Decreased Reendothelialization and Increased Neointima Formation With Endostatin Overexpression in a Mouse Model of Arterial Injury

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Background—Impaired endothelial regeneration contributes to arterial lesion formation. Endostatin is a specific inhibitor of endothelial cell growth and induces endothelial cell apoptosis. We examined the effect of endostatin overexpression on reendothelialization and neointima formation in a mouse model of arterial injury.

Methods and Results—Mice underwent femoral arterial denudation and received recombinant adenovirus, expressing either murine endostatin (n=19) or control adenoviral vector (n=12), by jugular vein injection. Endostatin gene transfer resulted in high serum levels of endostatin. Strong adenoviral gene expression of β-galactosidase–expressing control vector was detected in liver tissue and was absent in the injured arterial wall at 1 week. Deposits of endostatin protein were detected along the denuded arterial wall and were not seen in the noninjured contralateral artery at 1 week. Endostatin deposits were also absent in the injured artery of control vector–treated animals. Overexpression of endostatin led to decreased reendothelialization and increased apoptosis of luminal endothelial cells 2 and 4 weeks after arterial injury (P<0.05). In addition, endostatin overexpression resulted in increased neointima formation (P<0.05). Endothelial apoptosis and neointima area correlated positively with endostatin serum levels, whereas the degree of reendothelialization correlated negatively with endostatin serum levels (P<0.05). Furthermore, poor reendothelialization correlated with increased neointima formation (P<0.05).

Conclusions—In summary, decreased reendothelialization and enhanced endothelial apoptosis, in response to endostatin overexpression, were associated with increased neointima formation. These findings demonstrate that high serum levels of endostatin are capable of inhibiting endothelial regeneration and promoting arterial lesion growth in conditions of endothelial injury. (Circulation. 2003;107:1658-1663.)

Key Words: gene therapy • endothelium • apoptosis • arteries • restenosis

The lack of selective modulators of endothelial cell function has been a major limitation for the investigation of the role of endothelial cells in arterial lesion formation. However, this has changed with the recent discovery of specific angiogenesis inhibitors such as endostatin, a C-terminal fragment of collagen type XVIII, which selectively inhibits proliferation and induces apoptosis of endothelial cells.1-5 Antiangiogenic therapy has been shown to inhibit tumor growth in several cancer models and also the progression of chronic hypercholesterolemia–induced arterial lesions in apolipoprotein E–deficient mice.5,6 Therefore, a potential rationale exists for the inhibition of activated endothelial cells to prevent neovascularization in patients with atherosclerosis.7,8 Conversely, various experimental models have demonstrated that the damage and removal of luminal endothelium results in arterial lesion formation with a severity proportional to the duration of endothelial denudation.9,10 Furthermore, mechanical endothelial injury has been reported to be a key feature in coronary arteries of patients after percutaneous coronary intervention (PCI) and, together with other forms of endothelial damage, has been implicated in arterial lesion formation in patients.11-13

To better understand the effects of endostatin on reendothelialization and neointima formation, we overexpressed the endostatin gene in a mouse model of arterial injury. In this model, luminal endothelium-denuding injury was performed without disruption of the internal elastic lamina, leading to luminal endothelial regrowth and concurrent neointima formation. Endostatin gene delivery was performed by jugular vein injection, resulting in endostatin overexpression in the

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liver, with high serum levels of secreted endostatin. We hypothesized that high serum levels of endostatin might inhibit reendothelialization and increase neointima formation.

**Methods**

**Adenovirus Construction and Protein Purification**

Total RNA was extracted from mouse liver. Endostatin cDNA was generated by reverse transcription–polymerase chain reaction using appropriate primers. Subsequently, murine endostatin was linked to a rat insulin leader sequence and cloned into a recombinant adenovirus (ADV) shuttle vector pADV.hEF1-α (human elongation factor 1α) for the rescue of the recombinant adenovirus (ADV-expressing murine endostatin, ADV.mEND) as described previously. Viral particles were measured by absorption (OD260), and plaque-forming units were determined by standard agarose overlay plaque assay on 293 cells. Soluble recombinant endostatin was generated by infection of breast cancer cells (MOD) with ADV.mEND at a multiplicity of infection of 100 pfu. After 48 hours, the serum-free supernatant was harvested and sequentially concentrated over Centrulipin spin columns (molecular weight cutoff, 50 000 Da, followed by 30 000 Da; Amicon Inc.).

