Angiogenesis Inhibitors Endostatin or TNP-470 Reduce Intimal Neovascularization and Plaque Growth in Apolipoprotein E–Deficient Mice

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Background—Neovascularization within the intima of human atherosclerotic lesions is well described, but its role in the progression of atherosclerosis is unknown. In this report, we first demonstrate that intimal vessels occur in advanced lesions of apolipoprotein E–deficient (apoE \(^{−/−} \)) mice. To test the hypothesis that intimal vessels promote atherosclerosis, we investigated the effect of angiogenesis inhibitors on plaque growth in apoE \(^{−/−} \) mice.

Methods and Results—ApoE \(^{−/−} \) mice were fed a 0.15% cholesterol diet. At age 20 weeks, mice were divided into 3 groups and treated for 16 weeks as follows: group 1, recombinant mouse endostatin, 20 mg \( \cdot \) kg \( ^{−1} \) \( \cdot \) d \( ^{−1} \); group 2, fumagillin analogue TNP-470, 30 mg/kg every other day; and group 3, control animals that received a similar volume of buffer. Average cholesterol levels were similar in all groups. Plaque areas were quantified at the aortic origin. Median plaque area before treatment was 0.250 mm\(^2\) (range, 0.170 to 0.348; \( n = 10 \)). Median plaque areas were 0.321 (0.238 to 0.412; \( n = 10 \)), 0.402 (0.248 to 0.533; \( n = 15 \)), and 0.751 mm\(^2\) (0.503 to 0.838; \( n = 12 \)) for the endostatin, TNP-470, and control groups, respectively (\( P \leq 0.0001 \)). Therefore, endostatin and TNP-470 inhibited plaque growth during the treatment period by 85% and 70%. Intimal smooth muscle cell contents of plaques from control and treated mice were similar.

Conclusions—Prolonged treatment with either angiogenesis inhibitor reduced plaque growth and intimal neovascularization in apoE \(^{−/−} \) mice. Although the mechanism of plaque inhibition induced by these agents is not established, these results suggest that intimal neovascularization may promote plaque development.

Key Words: angiogenesis ■ atherosclerosis ■ apolipoproteins

In normal vessels, the microvascular network of vasa vasorum is confined to the adventitia and outer media. In vessels with atherosclerotic involvement, these networks become more abundant and extend into the intima of atherosclerotic lesions.\(^1,2\) Casting studies and confocal microscopy have shown that intimal vessels mostly branch from the native adventitial vasa vasorum.\(^3\) The proliferation rates of endothelial cells in plaque vessels range from undetectable to 43%, which indicates these vessels are found in various stages of development.\(^4\)

Plaque vessels are often found in areas rich in macrophages, T cells, and mast cells—cell types that can activate angiogenesis.\(^5−7\) Their close proximity to inflammatory infiltrates and the expression of adhesion molecules (such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin) on the endothelium of plaque vessels both suggest that these vessels may recruit inflammatory cells into lesions and initiate a positive-feedback mechanism.\(^8\) It is also conceivable that the supply of oxygen and nutrients provided via plaque vessels is a precondition for growth beyond a certain stage, after which diffusion from the artery lumen is insufficient to meet the metabolic demands of the plaque.

The clinical importance of plaque neovascularization is suggested by studies that show a higher prevalence of neovascularization in lesions with plaque rupture, mural hemorrhage, or unstable angina.\(^9,10\) Angiogenesis occurs in association with remodeling and protease activation in the surrounding tissues.\(^11,12\) Therefore, factors that stimulate plaque angiogenesis could also contribute to activities that promote plaque disruption, the event often responsible for myocardial infarction and ischemic stroke.

To test the hypothesis that intimal neovascularization promotes the progression of atherosclerosis, we investigated
whether treatments with potent angiogenesis inhibitors reduce plaque growth in an animal model of the disease. The limited availability of such agents has previously impeded the experimental verification of this hypothesis in significant numbers of animals.

