Vascular Endothelial Growth Factor Regulates Reendothelialization and Neointima Formation in a Mouse Model of Arterial Injury

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**Background**—The rate of reendothelialization is critical in neointima formation after arterial injury. Vascular endothelial growth factor (VEGF), a potent endothelial mitogen, has been advocated for accelerating endothelial repair and preventing intimal hyperplasia after percutaneous coronary interventions. However, the precise mechanism of action of VEGF treatment and the physiologic role of endogenous VEGF after arterial injury are not well described. To better understand the role of VEGF in arterial repair, we overexpressed both VEGF and a soluble, chimeric VEGF receptor (VEGF-trap), which binds free VEGF with high affinity, in a mouse model of arterial injury.

**Methods and Results**—Four groups of C57BL/6 mice underwent denuding endothelial injury 1 day after systemic injection of recombinant adenovirus expressing (1) VEGF, (2) VEGF-trap, (3) VEGF plus VEGF-trap, or (4) control adenovirus. Circulating levels of adenovirus-encoded proteins were significantly elevated after gene transfer. VEGF overexpression accelerated reendothelialization and increased luminal endothelial cell proliferation 2 weeks after arterial injury ($P<0.05$), resulting in decreased neointima formation at 4 weeks compared with control ($P<0.01$). Cotreatment with VEGF-trap completely sequestered free VEGF and abrogated the beneficial effect of VEGF overexpression. Interestingly, sequestration of endogenous VEGF by VEGF-trap overexpression alone also led to delayed reendothelialization at 2 weeks ($P<0.01$) and increased neointima formation at 4 weeks ($P<0.01$).

**Conclusions**—VEGF overexpression accelerated endothelial repair and inhibited neointima formation after arterial injury. Conversely, sequestration of exogenous and/or endogenous VEGF by VEGF-trap delayed reendothelialization and significantly increased neointima size. This demonstrates the therapeutic potential of VEGF but also emphasizes the important physiologic role of endogenous VEGF in vascular repair. (Circulation. 2004;110:2430-2435.)

**Key Words:** angiogenesis ■ endothelium ■ gene therapy ■ stenosis

The luminal endothelium plays a critical role in arterial lesion formation by regulating vascular permeability, cellular adhesion to the arterial wall, and the local production of growth-regulatory molecules.1-4 Injury to the luminal endothelium, either metabolic or mechanical, ultimately results in the development of arterial lesions. However, the contribution of endothelial cells to the arterial response-to-injury process is more complex: initial luminal endothelial cell repair is complemented by later intra-arterial wall neovascularization.5-8 Both processes are taking place in parallel; however, they should be carefully distinguished. In later stages of chronic atherosclerosis, neovascularization seems to be predominant, whereas in response to mechanical arterial injury, early luminal reendothelialization is the most important factor for determining the size and composition of arterial lesions.9

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We recently reported that overexpression of endostatin, a specific inhibitor of endothelial cell growth, significantly impaired reendothelialization and endothelial cell integrity after arterial injury.9 Endostatin overexpression not only reduced reendothelialization but also dramatically changed the growth of arterial lesions, increasing neointima size nearly 3-fold. These data underscore the strong relation between luminal endothelial cell biology and the response to injury of the arterial wall. Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells and has been
shown to induce endothelial cell proliferation and angiogenesis in vitro and in vivo.\textsuperscript{10–13} Conversely, VEGF-trap, a soluble, chimeric, high-affinity VEGF receptor that sequesters free VEGF, has been shown to suppress angiogenesis in vivo and appears to be more effective in inhibiting endothelial cell proliferation than alternative blockers of VEGF, including monoclonal antibodies.\textsuperscript{14} Studies have shown that therapeutic application of VEGF is capable of reducing neointima formation in different in vivo models of acute arterial lesion formation.\textsuperscript{2,15–17} Delivery of recombinant VEGF protein has also been shown to accelerate the development of an intact luminal endothelial monolayer after stent implantation. Taken together, these data suggest that VEGF delivery might play an important therapeutic role after acute mechanical vascular injury, such as in percutaneous coronary interventions. However, the precise mechanism of action of VEGF treatment, its reversibility through specific VEGF blockade, and the possible physiologic role of endogenous VEGF in arterial lesion formation have not been demonstrated.

To better understand the regulatory effect of exogenous and endogenous VEGF on reendothelialization and arterial lesion formation after mechanical arterial injury, we overexpressed VEGF and VEGF-trap, both alone and in combination, in a mouse model of arterial injury.