**In Vitro Bioactivity Assay and In Vivo Expression of Endostatin**

A proliferation inhibition assay with endostatin protein, purified from ADV.mEND conditioned supernatant, was performed on human umbilical vein endothelial cells (HUVECs) as previously described. HUVEC proliferation was measured by use of a tetrazolium-based assay (EZ4U kit, Biomedica Inc.). Endostatin levels in serum of mice treated with ADV.mEND and ADV control vector (ADV.DL312) were measured with commercially available ELISA kits (Accucyte).

**Mice**

Male C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) and housed at the Center for Laboratory Animal Sciences at The Mount Sinai Medical Center, New York. Mice received standard rodent chow (Mouse diet 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 (Washington, DC: National Research Council; National Academy Press, 1996).

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

C57BL/6 mice underwent femoral arterial injury receiving either ADV.mEND (n=29) or ADV.DL312 (n=20). Mice were anesthetized with pentobarbital sodium (40 mg/kg IP) (Nembutal, Abbott Laboratories). Removal of the endothelium of the common femoral artery using a surgical microscope was achieved by 3 passages of a 0.25-mm angioplasty guidewire (Advanced Cardiovascular Systems). The protocol and the degree of injury applied to the vessel wall have been standardized, validated, and described in detail in previous studies. After arterial injury, a neck incision was made, and the jugular vein was exposed for injection of ADV.mEND (100 μL; 1×10¹¹ particles) or ADV.DL312 (100 μL; 1×10¹¹ particles) with a 30-gauge needle.

**Tissue Preparation, Histology, and Immunohistochemistry**

Animals were euthanized 1, 2, and 4 weeks after gene transfer and arterial injury and perfusion-fixed with 4% paraformaldehyde in PBS at 100 mm Hg for 10 minutes, and their hindlimbs were excised en bloc. Specimens were fixed overnight in 4% paraformaldehyde in PBS and decalcified in 10% formic acid. Two cross sections 2 mm thick were cut from each hindlimb at the level of injury in the common femoral artery and processed for paraffin embedding. Sequential sections (4 μm thick) were stained with hematoxylin-eosin. Immunohistochemical staining of adjacent sections was performed with von Willebrand factor (Dako; 1:1000), intercellular adhesion molecule-1 (Seikagaku; 1:300), active caspase-3 (R&D Systems; 1:150), β-galactosidase (3prime Corp, 1:100), and murine endostatin (R&D Systems, 1:100). Tissue sections were quenched with 3% hydrogen peroxide, blocked with 1% BSA in PBS, and incubated with the primary antibodies at 37°C for 2 hours. After washing in PBS, bound primary antibody was detected using an appropriate biotinylated secondary antibody for 15 minutes at 37°C. Sections were washed in PBS, reacted with horseradish peroxidase–conjugated streptavidin, developed with 3,3′-diaminobenzidine, and counterstained with hematoxylin. Negative controls were prepared by substitution of the primary antibody with the respective control IgG.

**Computer-Assisted Morphometry**

Histomorphometric evaluation of 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 (software: Image Pro Plus 3.0.1). Endothelial cell coverage of the luminal surface was assessed by ×400 microscopic examination of sections with nuclear hematoxylin counterstain and staining for von Willebrand factor and intercellular adhesion molecule-1 when both an endothelial cell nucleus and immunostaining were present. Luminal endothelial cell apoptosis was measured accordingly by use of active caspase-3 antigen detection. Neointimal area was assessed by hematoxylin-eosin and Masson’s trichrome staining. No significant interobserver or intraobserver variations were noted.

**Statistical Analysis**

For data analysis, the SPSS/PC+ software was used. Data are given as mean±SEM. After testing for normal distribution and equality of variances with Levene’s F test, the independent-sample t test was used to compare reendothelialization, expression of active caspase-3 in luminal endothelial cells, and neointimal area. Endostatin serum concentrations in mice were compared by the Mann-Whitney rank-sum test because of lack of normal distribution of this variable. Correlation coefficients were determined by Pearson’s test. Probability values were 2-tailed and corrected for ties. Probability values of P<0.05 were considered significant.