The fumagillin analogue TNP-470 is a selective inhibitor of endothelial cell proliferation and migration and is under investigation in clinical trials as an anticancer agent. TNP-470 inhibits primary tumor growth and demonstrates 70% inhibition of angiogenesis in the corneal microcapillary assay. Endostatin, a C-terminal 20-kDa fragment of the basement membrane protein collagen XVIII, is a potent inhibitor of primary tumor growth and endothelial cell proliferation and migration.

In this report, we first demonstrate that intimal capillaries occur in advanced lesions of apolipoprotein E–deficient (apoE–/–) mice by immunohistochemistry with antibodies against CD31 and von Willebrand factor (vWF) and by transmission electron microscopy (TEM). We then show that long-term treatment with recombinant murine endostatin or TNP-470 significantly reduces the further growth of atherosclerosis without affecting cholesterol levels.

Methods

Animals and Experimental Design

Male apoE–/– mice (Jackson Labs, Bangor, Me) were fed a 0.15% cholesterol diet (Western type No. 88137, Teklad) from age 6 to 8 weeks. At 20 weeks of age, 10 animals were killed to evaluate the baseline extent of atherosclerosis. The remaining littermates were divided into 3 groups and treated for 16 weeks as follows: group 1 was treated with recombinant murine endostatin 20 mg·kg–1·d–1 by subcutaneous injection, a dose shown to produce >95% inhibition of primary tumor growth in mice14; group 2 was treated with the fumagillin analogue TNP-470 at 30 mg/kg SC every other day; and group 3 consisted of control animals that received a similar volume of saline (TNP-470 buffer) or PBS (endostatin buffer).

After 16 weeks of treatment, animals were euthanized with methoxyflurane. A blood sample was obtained from the right ventricle for the analysis of serum cholesterol, which was performed by an automated colorimetric assay on a Hitachi 917 instrument. The heart and aorta were perfused with 2% paraformaldehyde and dissected as described previously.16,17 Atherosclerotic plaques in the aortas were used for histology, and plaque areas were only determined at the aortic origin. However, to document the extent of disease in the total aorta, the unopened aortas were photographed.

The effects of these angiogenesis inhibitors on late-stage lesions were then compared with their effects on early lesions, in which intimal vessels were rarely observed. Animals were treated with endostatin or TNP-470 from age 32 to 48 weeks (late-stage experiment) or from 6 to 22 weeks (early-stage experiment). Plaque involvement was measured at the aortic origin. Because the aortas from early-stage animals were not used for immunohistochemistry in this experiment, the extent of atherosclerosis was also measured in the entire aorta (percent surface area of Sudan IV–stained lesions).17

Animal health and weight was monitored throughout treatment. The percentages of animals available for analysis in all studies were 92% (range, 86% to 100%), 89% (80% to 94%), and 91% (86% to 100%) for the endostatin, TNP-470, and control groups, respectively. Chronic dermatitis and dental malocclusion were the most common reasons for exclusion.

Immunohistochemistry

To detect intimal capillaries, the hearts and portions of the descending aorta with substantial lesions were embedded in paraffin, sectioned (10 μm), digested with 0.02% protease XXIV (Sigma) for 6 minutes at room temperature, and incubated with rabbit polyclonal anti-human vWF antibody (Dako; 1:500 dilution) or rat monoclonal anti-mouse CD31 antibody (Pharmigen; 20 μg/mL). When acetone-fixed frozen sections were stained, the CD31 antibody was used at 5 μg/mL. The bound antibodies against vWF or CD31 were detected with biotinylated goat anti-rabbit or rabbit anti-mouse antibodies (Vector; 1:250 dilution) and the avidin-biotin peroxidase complex (ABC standard kit, Vector). A red reaction product was produced with 3-amin-9-ethyl carbazole substrate (AEC; Dako), and sections were counterstained with Gill’s hematoxylin (Sigma). Vascular sections incubated with nonimmune serum served as negative controls. Positive staining of the endothelium on the lumen and in adventitial capillaries served as an internal control. Intimal vessels were identified under high power (×400) and counted when both an endothelial cell nucleus and lumen were seen and when the vessel was also observed in an adjacent section.