**Methods**

**ADV Construction**

Total RNA was extracted from human umbilical vein endothelial cells (HUVECs), and human (h) VEGF-165 cDNA was generated by reverse transcription–polymerase chain reaction with appropriate primers. hVEGF was subsequently linked to a rat insulin leader sequence and cloned into a recombinant adenovirus (ADV) shuttle vector pXCJL.1 downstream of a Rous sarcoma virus promoter. Recombinant adenovirus (ADV.hVEGF-165) was rescued by standard calcium phosphate co-transfection of the ADV shuttle vector with the ADV backbone vector pBHG10 in 293 cells. Viral plaques were isolated and tested for transgene expression by measurement of hVEGF levels in conditioned supernatants with use of a commercially available ELISA kit (Quantikine, R&D Systems). A high-expressing single plaque was amplified in 293 cells, and virus was purified by 2 rounds on a CsCl gradient. Viral particles were measured by absorption (optical density at 260 nm), and plaque-forming units were determined by standard agarose overlay plaque assay on 293 cells. The specific structure and properties of the VEGF receptor chimera (VEGF-trap\textsubscript{hVEGF-165}) encoded by ADV.R1R2-Fc (ADV.VEGF-trap) have previously been described.\textsuperscript{14}

**In Vitro Bioactivity Assay of VEGF and VEGF-Trap**

HUVEC proliferation and viability were assessed as previously described by O’Reilly et al.\textsuperscript{18} In brief, HUVECs were serum-starved over night and then incubated with a 2-fold dilution series of VEGF-trap–conditioned supernatant and RPMI containing 10% fetal calf serum plus 2 ng/mL fibroblast growth factor. HUVEC proliferation was measured after 48 hours by a tetrazolium-based assay (cell proliferation kit 1 MTT; Roche Mannheim).

**Mice**

Male C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) and housed at the Center for Laboratory Animal Sciences at Mount Sinai Medical Center, New York, NY. Mice received standard rodent chow (mouse diet No. 5015, PMI Nutrition International) and tap water ad libitum. Procedures and animal care were approved by the Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC: National Academy Press; 1996).

**Endothelial Denudation Injury of Mouse Femoral Artery and ADV Delivery**

C57BL/6 mice were anesthetized by isoflurane/O\textsubscript{2} (1:1, vol/vol) inhalation anesthesia. Denudation of the endothelium of the common femoral artery was achieved by 3 passages of a 0.25-mm angioplasty guide wire (Advanced Cardiovascular Systems). The protocol, as well as the degree of injury applied to the vessel wall, has been standardized, validated, and described in detail in previous studies.\textsuperscript{9,19} One day before arterial injury, a neck incision was made, and the jugular vein was exposed for injection of (1) ADV.hVEGF (10\textsuperscript{10} particles, n = 15); (2) ADV.VEGF-trap (10\textsuperscript{11} particles, n = 15); (3) ADV.hVEGF and ADV.VEGF-trap (10\textsuperscript{10} and 10\textsuperscript{11} particles, respectively; n = 17); and (4) ADV.DL312 (10\textsuperscript{11} particles, n = 20) with a 30-gauge needle. Total viral particle number was held constant in each group by addition of control vector.

**VEGF and VEGF-Trap Measurements**

hVEGF levels in conditioned supernatants and serum were measured with a commercially available ELISA kit (Quantikine R&D Systems). VEGF-trap levels were measured by Regeneron Pharmaceuticals, Inc, as previously described by Holash et al.\textsuperscript{14} Free VEGF-trap was identified by using a VEGF capture and an anti-human Fc reporter system, whereas VEGF-bound VEGF-trap levels were determined by using an anti-VEGF antibody and an anti-human Fc reporter system.

**Tissue Preparation, Histology, and Immunohistochemistry**

Animals were euthanized in a CO\textsubscript{2} chamber according to American Veterinary Medical Association guidelines 2 and 4 weeks after gene transfer and arterial injury and perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline at 100 mm Hg for 10 minutes; their hindlimbs were excised en bloc. Specimens were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline and decalcified in 10% formic acid. Two 2-mm-thick cross sections were cut from each hindlimb at the level of injury in the common femoral artery and processed for paraffin embedding. Sequential sections (4 \textmu m thick) were stained with hematoxylin-eosin. Immunohistochemical staining of adjacent sections with intercellular adhesion molecule-1 (ICAM-1, Seikagaku; 1:300), von Willebrand factor (vWF, Dako; 1:100), and Ki67 (Dako, 1:50) antibodies was performed as described previously.\textsuperscript{9}

**Computer-Assisted Morphometry**

Histomorphometric evaluation of the arterial response to injury was performed at 2 and 4 weeks by investigators blinded to the study design. A computer-assisted planimetry system was used (Image Pro Plus 3.0.1 software). Endothelial cell coverage of the luminal surface was assessed by \times 400 microscopic examination of sections with a nuclear hematoxylin counterstain and staining for ICAM-1/vWF when both an endothelial cell nucleus and immunostaining were present. Neointimal area was assessed by hematoxylin-eosin and Masson’s trichrome staining. No significant interobserver or intraobserver variations were noted.