**Results**

**Biological Activity of Adenovirally Expressed Endostatin In Vitro**

An E1-deleted recombinant adenovirus was generated expressing a secreted form of endostatin under the control of the human elongation factor-1α (EF-1α) promoter (Figure 1A). The biological function of the recombinant endostatin protein, purified from serum-free supernatant of adenovirally transduced cells, was tested in a proliferation inhibition assay on basic fibroblast growth factor–stimulated HUVECs. A positive correlation between the endostatin concentration and the percent of proliferation inhibition of HUVECs was demonstrated. At the highest endostatin concentration (2000 ng/mL), HUVEC proliferation was inhibited up to 87.5% (Figure 1B).

**Adenovirus-Mediated Transgene Expression In Vivo and In Situ Detection of Endostatin**

Strong adenoviral transgene expression was demonstrated by β-galactosidase immunostaining only in the mouse liver but not at the site of arterial injury 1 week after jugular vein injection of β-galactosidase control vector and arterial injury (Figure 2).
Endostatin gene transfer by single intravenous injection of $10^{11}$ particles of ADV.mEND resulted in strongly increased endostatin serum concentrations compared with controls at 1 week ($10^{11}$ versus $10^9$ ng/mL; $P<0.01$) and at 4 weeks ($317$ versus $88$ ng/mL; $P<0.01$) (Figure 3).

A statistically significant increase in endogenous endostatin levels was observed when preinjury levels (24.1 ng/mL) were compared with endogenous levels in the control vector group at 1 week ($10^9$ versus $88$ ng/mL; $P<0.01$) and 4 weeks ($88$ ng/mL; $P<0.01$) after femoral arterial injury.

Endostatin levels before and after arterial injury without virus injection (n=5) were also examined to exclude a potentially confounding effect by the adenovirus itself. A significant increase of endogenous endostatin was also observed ($P<0.05$).

Strong endostatin protein immunostaining was observed along the denuded vascular surface at the time of high endostatin serum levels 1 week after ADV.mEND injection and arterial injury (Figure 4A). In contrast, noninjured contralateral arteries with intact endothelial coverage did not exhibit luminal deposition of endostatin (Figure 4B). Furthermore, endostatin protein deposition was not detected at the denuded surface of the injured arteries of control vector–treated animals at 1 week (Figure 4C). The elastic media stained weakly for endostatin in all specimens, as has been reported previously by Sasaki et al. Furthermore, hepatocellular endostatin staining indicating synthesis of endostatin protein by liver cells after adenovirus transduction is shown in Figure 4D.
Effects of Endostatin Overexpression on Reendothelialization and Apoptosis of Luminal Endothelial Cells

Morphometric analysis of von Willebrand factor immunostaining along the luminal surface of injured arteries showed significantly decreased reendothelialization of the denuded vascular surface with ADV.mEND treatment compared with control (2 weeks: 27±2% versus 54±4%, P<0.01; 4 weeks: 69±4% versus 84±4%, P<0.01) (Figures 5A and 6A). Interestingly, the degree of reendothelialization correlated inversely with endostatin serum levels of adenovirus-treated animals (r=−0.427; P<0.01).

A significant increase of luminal endothelial cell apoptosis, as indicated by expression of activated caspase-3 antigen, was observed in injured arteries of endostatin-overexpressing animals compared with controls (2 weeks: 23±6% versus 1±1%, P<0.01; 4 weeks: 12±3% versus 4±3%, P<0.01) (Figures 5B and 5C, and 6B). This finding is consistent with the inhibition of luminal endothelial regrowth by endostatin. Indeed, a significant positive correlation between the expression of active caspase-3 in luminal endothelial cells and the serum concentration of endostatin was observed (r=0.428; P<0.01).

Effects of Endostatin Overexpression on Neointima Formation

Morphometric analysis showed a substantial increase in neointima area of animals treated with ADV.mEND compared with ADV.DL312 treatment at both time points (2 weeks: 0.94±0.14×10^-2 versus 0.51±0.25×10^-2 mm^2, P=NS; 4 weeks: 1.16±0.14×10^-2 versus 0.75±0.08×10^-2 mm^2; P<0.05) (Figures 7 and 8). In fact, the neointima area of injured arteries correlated positively with endostatin serum concentration (r=0.519; P<0.01), and at 4 weeks, the degree of reendothelialization correlated inversely with neointima formation (r=−0.529, P<0.01).