Smooth muscle cells were identified with a monoclonal IgG2a antibody against human smooth muscle cell α-actin (Dako, M0851). Sections were immersed in 0.1 mol/L sodium citrate, pH 6.0, and heated for 10 minutes in the microwave. Primary antibody was bound to sections at 3 μg/mL and then visualized with biotinylated mouse IgG2a antibody (1:200 dilution, Amersham PN1181) and the ABC method described above. The adjacent medial layer of vessels served as positive control.

Transmission Electron Microscopy

Aortas with atherosclerotic lesions were isolated from retired breeder apoE–/– mice that were fed the Western diet for 20 weeks. Extensive lesions in the descending aorta were divided transversely into 2 segments. One segment was frozen and cryosectioned for CD31 immunohistochemistry; the other was processed for TEM. Only lesions that screened positive for intimal vessels were analyzed on an electron microscope (EM 10, Zeiss).

Recombinant Murine Endostatin and TNP-470 Treatments

Recombinant murine endostatin was prepared as described with the expression plasmid TB01#8 transformed into the Escherichia coli strain BL21-DE3.14,15 Induction results in a fusion protein with the amino acid sequence MARRASVGTDHHHHH at the N-terminus followed by the sequence of endostatin, which corresponds to the C-terminus, 184 amino acids of mouse collagen XVIII. Murine endostatin was purified under denaturing conditions on an Ni2+-NTA column (Qiaper II Expressionist Handbook, Qiagen). Purity was analyzed by SDS-PAGE. Before use in apoE–/– mice, endostatin batches were tested for inhibition of Lewis lung carcinoma growth in C57Bl/6J mice.14

The apoE–/– mice received endostatin 20 mg/kg SC every day. Animals tolerated the injections and showed regular weight gain, activity levels, and no ulceration at the injection sites.

The fumagillin analogue TNP-470 was donated for these studies by TAP Holdings, Deerfield, Ill. An injection solution of TNP-470 (3 mg/mL) was freshly prepared and mice received 30 mg/kg SC every other day. Control animals received a similar volume of saline or endostatin buffer.

Plaque Morphometry

Plaque Area

To determine the extent of atherosclerosis at the aortic origin, 40 serial sections (8 to 10 μm thick) of the aortic sinuses were collected on 10 slides.18 Every second slide was stained with hematoxylin and eosin for morphometry. The rest were used for immunohistochemistry to count intimal vessels and determine intimal smooth muscle cell contents. Plaque images were captured at ×100 magnification with a Hitachi HV-C20 3CCD digital camera and measured with the Leica Q500 MC image-analysis program.20 Lesion area for each animal was reported as the mean intimal cross-sectional area (in mm2). In the early-stage experiment, plaque involvement in the entire aorta was also measured by image analysis as the percentage of aortic surface area covered with lesions.17,20
Plaque Cell Density
Cell densities in aortic sinus plaques were evaluated to determine whether endostatin or TNP-470 treatments affected lesion cellularity. Cell nuclei in the intima of the aortic sinus plaques were counted at 3 midlesion levels (slides 4, 6, and 8). Cell density was reported as the number of cells per intimal area (cells/mm²).

Intimal Smooth Muscle Cell Content
To determine whether angiogenesis inhibitor treatments affected smooth muscle cell migration or proliferation in lesions, 2 slides from the middle of the aortic sinus were stained for smooth muscle cell α-actin. For each animal, the numbers of intimal smooth muscle cells and total cell nuclei were counted in 8 nonoverlapping fields. A smooth muscle cell was counted only if it stained for smooth muscle cell α-actin and its cell nucleus was seen. The mean percentage of smooth muscle cells relative to total cells was determined for each animal.

Statistical Analysis
The inhibitory effects on median plaque growth induced by treatment with TNP-470 and endostatin were compared with the Kruskal-Wallis test. Data presented in Tables 1 and 2 were evaluated with the Fisher exact test. Intimal cell density and smooth muscle cell content of lesions from animals in treatment and control groups were compared by nonparametric ANOVA analysis with adjustment for multiple measurements from each animal (Table 3).