**Statistical Analysis**

Data were analyzed for significant differences by ANOVA, followed by appropriate post hoc tests according to gaussian distribution rules. Differences were considered to be significant at P<0.05. The tests were performed with SPSS, version 6.1, for the Apple Macintosh computer (SPSS, Inc). All values are given as the mean±SEM. hVEGF and VEGF-trap serum concentrations in mice were compared by the Mann-Whitney rank-sum test because this variable was not normally distributed. Correlation coefficients were determined by Pearson’s test.
In Vitro Bioactivity of Adenovirally Expressed hVEGF and VEGF-Trap

The biologic function of hVEGF, VEGF-trap, and control vector (ADV.DL312), expressed in the supernatants of ADV.hVEGF-, ADV.VEGF-trap–, and ADV.DL312-transduced JC cells, was tested in a proliferation assay on HUVECs. ADV.hVEGF-conditioned medium enhanced HUVEC proliferation and viability in a dose-dependent manner at concentrations of 50%, 25%, 12%, 6%, and 3% supernatant (Figure 1). ADV.VEGF-trap–conditioned medium, on the other hand, inhibited HUVEC proliferation also in a dose-dependent manner and totally inhibited cell growth at a concentration of 50%. Supernatant from control virus affected neither HUVEC proliferation nor viability.

ADV-Mediated Transgene Expression In Vivo

Intravenous injection of $10^{11}$ particles of ADV.hVEGF resulted in lethal serum concentrations of hVEGF. We determined that intrajugular injection of $10^{10}$ viral particles of ADV.hVEGF was the maximal tolerable dose, with a mortality rate of ~30%. This viral dose resulted in significantly elevated systemic hVEGF levels at 1 and 2 weeks after injection (1 week, 400±110 pg/mL and 2 weeks, 124±11 pg/mL). Injection of ADV.VEGF-trap ($10^{11}$ viral particles) resulted in the rapid appearance of high concentrations of VEGF in the circulation, with levels remaining elevated until 4 weeks after infection (1 week, 1170±180 μg/mL; 2 weeks, 1960±447 μg/mL; and 4 weeks, 1442±208 μg/mL; Figure 2). At the peak of VEGF expression at 1 week, high circulating levels of VEGF/VEGF-trap complex could be measured in the coinjected group (VEGF plus VEGF-trap); 11.77±0.69 versus 2.3±0.19 μg/mL in the VEGF-trap–only group, indicating efficient binding of circulating VEGF by VEGF-trap. Accordingly, circulating free VEGF levels could not be detected either in the coinjected (ADV.VEGF-trap plus ADV.hVEGF) group or in the VEGF-trap–only group owing to the high affinity of the soluble receptor for the ligand (Kd of 1 pmol/L). Injection of $10^{11}$ ADV.VEGF-trap did not produce any observable adverse side effects (mortality or any other clinical signs of distress/morbidity), and more important, when co injected with ADV.VEGF, it completely abrogated the VEGF-induced mortality.

To control for vessel size, we compared total vessel area between treatment groups and controls. All groups were similar in arterial size. To assess potential nonspecific virus-induced effects, we also compared injured arteries of saline-treated and control vector–treated animals, and no significant differences (neointima formation, vessel geometry, etc) were observed (data not shown).

Effect of VEGF and VEGF-Trap Overexpression on Reendothelialization

Morphometric analysis of ICAM-1 and vWF immunostaining along the luminal surface of injured arteries showed significantly increased reendothelialization of the denuded vascular surface 2 weeks after arterial injury with ADV.hVEGF treatment compared with control (76%±8 vs 60%±13; P<0.05; Figure 3). The positive effect of ADV.hVEGF on reendothelialization was completely reversed by coexpression of ADV.VEGF-trap (ADV.VEGF-Trap plus ADV.hVEGF treatment, 76%±8% vs 47±14.6%, P<0.01; Figure 3D). Importantly, inhibition of endogenous VEGF signaling by overexpression of VEGF-trap alone led to a significant decrease in the rate of reendothelialization of the luminal surface at both 2 weeks (25±4.8% vs 60±12.6%, P<0.01; Figure 3D) and 4 weeks (62±14.5% vs 84±12.4%, P<0.01) after arterial injury compared with control groups.

