Angiogenesis Is Impaired by Hypercholesterolemia
Role of Asymmetric Dimethylarginine

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Background—Many angiogenic factors require endothelium-derived nitric oxide (NO) to exert their effects. Recently, an endogenous competitive antagonist of NO synthase has been characterized: asymmetric dimethylarginine (ADMA). Elevated plasma levels of ADMA reduce NO synthesis in hypercholesterolemia. Accordingly, we hypothesized that hypercholesterolemia impairs angiogenesis by an ADMA-dependent mechanism.

Methods and Results—Angiogenesis was assessed with the use of a disk angiogenesis system implanted subcutaneously in normal (E⁺) mice or apolipoprotein (apo)E-deficient hypercholesterolemic (E⁻) mice. After 2 weeks, the disks were removed, and the fibrovascular growth area was used as an index of angiogenesis. Basal and fibroblast growth factor–stimulated angiogenesis was impaired in E⁻ mice, associated with an elevation in plasma ADMA. Oral administration of L-arginine reversed the impairment of angiogenesis in E⁻ mice. By contrast, oral administration of L-nitroarginine (an exogenous antagonist of NO synthase) reduced angiogenesis. When added directly to the disk, ADMA dose-dependently inhibited basal and fibroblast growth factor–induced angiogenesis, an effect that was reversed by oral administration of L-arginine.

Conclusions—The derangement of the NO synthase pathway that occurs in hypercholesterolemia is associated with an impairment of angiogenesis. The lipid-induced impairment of angiogenesis can be reversed by oral administration of L-arginine and can be mimicked in normocholesterolemic animals by administration of an NO synthase antagonist. The data are consistent with the hypothesis that ADMA is an endogenous inhibitor of angiogenesis. (Circulation. 2000;102:1414-1419.)

Key Words: angiogenesis ■ nitric oxide ■ hypercholesterolemia

Endothelium-derived nitric oxide (NO) plays an important role in the actions of angiogenic factors. A number of angiogenic factors upregulate the expression of endothelial NO synthase (eNOS) and stimulate the release of endothelial NO. Vascular endothelial growth factor (VEGF) stimulates the release of NO from cultured human umbilical venous endothelial cells and upregulates the expression of eNOS. Vascular segments of rabbit thoracic aorta release NO in response to VEGF; preincubation with L-arginine increases basal and VEGF-stimulated NO release by 2-fold. Similar observations have been made when the angiogenic stimulus was transforming growth factor-β or basic fibroblast growth factor (bFGF). In the rabbit cornea model of angiogenesis, VEGF-induced angiogenesis is blocked by L-NAME.

An endogenous antagonist to NOS has recently been described. Asymmetric dimethylarginine (ADMA) is an arginine analogue that competes with L-arginine for NO synthase. The competitive inhibition of NOS by ADMA can be reversed by supplemental L-arginine. Because NO appears to play a role in angiogenesis and because in certain disorders (such as hypercholesterolemia) plasma ADMA is sufficiently elevated to interfere with NO synthesis, we hypothesized that ADMA may be an endogenous antiangiogenic factor. Accordingly, the current study was designed to determine if the elevation of plasma ADMA in hypercholesterolemia mice impairs angiogenesis.

Methods

Animals Twenty-four- to 26-week-old female, wild-type (E⁺; n=10) and apolipoprotein (apo)E-deficient hypercholesterolemic (E⁻; n=10) C57BL/6J mice were used to examine the systemic effects of
Eight- to 10-week-old female wild-type C57BL/6J (n = 40) mice were used to examine the local effects of ADMA. The E mice were produced and maintained as previously described. Preparation of the Disk