Results
ApoE −/− Mice Have Intimal Neovascularization
Advanced atherosclerotic lesions of apoE −/− mice were first examined for the presence of intimal capillaries. Intimal vessels were identified by immunohistochemistry with antibodies against the endothelial cell markers CD31 (Figure 1, A through D) or vWF (Figure 1, E and F). Thin-walled, capillary-like vessels were observed in lesions harvested from the descending aorta (Figure 1, A through E) and the aortic sinus (Figure 1F). Intimal neovascularization was also observed when advanced atherosclerotic lesions were examined by TEM (Figure 2A). Increased numbers of capillaries (Figure 2B) and endothelial cells with signs of early lumen formation (Figure 2C) were seen in the adventitia adjacent to atherosclerotic lesions.

The incidence of lesions with intimal vessels was generally low in apoE −/− mice but increased in more extensive lesions. No vessels were observed in fatty streak lesions and only a few in lesions <250 μm thick. Intimal vessels were detected in 15 (13%) of 114 advanced aortic lesions from cholesterol-fed apoE −/− mice aged 36 to 60 weeks (Table 1). The majority of plaques that contained intimal vessels (13 of 15; 87%) were >250 μm thick. The incidence of intimal vessels in this group was increased 9-fold compared with 100- to 250-μm-thick lesions (P<0.0005).

Plaque Growth Is Reduced by Long-Term Treatment With Endostatin or TNP-470
To demonstrate a role of plaque vessels in the progression of atherosclerosis, apoE −/− mice were treated with an angiogenesis inhibitor, either endostatin or TNP-470, for a prolonged period. A prolonged treatment schedule was chosen because of uncertainty about the onset of neovascularization in murine lesions. On the basis of our initial survey of mice younger than 20 weeks, plaques thicker than 250 μm were...
infrequent. We therefore induced substantial atherosclerosis by feeding apoE \(^{-/-}\) mice a diet that contained 0.15% cholesterol, and at 20 weeks, we initiated treatments for 16 weeks.

The median aortic origin plaque area in 10 mice killed at baseline was 0.250 mm\(^2\) (range, 0.170 to 0.348 mm\(^2\); \(n=10\)), represented by the dashed line in Figure 3. The remaining 3 groups of animals were treated with endostatin, TNP-470, or buffer as described in Methods. The median plaque areas were 0.321 (0.238 to 0.412; \(n=10\)), 0.402 (0.248 to 0.533; \(n=15\)), and 0.751 mm\(^2\) (0.503 to 0.838; \(n=12\)) for the endostatin, TNP-470, and control groups, respectively \((P<0.0001)\). Despite the considerable variability in plaque areas, there was little overlap between the treatment and control groups (Figure 3). Because the extent of lesions before treatment was 0.250 mm\(^2\), long-term treatment with endostatin or TNP-470 appeared to inhibit plaque growth by 85% and 70%, respectively. Percent inhibition was calculated by the formula \(100\times(\text{median plaque area treated} - 0.25)/\text{median plaque area control} - 0.25)\).

The cholesterol levels in treatment and reference groups were not different (Figure 4). Median cholesterol levels at the time the animals were killed were 877 (range, 542 to 1045; \(n=10\)), 823 (461 to 1164; \(n=15\)), and 918 mg/dL (558 to 1366; \(n=12\)) in the endostatin, TNP-470, and control groups, respectively \((P=0.62)\). In a subset of animals, cholesterol levels were similar before and after 4 weeks of treatment.

Median weights after treatment were 36 (31 to 37), 31 (24 to 34), and 35 g (26 to 49) for the endostatin, TNP-470, and control groups, respectively. All animals increased weight during treatment, but TNP-470–treated animals gained only 4 g \((P<0.001)\) compared with 8 g for control and 9 g for endostatin-treated animals.