With regard to neointima/media ratio, however, no statistical significance was found at 4 weeks (1.54±0.24 versus 1.14±0.15; P=NS), reflecting a concomitant increase in media size with endostatin treatment (0.92±0.08×10^-2 versus 0.77±0.07×10^-2 mm^2; P=NS). It is important to note that at this time point, no significant difference in total vessel area was observed between the endostatin and control vector–treated groups (4.66±0.48×10^-2 versus 4.66±0.28×10^-2 mm^2; P=NS). No histological signs of inflammation in the vessel wall were seen in the treatment groups at either 2 or 4 weeks. The neointima exhibited a predominantly fibrous and smooth muscle cell–rich phenotype. Likewise, no significant differences were observed between the control vector treatment group and injured arteries without treatment (natural history of arterial response to injury; data not shown).

Figure 5. Immunohistochemistry of luminal endothelium 2 weeks after arterial injury. Identification of endothelial cells. A, Regrowing endothelial cells present at luminal surface of femoral arteries at 2 weeks after arterial injury stained for von Willebrand factor (arrows); ×400. Detection of apoptosis: B, Endostatin treatment: clearly demarcated brown-stained endothelial cells (arrows) indicate apoptosis by expression of active caspase-3 protein; ×600. C, Control vector treatment: absence of active caspase-3 staining of endothelial cells (arrows); ×600. Ad, adventitia; Me, media; Lu, lumen. Magnification: A, ×400; B and C, ×600.

Figure 6. Comparison of reendothelialization and luminal endothelial cell apoptosis in endostatin treatment (ADV.mEND) and control (ADV.DL312) groups at 2 and 4 weeks after arterial injury and adenoviral injection. A, Difference between reendothelialization as percentage of luminal circumference of artery covered by endothelium (independent-sample t test). B, Proportion of active caspase-3–expressing endothelial cells in luminal endothelium (independent-sample t test).

Figure 7. Hematoxylin-eosin staining demonstrating effects of endostatin overexpression on neointima formation at 4 weeks after arterial injury. A, Distinct large neointima (Ni) (arrow) can be seen with endostatin vector treatment. As in all lesions analyzed in this study, internal elastic lamina is intact, as demonstrated by Masson’s trichrome staining of adjacent section (data not shown). B, Neointima of control vector–treated animals is considerably smaller than in A. Magnification ×200.
Discussion

In this study, we have shown that overexpression of the angiogenesis inhibitor endostatin can interfere with reendothelialization and endothelial cell integrity after arterial injury. Furthermore, mice overexpressing endostatin also exhibited an increased neointima formation on endothelial denudation.

The biological activity of murine endostatin from the supernatant of transduced cells was demonstrated in vitro (Figure 1). Endostatin gene transfer in vivo led to a strong and sustained transgene expression with endostatin secretion from liver resulting in high circulating levels of endostatin in mice. The endostatin serum concentrations were found to be significantly elevated in the ADV.mEND-treated animals compared with controls at 1 week (1011 ± 6 versus 109 ± 12 ng/mL; P < 0.01) and at 4 weeks (317 ± 35 versus 88 ± 35 ng/mL; P < 0.01) (Figure 3). Interestingly, injury alone, with or without control vector administration, also resulted in a small but significant increase in the basal endogenous endostatin levels at 1 week (24.1 ± 3 versus 109 ± 12 ng/mL; P < 0.01).

Deposition of endostatin was demonstrated in vivo at the denuded arterial segment and not at the site of intact luminal endothelium in the noninjured contralateral artery of the same endostatin-overexpressing animal (Figure 4). It is important to note that strong β-galactosidase expression was observed only in liver tissue and not in the injured arteries at 1 week after injection of β-galactosidase–expressing adenovirus and arterial injury (Figure 2). This excludes local arterial synthesis of endostatin protein and suggests that circulating endostatin is capable of adhering to exposed subendothelial structures. Endostatin could not be detected by immunostaining along the denuded endothelial surface of control vector–treated animals 1 week after injury.

