants, but this effect was not significant (Figure 5). CD31-positive cells were not detected in the media and, apart from luminal staining of the endothelium, were also absent from the neointima.

**Cell Adhesion Molecule Expression in VEGF-Transduced Collared Arteries**

Upregulation of the endothelial cell adhesion molecule, VCAM-1, is thought to play a central role in mediating increased monocyte adhesion to the endothelium and transen-
dothelial monocyte migration leading to neointimal accumulation of macrophages. VEGF-mediated modulation of endothelial VCAM-1 expression is therefore a possible mechanism that could explain the marked decrease in macrophage accumulation observed in the VEGF-transduced collared arteries of cholesterol-fed rabbits.

Endothelial expression of VCAM-1 was strikingly increased in lacZ-transduced collared arteries from cholesterol-fed rabbits compared with the sham-operated contralateral control arteries in the same animals (Figure 6). VCAM-1 staining in the lacZ-transfected arteries of cholesterol-fed rabbits was also evident within the media and more sparsely in the adventitia. ICAM-1 was constitutively expressed on the endothelia of sham-operated arteries in cholesterol-fed rabbits, and collaring caused no significant increase in endothelial ICAM-1 expression. In the VEGF-transduced collared arteries of cholesterol-fed rabbits, endothelial VCAM-1 staining was markedly reduced, particularly where neointimal thickness exhibited the most striking decrease, and was also noticeably decreased in the media (Figure 6). Quantification of VCAM-1 immunostaining showed that the total percentage of the endothelium positive for VCAM-1 immunostaining was significantly reduced in VEGF-transduced arteries compared with lacZ (Figure 6). A similar marked significant reduction in VCAM-1 staining in VEGF-transduced arteries was found when total neointimal VCAM-1 staining was quantified. VEGF gene transfer did not significantly affect endothelial ICAM-1 immunostaining in hypercholesterolemic rabbits.

Discussion

The major finding of this study is that periadventitial gene transfer of VEGF<sub>164</sub> inhibited collar-induced arterial intimal thickening and macrophage accumulation in cholesterol-fed rabbits. The fact that macrophage infiltration into carotid arteries was not detected in sham-operated vessels in hypercholesterolemic rabbits indicates that the neointimal accumulation of macrophages in collared arteries is critically dependent on intimal thickening as well as high blood cholesterol. Therefore, it can be inferred that VEGF gene transfer inhibits macrophage accumulation in cholesterol-fed rabbits, partly through an indirect neointima-decreasing effect. However,
the finding that neointimal macrophage density was also significantly reduced in VEGF-transfected arteries suggests that VEGF may either directly or indirectly inhibit macrophage infiltration into the subendothelial region through other mechanisms. Because VEGF gene delivery did not significantly increase vessel wall neovascularization in collared arteries, our results suggest that the inhibitory effects of VEGF on intimal thickening and macrophage influx are largely independent of its ability to stimulate angiogenesis. The weak angiogenic response induced in VEGF-transduced arteries is likely to be because of a low intravascular concentration of VEGF resulting from the low efficiency of liposome-mediated VEGF gene delivery and the impairment of angiogenesis by hypercholesterolemia.24