Significant inhibitions of plaque growth by endostatin and TNP-470 were also observed in a second experiment that treated mice from ages 20 to 36 weeks. Median plaque areas were 0.475 (0.308 to 0.598; \(n=12\)), 0.370 (0.181 to 0.521; \(n=12\)), and 0.690 mm\(^2\) (0.591 to 0.720; \(n=12\)) for the animals in the endostatin, TNP-470, and control groups, respectively \((P<0.001)\).

<table>
<thead>
<tr>
<th>TABLE 1. Intimal Vessels in Advanced Lesions</th>
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<tr>
<td>Maximum Intimal Depth</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>&gt;250 (\mu m)</td>
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<tr>
<td>100–250 (\mu m)</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
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\(P<0.0005\), Fisher’s exact test.

Figure 3. Size of lesions at aortic origin after treatment with angiogenesis inhibitors in mice aged 20 to 36 weeks. Control (○), endostatin (△), and TNP-470 (□) animals were treated for 16 weeks as described in Methods. Dashed line centered at 0.25 mm\(^2\) represents median plaque area of aortic sinus lesions measured in a cohort \((n=10)\) analyzed at 20 weeks.
For these experiments, plaque measurement at the aortic origin was selected to detect a change in plaque thickness rather than surface area. However, photographed images of the entire aorta, taken before the aortas were dissected for histology, also demonstrated a noticeable reduction of plaque surface area in endostatin or TNP-470–treated animals (not shown).

**Inhibition of Plaques by Endostatin or TNP-470 Is Less Prominent in Early Lesions**

It was then evaluated whether endostatin and TNP-470 were as effective in animals with predominantly advanced lesions as in animals with predominantly early lesions. In this experiment, cholesterol-fedapoE 
\( ^{-/-} \) mice were treated with endostatin or TNP-470 from age 32 to 48 weeks. Plaque areas at the aortic origin were determined after the same 16-week treatment period (Figure 5). Median plaque areas for the endostatin, TNP-470, and control groups were 0.422 (0.283 to 0.637; \( n = 12 \)), 0.448 (0.260 to 0.566; \( n = 14 \)), and 0.584 mm\(^2\) (0.426 to 0.911; \( n = 13 \)), respectively. The inhibitions of plaque growth were smaller than in the previous experiments but still significant (\( P = 0.002 \)). Again, cholesterol levels were similar in all groups (\( P = 0.13 \)). Final weights were 39 (32 to 50), 33 (25 to 37), and 35 g (32 to 44) for the endostatin, TNP-470, and control mice, respectively (first level test \( P \leq 0.0001 \); TNP-470 versus control, \( P = 0.003 \); endostatin versus control, \( P = 0.04 \), not significant by adjusted probability value 0.017).

The effect of endostatin or TNP-470 treatment was then evaluated in mice that had primarily early lesions without significant intimal neovascularization. ApoE 
\( ^{-/-} \) mice were started on the diet at 6 weeks and divided into endostatin, TNP-470, and control groups. After treatment for 16 weeks, lesions in the entire aorta were not very extensive, and no significant difference was seen between treatment and control groups (Figure 6; \( P = 0.88 \)). Median percent plaque areas were 9.6% (6.2% to 16.4%; \( n = 10 \)), 11.7% (4.4% to 16.6%; \( n = 8 \)), and 14.4% (5.4% to 16.1%; \( n = 12 \)) for the endostatin, TNP-470, and control animals, respectively. Plaque measurements at the aortic origin also showed no difference (not shown). Therefore, the inhibitory effects of endostatin and TNP-470 were less pronounced during early atherogenesis.

**Incidence of Intimal Vessels in Endostatin-or TNP-470–Treated Lesions Is Reduced**

Aortic sinus plaques isolated from control and treated animals were examined for the presence of intimal vessels. The percentage of plaques that contained any intimal vessels was significantly smaller in treated mice (5% for endostatin, \( P = 0.032 \); 0% for TNP-470, \( P = 0.003 \)) than in controls (29%) (Table 2).