Effect of VEGF and VEGF-Trap Overexpression on Luminal Endothelial Cell Proliferation

To confirm the selective effects of VEGF on endothelial cell proliferation, we performed Ki67 immunostaining in all groups. As shown in Figure 4, proliferation of the luminal endothelial cells was significantly increased on treatment with VEGF compared with controls (2 weeks, 31±1.8% vs 17.8±1.2% proliferation, respectively; P<0.01), while VEGF-trap alone or in combination with VEGF resulted in a significant decrease in the rate of luminal endothelial cell proliferation (12.5±1.2% vs 17.8±1.2%, P<0.01).

Results

In Vitro Bioactivity of Adenovirally Expressed hVEGF and VEGF-Trap

The biologic function of hVEGF, VEGF-trap, and control vector (ADV.DL312), expressed in the supernatants of ADV.hVEGF–, ADV.VEGF-trap–, and ADV.DL312-transduced JC cells, was tested in a proliferation assay on HUVECs. ADV.hVEGF-conditioned medium enhanced HUVEC proliferation and viability in a dose-dependent manner at concentrations of 50%, 25%, 12%, 6%, and 3% supernatant (Figure 1). ADV.VEGF-trap–conditioned medium, on the other hand, inhibited HUVEC proliferation also in a dose-dependent manner and totally inhibited cell growth at a concentration of 50%. Supernatant from control virus affected neither HUVEC proliferation nor viability.

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Conversely, VEGF-trap treatment, alone and in combination with VEGF, inhibited luminal endothelial cell proliferation compared with controls (VEGF-trap at 2 weeks, 0% vs 7±1.4%, P<0.01; 4 weeks, 0% vs 2.3±0.7%, P=NS; VEGF-trap+VEGF at 2 weeks, 0% vs 7±1.4%, P<0.01; 4 weeks, 1.6% vs 2.3±0.7%, P=NS; Figure 4).

Effect of VEGF and VEGF-Trap Overexpression on Neointima Formation
Hematoxylin and eosin staining and subsequent morphometric analysis of the treatment groups revealed significant differences in the size of neointima 4 weeks after arterial injury (Figure 5). Animals treated with ADV.hVEGF exhibited significantly smaller neointima compared with controls (2.5±1.5 vs 7.5±2.6×10⁻³ mm², P<0.01), whereas treatment with ADV.VEGF-trap, either in combination with ADV.VEGF (21.4±16 vs 7.5±2.6×10⁻³ mm², P<0.01) or alone (17.3±7 vs 7.5±2.6×10⁻³ mm², P<0.01), resulted in a dramatically increased neointima (Figure 6).

Interestingly, whereas VEGF-trap reversed the effect of VEGF overexpression in the combination group, ADV.VEGF-trap treatment alone also significantly increased neointima size compared with controls. At 4 weeks, the degree of reendothelialization was correlated inversely with neointima formation in ADV.VEGF-trap and ADV.VEGF-trap plus ADV.hVEGF vector treatment groups (r=-0.553, P<0.05 and r=-0.584; P<0.01; respectively), whereas in the ADV.hVEGF treatment group, the endothelium had completely regrown and the neointima was almost absent. The increase in neointima size in the VEGF-trap group compared with VEGF treatment alone was caused by neoin-
versely, sequestration of exogenous and/or endogenous VEGF by VEGF-trap delayed reendothelialization and dramatically increased neointima size.

We and others have examined the relation between reendothelialization and neointima formation in various models of endothelial injury.6,9,20 It is becoming increasingly clear that the rate of luminal endothelial repair is a critical modulator of arterial lesion formation after injury. In this study, we examined the effects of circulating VEGF on reendothelialization after mechanical endothelial denudation and consequently, on the degree of neointima formation. To specifically evaluate the role of exogenous and endogenous VEGF in a mouse model of arterial injury, we looked at (1) high circulating VEGF levels (ADV VEGF-overexpression), (2) physiologic VEGF levels (control vector treatment), and (3) complete abrogation of circulating VEGF levels (VEGF-trap overexpression alone or in combination with VEGF-ADV vector treatment).

To this end, ADV vectors expressing either VEGF or a specific inhibitor of VEGF (VEGF-trap) were developed and intravenously administered to C57/BL6 mice 1 day before arterial injury.9,19 Owing to the hepatotropic property of ADV in mice, the transgenes were overexpressed in the liver and not at the site of endothelial denudation and arterial injury, as done previously.9 This resulted in significantly elevated circulating protein levels (VEGF and/or VEGF-trap) compared with controls.