Although a number of molecules expressed on activated endothelial cells have been proposed to interact with endostatin, none have been shown to be the “endostatin receptor.”15,16 In our model, endostatin, a cleavage product of the basement protein collagen XVIII, is deposited initially along the exposed internal elastic lamina after endothelial denudation, most likely through nonspecific adherence. There is no deposition of endostatin in areas with intact endothelium. The deposited endostatin at the site of endothelial denudation may have inhibited reendothelialization by interfering with regrowing endothelial cells.

Endostatin overexpression decreased luminal reendothelialization by 50% at 2 weeks and to a lesser, although still statistically significant, extent at 4 weeks after arterial injury compared with the control vector group (Figures 5A and 6A). Moreover, apoptosis of luminal endothelial cells was shown to be increased >10-fold at 2 weeks and 3-fold at 4 weeks compared with controls (Figures 5, B and C, and 6B).

Of note, endostatin serum levels correlated inversely with the degree of reendothelialization and positively with the amount of apoptotic luminal endothelial cells at 2 and 4 weeks. These data demonstrate that endostatin inhibited endothelial cell growth in a concentration-dependent manner not only in vitro but also in vivo, which is mediated, at least in part, by the induction of programmed cell death in regrowing luminal endothelial cells. Concurrent with the previously published reports, we detected no signs of apoptosis other than in luminal endothelial cells, making direct effects of endostatin on other intimal cells unlikely.2,5

The vascular endothelium lines the inner surface of blood vessels at the interface between circulating blood and the vascular wall and serves as an important autocrine and paracrine regulator of vascular wall cell biology.17,18 Acute, local removal of the inner vascular endothelial surface leads to a well-characterized response-to-injury reaction: regrowth of activated endothelium and accumulation of α-actin–positive intimal cells, resulting in an arterial lesion.19 Indeed, attenuated neointimal thickening has been reported in several studies in the rat carotid artery in which different strategies were used to improve endothelial cell function and to facilitate reendothelialization after balloon denudation injury (eg, vascular endothelial growth factor therapy; blockage of the angiogenesis inhibitor thrombospordin-1).20,21

Therefore, it was of particular interest to us to investigate whether the inhibition of reendothelialization and the increased apoptosis of luminal endothelial cells observed with high serum levels of endostatin were also associated with changes in neointima formation. Interestingly, high serum levels of endostatin increased neointima size in a dose-dependent manner starting at 2 weeks and resulting in a significantly larger neointima at 4 weeks compared with control vector treatment. This finding suggests a possible link between the integrity of the luminal endothelium and the degree of neointima formation. This observation is important because it shows that, under the condition of ongoing luminal endothelial repair, high systemic levels of an angiogenesis inhibitor promote, rather than inhibit, the progression of growing arterial lesions. This indicates that potential high-dose antiangiogenic therapy may interfere with acute endothelial repair in patients after PCI.

These observations were further corroborated by the fact that the neointima area showed a statistically significant negative correlation with the degree of reendothelialization, demonstrating that prolonged loss of endothelial integrity was indeed related to increased neointima formation. Further evidence for the luminal endothelium as a potential modula-
tor of neointima formation stemmed from our observation that the difference in luminal endothelial coverage and apoptosis between endostatin-treated and control-vector–treated arteries was greatest at 2 weeks after arterial injury, whereas no significant difference was present as yet in the neointimal area. At 4 weeks, together with falling levels of endostatin serum concentration, the gap in luminal endothelial coverage and apoptosis was closing; however, the differences in neointima area in endostatin-treated compared with control-vector–treated animals became statistically significant. These data strongly support the hypothesis that the increase in neointima formation associated with endostatin overexpression is not related to direct effects of endostatin but rather reflect the sequelae of the inhibition of luminal endothelial regeneration. Further studies are necessary, however, to prove a direct causality between endothelium-mediated effects and arterial lesion formation.

In conclusion, the effects of sustained endostatin overexpression on injured arteries in this model might characterize endostatin as a molecule that at high concentrations is capable of inducing a state of insufficient luminal endothelial regeneration associated with increased neointima formation. The biological importance of the small but significant increase of the endogenous endostatin levels after arterial injury warrants further investigation.

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