**Smooth Muscle Cell Content and Cellular Density Of Lesions**

The magnitude of plaque inhibition observed with endostatin or TNP-470 raised the possibility that these agents not only

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aortic Sinus Plaque, n</th>
<th>Neovascularization (+)*</th>
<th>( P )†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>7 (29%)</td>
<td></td>
</tr>
<tr>
<td>Endostatin</td>
<td>22</td>
<td>1 (5%)</td>
<td>0.032</td>
</tr>
<tr>
<td>TNP-470</td>
<td>27</td>
<td>0</td>
<td>0.003</td>
</tr>
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*Plaques with \( \geq 1 \) intimal capillary were counted positive (see Methods).
†Fisher’s exact test.
affect plaque angiogenesis but may also affect other cell types found within atherosclerotic lesions. To evaluate whether TNP-470 and endostatin influence smooth muscle cell migration and proliferation in atherosclerotic lesions, the smooth muscle cell contents of lesions from treatment and control mice were compared (Table 3). The median smooth muscle cell contents of aortic sinus lesions from control mice were 20.4% and were similar for endostatin-treated (19.4%; P=0.20) and TNP-470–treated mice (19.8%; P=0.55).

The cellular density of atherosclerotic lesions from treated and control mice were determined at 3 levels of the aortic sinus, as described in Methods. The calculated median cell densities were similar between the control and TNP-470 groups but were increased in the endostatin group (1645 cells/mm²; P=0.0005).

**Discussion**

The present study demonstrates for the first time that atherosclerotic lesions in the aorta of apoE−/− mice contain intimal vessels, as do human lesions. This observation is remarkable because the absolute dimensions of murine lesions are much smaller. The incidence of intimal vessels in advanced murine lesions was 13%, whereas the incidence in human lesions has been reported at 40% to 53%. Prior studies showed infrequent vasa vasorum in normal mouse aortas. Comparisons of the lamellar structure across many species suggested that a minimal vessel wall thickness was required for the presence of vasa vasorum. Intravital studies of tissue oxygenation in tumors demonstrated that significant hypoxia and acidosis occur when the distance from a capillary exceeds 100 μm. Given the thin media of the murine aorta, only relatively large lesions may exceed the limit at which growing plaques require additional sources of perfusion beyond the artery lumen and adventitial vessels.

Our results are consistent with this assumption. However, although the intimal thickness of many plaques from apoE−/− mice exceeded 100 μm, only 13% of these lesions showed intimal neovascularization. It is possible that the metabolic requirements of plaque tissues depend on lesion composition and differ from tumors. In addition, the contribution from adventitial vessels was not measured.

The vessel density in vascularized plaques ranged from 1 to 17 capillaries per high-power field, but the vessel number in these lesions did not correlate with intimal thickness. For example, 1 plaque (Figure 1A) had >30 vessels and a maximal intimal thickness of 312 μm, whereas a 486-μm-thick lesion (not shown) had 8 to 10 capillaries. It is likely that factors other than size, such as cell density, leukocyte infiltrates, and matrix composition, also influence the development of plaque vessels. Despite this lack of a linear correlation, plaque size may be an indicator of the presence of intimal vessels, because their incidence in lesions >250 μm was increased 9-fold compared with smaller lesions.

The observation of intimal vessels in lesions from apoE−/− mice provided the opportunity to test whether potent angiogenesis inhibitors affect the progression of atherosclerosis in these animals. The results of 2 separate experiments conducted on animals treated from ages 20 to 36 weeks showed significant inhibition of atherosclerosis with no effect on cholesterol levels (Figure 3). Compared with animals killed at baseline, endostatin and TNP-470 treatments inhibited atherosclerosis by 85% and 70%, respectively. In the second experiment, either treatment significantly inhibited plaque growth, but the degree of inhibition by endostatin was less than that by TNP-470.

Significant inhibition of plaque growth by endostatin or TNP-470 was seen even when the treatment was delayed until 32 weeks, although the degree of inhibition was smaller (Figure 5). One potential explanation for the smaller inhibition could be that the plaque growth rate from age 20 to 36 weeks is different than that from age 32 to 48 weeks. Second, both endostatin and TNP-470 are reversible inhibitors of endothelial cell proliferation and appear to exert few effects on quiescent nonproliferating endothelium. Therefore, the effects of these inhibitors on plaques with established intimal vessels might be different than those on plaques that develop intimal neovascularization during the treatment period.