We previously reported that VEGF-trap binds its ligand (VEGF) with exceptionally high affinity (Kd of 1 pmol/L).14 This finding was reflected in the present study by the marked increase of circulating VEGF/VEGF-trap complexes after coinjection of ADV.VEGF and ADV.VEGF-trap (Figure 2). Therefore, VEGF-trap overexpression resulted in the complete sequestration of both ADV-overexpressed and endogenous VEGF in serum. In addition, we confirmed the biologic activity of our VEGF and VEGF-trap transgenes present in the conditioned supernatants of ADV-transduced cells by assessing stimulation/inhibition of endothelial cell proliferation in vitro (Figure 1).

Systemic overexpression of VEGF with high serum transgene levels selectively induced luminal endothelial cell proliferation and significantly accelerated reendothelialization at 2 weeks after injury compared with controls, resulting in significantly reduced neointima formation at 4 weeks (Figures 3 through 6). This finding is consistent with reports showing that VEGF is a powerful endothelial mitogen capable of stimulating reendothelialization and attenuating neointimal thickening in response to arterial injury.2,15–17

The beneficial therapeutic effect of VEGF on the rate of reendothelialization was inhibited by co-overexpression with the specific inhibitor of VEGF (VEGF-trap), reducing luminal endothelial cell proliferation and resulting in a dramatic increase of neointima size (hypertrophy; Figures 3 through 6). The degree of reendothelialization indeed was correlated inversely with the size of the neointima, demonstrating the effects of prolonged loss of endothelial integrity on vascular repair. These results are consistent with our previous report showing that an endothelial cell–specific inhibitor, endostatin, also resulted in inhibition of reendothelialization, together with a subsequent increase of neointima size.9
Most importantly, however, was the observation that the inhibition of reendothelialization and the increase in neointima size by cotreatment with VEGF and VEGF-trap far surpassed the respective values of the control group, indicating that in addition to a mere reversal of the therapeutic effect of exogenously added VEGF, something else must have occurred. We hypothesize that inhibition of endogenous VEGF was the only distinguishing variable between the VEGF-plus–VEGF-trap–treated and the control vector–treated groups. Endogenous VEGF levels could therefore potentially be accounted for the observed difference between the groups.

In light of this novel and important finding and in contrast to previous studies that focused on the pharmacologic efficacy of VEGF to promote reendothelialization after arterial injury,6,21 we wanted to investigate the physiologic role of endogenous VEGF in endothelial repair and in the arterial response-to-injury process. To this end, we studied the effect of VEGF-trap overexpression alone and hence, the sequestration of endogenous VEGF, on reendothelialization and neointima formation. Overexpression of VEGF-trap alone indeed significantly inhibited luminal endothelial cell proliferation and inhibited reendothelialization by almost 70% compared with VEGF therapy (25% vs 76%) and by 58% compared with controls (25% vs 60%; Figures 3 and 4), ultimately resulting in a significantly enlarged neointima in the VEGF-trap–treated animals at 4 weeks (Figures 5 and 6).

These data are reflective of the pivotal role of the small amount of endogenous VEGF in the repair of luminal endothelium after arterial injury. The inhibition of reendothelialization by VEGF blockade (VEGF-trap) and the dramatic subsequent increase of neointima formation after endothelial denuding injury is also concordant with our previously published report on the endothelial cell inhibitor, endostatin,9 and supports the important link between the integrity of the luminal endothelium and the degree of neointima formation. Taken together, VEGF overexpression accelerated luminal endothelial repair and inhibited neointima formation after arterial injury compared with controls. Conversely, sequestration of exogenous and/or endogenous VEGF by VEGF-trap delayed reendothelialization and dramatically increased neointima size, implicating the VEGF pathway in control of the arterial repair process and particularly pointing to the critical role of endogenous VEGF levels, which to date has not been reported. It is conceivable that delayed reendothelialization may initiate a complex cellular and molecular cascade that eventually results in a change of neointima size. The different reendothelialization rates between the groups may be responsible for differences in homing of circulating cells (stem/progenitor cells, peripheral blood mononuclear cells, etc), in exposure time of subendothelial structures to circulating growth factors, and in endothelial cell–dependent rates of nitric oxide production and its downstream effect on neointima size. The individual contribution of these factors to the response to vascular injury remains to be investigated.

Disclosure

Dr. Rudge and Wiegand are employees of Regeneron Pharmaceuticals, Inc, which has a proprietary interest in developing and commercializing the VEGF trap.

References


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