The disk angiogenesis system is a disk (11 mm in diameter and 1 mm in thickness) made of a polyvinyl alcohol sponge (Kanebo PVA, Rippey Co). Nitrocellulose cell-impermeable filters (Millipore) are affixed to each side of the sponge with Millipore glue No. 1 (xx70000.00, Millipore). As a result, cells (and thus vessels) penetrate or exit only through the rim of the disk. To study the direct effect of an angiogenic or an antagonist substance, these agents are added directly to the disk. A 1.5-mm core (pellet) is cut from the disk center. Both the pellets and disks are sterilized before assembly. The pellet is loaded with 20 mL of vehicle (PBS, Sigma, Chemical Co) or 20 mL of the desired solution of drug and air-dried. To determine the local effects of ADMA, 40 mg or 400 mg of ADMA (Sigma) was dissolved in 20 mL PBS. bFGF (Scios) (20 mg) was diluted into 20-L solutions. The pellet is coated with ethylene-vinyl acetate copolymer (Elvax, Dupont, Chemcentral Corp) to provide for slow release of the solution from the pellet into the disk. The pellet is inserted into the disk before sealing the disk with the Millipore filters. In this study, some animals also received oral l-arginine (LA, 6 g/100 mL, Sigma) or l-nitro-arginine (LNA, 6 mg/100 mL, Sigma) in their drinking water. Implantation of the Disk

The mice were anesthetized with 4% chloral hydrate (intraperitoneal administration, 0.1 mL/10 g body wt). The flanks and posterior surface of the thorax were shaved and cleaned with 70% isopropyl alcohol. A 2-cm incision was made in the flank, and blunt dissection through the subcutaneous tissue produced a channel into which the PBS-moistened disk was inserted. The skin was closed with 5-0 silk suture. Experimental Protocol

The following studies were performed to determine the effect of hypercholesterolemia on angiogenesis and the role of ADMA in the lipid-induced impairment of angiogenesis. Specifically, the following hypotheses were tested: (1) hypercholesterolemia impairs basal and stimulated angiogenesis; (2) these effects of hypercholesterolemia can be reversed by LA; and (3) these effects of hypercholesterolemia can be mimicked in normal animals by treating them systemically or locally with an inhibitor of NOS. Disks containing vehicle were implanted into E mice and E mice of the following 4 groups: E (n = 5); E (n = 5); E + bFGF (20 mg/100 mL drinking water, n = 5); and E + LNA (6 mg/100 mL drinking water, n = 5). Subsequently, 8- to 10-week-old E mice were separated into 8 groups. Disk angiogenesis systems were implanted subcutaneously with disk pellets containing vehicle (PBS, n = 5); ADMA (40 mg, n = 5); ADMA (400 mg, n = 5); bFGF (20 mg, n = 5); or bFGF (20 mg) + ADMA (400 mg, n = 5). In another set of mice, disks were implanted containing vehicle (n = 5), ADMA (400 mg, n = 5), or bFGF (20 mg) + ADMA (400 mg, n = 5); and in this set of mice, LA (6 g/100 mL) was placed in the drinking water throughout the duration of the study.

Disk Removal and Preparation

Two weeks after disk implantation, the mice were given an overdose of 4% chloral hydrate and cervical dislocation. The disk was removed through an incision in the skin at the implantation site. Attached tissue was detached from the disk, and one filter was separated from the disk. The disks were then fixed in 10% formalin and embedded in paraffin. Subsequently, 5-μm sections were made in a plane through the center of the disk and parallel to the disk surface.

Quantification of Results

Implantation of the disk causes the ingrowth of fibrovascular tissue (also known as granulation tissue). Previous studies have demonstrated that the area occupied by fibrovascular growth is directly proportional to the total area of the disk occupied by blood vessels, at a given stage of growth. Therefore, the measurement of such total area was used as an index of angiogenesis. To study the histological characteristics of the fibrovascular growth area, the disk sections were stained with hematoxylin and eosin for light microscopy. For quantification of the total area of fibrovascular growth, the sections were stained with toluidine blue. The fibrovascular tissue stains deeply with toluidine blue and on gross inspection appears purple-black in color, whereas the disk matrix appears gray. With the use of a videomicroscope and a computer-assisted digital image analysis system (NIH Image 1.59b9), the entire area of the matrix supporting fibrovascular growth (fibrovascular growth area) in the toluidine-stained section was calculated (expressed in mm²). Vascular Continuity Assessment