Interestingly, we saw little effect when treatments were performed during early stages of atherosclerosis (Figure 6). In apoE−/− mice fed the Western diet, fatty streaks are typically seen from 8 to 20 weeks, and plaques with smooth muscle cells are initially observed at 15 weeks. Our results therefore suggest that neither endostatin nor TNP-470 significantly affected foam cell and early fibromuscular lesions.

When lesions from endostatin- or TNP-470–treated animals were examined, few intimal vessels were observed. However, this correlation does not prove that inhibition of plaque growth occurred because intimal neovascularization was decreased. The reduced neovascularization could merely be a consequence of reduced plaque size rather than an effect of angiogenesis inhibitors and the cause of reduced atherosclerosis.

The process of atherosclerosis involves multiple factors that control inflammation, cell proliferation and migration, cholesterol metabolism, and interactions between cells, blood, and matrix. It is possible these agents altered the functional characteristics of the endothelium that influence leukocyte adhesion, transmigration, or activation. TNP-470 has been shown to enhance E-selectin expression, but this would not be predicted to inhibit lesion development. Smooth muscle cell proliferation and migration are inhibited by TNP-470 in vitro, but this requires doses that are 30- to 70-fold greater than the doses for endothelial cells. Despite these in vitro findings, no significant differences in the smooth muscle cell contents of lesions from treated and control animals were observed in the present study.
Angiogenesis Inhibitors Reduce Atherosclerosis

Currently, only limited information on the effects of TNP-470 and endostatin is available. TNP-470 regulates cyclin activity in endothelial cells and forms a covalent bond to methionine aminopeptidase-2, a cobalt-dependent metalloprotease. Additional investigations of the mechanisms of action for TNP-470 and endostatin may therefore provide insights toward understanding their effects on plaque growth.

Although the present study cannot provide conclusive evidence for causality, the combined findings provide strong support for the hypothesis that intimal vessels contribute to the progression of atherosclerosis. First, similar inhibition of atherosclerosis was observed with 2 very different agents that share a potent inhibitory effect on endothelial cell proliferation. Second, the inhibition of plaque growth by these agents was associated with a decreased incidence of intimal neovascularization. Finally, these inhibitors showed little effect during early stages of plaque development, when intimal neovascularization was unlikely to occur. The endothelium of plaque vessels may be qualitatively different from the arterial endothelium that covers the plaque. In future studies, characterization of these potential differences may distinguish pathways for leukocyte exchange in the plaque that are selective for the plaque microvasculature.

If studies in other models confirm our present observations, new treatments directed at intimal vessels might be considered to augment established interventions that reduce atherosclerosis. Clinical trials of angiogenesis inhibitors for the treatment of tumors, macular degeneration, and other diseases characterized by neovascularization may provide opportunities to evaluate the effects of these agents on concurrent atherosclerosis. Finally, the recent demonstrations of therapeutically targeted angiogenesis in ischemic myocardium and peripheral limbs raise questions as to whether endothelial cell growth factors will promote plaque angiogenesis, growth, or vulnerability. Exogenous endothelial cell growth factors might not induce such effects because these factors are already produced in plaque tissue.

Acknowledgments

Studies were supported by a Physician Scientist Award (K11 HL-02563) from the National Heart, Lung, and Blood Institute and the Harvard Medical School 50th Anniversary Program (K.S.M.). We thank Thomas Boehm for advice on endostatin preparation, Dipak Panigrahy for testing endostatin preparations, Allison Brown for assistance with image analysis, Kerstin Bahr for TEM, and Elizabeth Allred for statistical analyses. We are grateful to Peter Libby and Joyce Bischoff for helpful discussions and review of this manuscript.

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_Circulation_. 1999;99:1726-1732
doi: 10.1161/01.CIR.99.13.1726

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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