Decreased VCAM-1 expression may partly be responsible for the inhibition of neointimal macrophage influx in VEGF-transduced arteries. In contrast to ICAM-1, which is expressed on the arterial endothelium in mice and rabbits with normal cholesterol levels, the cytokine-inducible cell adhesion molecule, VCAM-1, is upregulated in the arterial endothelium at atherosclerosis-prone sites in hypercholesterolemic rabbits and mice.25 A critical role for VCAM-1 in early atherosclerosis was revealed by studies showing that atherosclerotic lesion formation was impaired in low-density lipoprotein receptor-deficient mice expressing greatly reduced levels of VCAM-1 but was not affected by ICAM-1 deficiency.23 Consonant with previous studies, we found that VCAM-1 expression was induced in the endothelia of collared arteries in hypercholesterolemic rabbits. VEGF gene transfer caused a marked decrease in endothelial VCAM-1 expression compared with lacZ-transfected arteries that was particularly marked where the neointima-reducing effect of VEGF was greatest. VEGF did not significantly alter VCAM-1 mRNA or protein levels in cultured endothelial cells, suggesting that VEGF is unlikely to exert a direct inhibitory effect on VCAM-1 expression in vivo (A. Stannard and I. Zachary, unpublished findings). The mechanism of the reduced VCAM-1 expression we have observed in VEGF-transduced arteries is unclear, but we infer from the inability of VEGF to inhibit VCAM-1 expression in cultured cells that this is likely to be complex and indirect, involving interactions between VEGF, the effects of high blood cholesterol, endothelial cells, and monocyte/macrophages. Low-density lipoprotein increases VCAM-1 expression in endothelial cells, whereas lipid-lowering drugs reduce endothelial VCAM-1 expression in atheroma in hypercholesterolemic rabbits.27 Interestingly, we found that VEGF attenuates low-density lipoprotein uptake by HUVECs (S. Shaﬁ and I. Zachary, unpublished ﬁndings), lending plausibility to the notion that VEGF might indirectly regulate VCAM-1 expression through a long-term effect on low-density lipoprotein interactions with the endothelium. This possibility warrants further investigation. Regardless of the mechanism(s) involved, given that VCAM-1 plays a key role in early atherosclerotic lesion formation and monocyte adhesion to the endothelium, our results indicate that impairment of endothelial VCAM-1 expression is at least partly responsible for the reduced neointimal macrophage content of VEGF-transduced arteries.

The role of VEGF in the pathogenesis of vessel wall disease has been a matter of considerable debate. Some studies have suggested that either angiogenesis28 or VEGF-dependent recruitment of monocyte/macrophages29,30 can promote atherosclerosis. The model of collar-induced neointima formation in hypercholesterolemic rabbits is distinct from those in which VEGF was reported to exhibit proatherosclerotic effects, and this may partly account for the divergence of our results from previous ﬁndings. In addition, local delivery of the VEGF gene directly into the collared carotid artery may produce biological effects distinct from those induced by administration of VEGF protein23 or soluble Flt-1 gene31 at sites distant from the biological target tissue. Another crucial determinant of the biological effect of VEGF in vivo is likely to be local concentration. It is possible that proatherosclerotic effects of VEGF may require at least transiently high systemic levels of VEGF and be mediated indirectly via nonendothelial actions of VEGF. VEGF stimulates monocyte chemotaxis via Flt1 receptors,31 and enhancement of plaque progression induced by intraperitoneal VEGF delivery was associated with mobilization of bone marrow endothelial progenitor cells and monocyte/macrophages.29 The lower-efficiency gene transduction resulting from liposome-mediated gene transfer, as used in the present study, is likely to generate relatively modest VEGF levels that may be sufﬁcient to cause neointima reduction via a bystander effect but insufficient to produce other biological effects such as angiogenesis. In support of this contention, our recent results show that adenosinemediated VEGF delivery to collared arteries in cholesterol-fed rabbits produced a striking adventitial neovascular response but had little effect on intimal thickening or macrophage accumulation (Khurana et al, unpublished results).

We hypothesized that VEGF can elicit a local arterioprotective effect through its abilities to promote endothelial functions such as nitric oxide and prostacyclin production, which inhibit VSMC proliferation, and antagonize endothelial leukocyte adhesion.28 The finding that local VEGF gene transfer can reduce intimal thickening and macrophage accumulation in the hypercholesterolemic rabbit identiﬁes a novel mechanism through which VEGF can exert arterioprotective effects in vivo, and thus advances our previous ﬁnding that periadventitial VEGF gene transfer inhibits collar-induced neointima formation.9 Furthermore, a growing body of evidence supports the concept that VEGF can elicit diverse protective effects in blood vessels independent of angiogenesis, including the regulation of thrombogenic potential,32 nitric oxide-dependent attenuation of leukocyte adhesion and transmigration in mesenteric venules,33 protection against endothelial toxicity induced by oxidized low-density lipoprotein,34 and upregulation of decay accelerating factor, which counteracts complement-mediated cell injury.35 Given that increased VCAM-1 expression and macrophage inﬁltration are implicated in vein graft intimal thickening in hypercholesterolemic rabbits,36,37 and that hypercholesterolemia is a major risk factor for human vein graft atherosclerosis,38 these results suggest that local periadventitial VEGF gene transfer may be therapeutically useful for preventing vein graft failure.
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