Animals were anesthetized with 4% chloral hydrate (0.1 mL/10 g body wt IP). An incision was made in the ventral midline of the neck. The left carotid artery was secured by two 4-0 silk sutures. An incision was made in the carotid artery, and a 15-cm length of PE-10 tubing (Beckton Dickinson) was introduced and advanced to the ascending aorta just distal to the aortic valve. Approximately 1.0 mL of luconyl blue dye was then slowly injected. Microscopic examination of disks removed from these animals revealed microvessels lined by a single layer of endothelium and erythrocytes contained within their lumen. Luconyl blue dye was clearly observed throughout the vessels in the disk, indicating their continuity with the systemic vasculature. Determination of LA and Dimethylarginine Levels

Arterial blood was collected from E and E mice. The plasma concentrations of LA and Nω,Nω-dimethylarginine (asymmetric dimethylarginine, ADMA) were measured by high-performance liquid chromatography as previously described by a novel enzymatic assay based on the affinity of dimethylarginine dimethylhydrolase (DDAH) for ADMA (unpublished observations).

Data Analysis

All data are given as mean ± SEM. Statistical significance was tested with an unpaired, 2-tailed t test for comparisons between groups. Statistical significance was corrected for multiple comparisons with the Bonferroni procedure, per recommendation of Dr Regina Nuzzo at the Department of Statistics at Stanford University, and was accepted for probability at the level of <0.05.

Results

Effect of Hypercholesterolemia on Angiogenesis: Role of ADMA

We have previously documented a reduction in vascular NO biosynthesis in apoE-deficient mice. In the current study, we found that hypercholesterolemic (E) mice had significantly higher ADMA plasma levels than normocholesterolemic (E) control mice (1.9 ± 0.6 versus 1.2 ± 0.2 μmol/L, respectively, P = 0.04). By contrast, there was no significant difference in plasma LA concentrations (41.6 ± 22.4 and 38.8 ± 29.5 μmol/L, E versus E, P = NS). The elevation of plasma ADMA was associated with an impairment of angiogenesis. Specifically, the fibrovascular growth area in the disks implanted in hypercholesterolemic E mice was approximately half of that in the disks from the E animals (5.9 ± 2.1 versus 10.8 ± 2.2 mm², P < 0.01) (Figure 1). Furthermore, fibrovascular growth in response to bFGF was reduced.
by half in the hypercholesterolemic mice (10.1±2.0 versus 23.3±4.0 mm², P<0.01).

In hypercholesterolemic animals and humans, the impairment of NO biosynthesis and endothelium-dependent vasodilation is reversible by administration of the NO precursor L-Arg.18–23 Accordingly, we administered LA in the drinking water (6 g/100 mL) to the apoE-deficient mice (E−/LA). L-Arginine augmented fibrovascular growth in E− mice to approximately twice that of the E− untreated animals (11.1±2.4 versus 5.9±2.1 mm², P<0.01). Oral administration of the NOS antagonist LNA mimicked the effect of systemic elevation of ADMA, reducing the fibrovascular growth area to approximately half that of the vehicle control (10.8±2.2 versus 6.0±0.4 mm², P<0.01).

Systemic elevations of the NOS inhibitor may have other effects (eg, hemodynamic effects) that could indirectly influence angiogenesis. Accordingly, the subsequent studies were performed to assess the local vascular effects of ADMA.

Local Effects of ADMA on Angiogenesis

We examined the local effects of ADMA on basal and bFGF-induced angiogenesis. Pellets loaded with ADMA (40 or 400 μg) for delayed local release were placed in the center of the sponge disks and implanted subcutaneously in normal mice. We observed that local administration of ADMA inhibited angiogenesis in a dose-dependent manner (12.2±1.0, 7.2±1.6, and 4.3±1.2 mm²; vehicle versus ADMA 40 μg versus ADMA 400 μg) (Figure 2 and Figure 3). We further observed that the antiangiogenic effect of local ADMA was completely reversed with dietary LA (19.8±2.6 versus 4.3±1.2 mm², P<0.01).

We determined whether local administration of ADMA may oppose the angiogenic effect of bFGF. FGF increased fibrovascular growth by 2-fold (10.8±2.2 versus 23.3±4.0 mm², vehicle versus bFGF-loaded disks, respectively; P<0.01) (Figure 4). When 400 μg ADMA was coadministered with bFGF, bFGF-induced angiogenesis was significantly reduced (23.3±4.0 versus 12.3±4.0 mm² P<0.01). The inhibitory effect of local ADMA on bFGF-induced growth, however, was reversed by the dietary administration of LA (12.3±4.0 versus 30.5±3.6 mm², P<0.01).

Discussion

In the present study, we found that the plasma level of the endogenous inhibitor of NOS (ADMA) is elevated in apoE-deficient, hypercholesterolemic mice. Locally or systemically elevated levels of ADMA impair both basal and bFGF-induced angiogenesis. The inhibitory effect of hypercholesterolemia on angiogenesis is mimicked by the exogenous NOS antagonist LNA in normal wild-type mice and is reversible by oral supplementation with the NO precursor LA. These observations indicate that angiogenesis may be impaired by a lipid-induced disruption of NO synthesis.

The mechanism(s) by which NO promotes angiogenesis are not fully elucidated. NO may exert its effect as an endothelial survival factor, inhibiting apoptosis.24,25 and or enhancing endothelial cell proliferation.26,27 Alternatively, NO may enhance endothelial migration28–28 by stimulating endothelial cell podokinesis,29 by enhancing the expression of αvβ3,28 and/or by increasing dissolution of the extracellular matrix through the bFGF-induced upregulation of urokinase-type plasminogen activator.27 Finally, the hemodynamic effects of this potent vasodilator may play a role in its angiogenic effects. It is known that increased flow (induced by prazosin) in the skeletal microcirculation is associated with increased endothelial cell proliferation (as indicated by uptake of bromodeoxyuridine by capillary endothelial cells).30

In conditions in which NO bioactivity is reduced, angiogenesis is attenuated. Vascular explants from rabbit thoracic aorta or human coronary artery manifest capillary-like out-
growth when placed into a collagen matrix that is inhibited by oxidized LDL cholesterol, an agent also known to reduce NO bioactivity. In hypercholesterolemic rabbits, endothelium-dependent NO-mediated vasodilation is blunted, as is the angiogenic response to hindlimb ischemia. More definitively, the angiogenic response to hindlimb ischemia is impaired in the eNOS-deficient mice, an effect that cannot be reversed by VEGF. These data indicate that NO plays a critical role in angiogenesis and are consistent with our hypothesis that ADMA is an endogenous antiangiogenic factor.

ADMA is derived from the methylation of internal arginine residues in protein. When these methylated proteins are hydrolyzed, ADMA is released. Studies of isolated vessels and cultured endothelial cells suggest that ADMA concentrations between 1 and 10 μmol/L inhibit endothelium-dependent vasodilation and vascular NOS activity. Endothelium-dependent NO-mediated vasodilation is attenuated in regenerating endothelial cells, where levels of intracellular ADMA are elevated 3-fold in comparison to normal cells. Plasma levels of ADMA are elevated in animals and/or humans with hypercholesterolemia, diabetes mellitus, hypertension, homocystinemia, tobacco use, aging, or congestive heart failure. A recent study in humans from our group revealed a positive correlation between the plasma LA/ADMA ratio and NO-dependent vasodilation as well as between this ratio and urinary nitrate excretion.

Increased endogenous formation, reduced clearance, or impaired metabolic degradation may increase plasma ADMA levels. Dimethylarginines derived from the degradation of methylated protein are excreted through the kidneys and accumulate in chronic renal failure. ADMA is also metabolized to citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Inhibition of DDAH causes a gradual vasoconstriction of vascular segments, which is reversed by LA. Recently, we have found that DDAH activity in vascular cells is impaired by hypercholesterolemia. Oxidized LDL cholesterol but not native LDL cholesterol caused ADMA to accumulate in the conditioned medium of endothelial cells. This effect was associated with a decline in the activity (but not expression) of DDAH. Similarly, DDAH activity was impaired in segments of thoracic aorta from hypercholesterolemic rabbits.

**Figure 3.** Histogram of fibrovascular growth in disks implanted in normal mice. When ADMA is added locally to disk, angiogenesis is impaired, an effect that can be reversed with oral administration of LA. Dietary LA alone enhances fibrovascular growth. Each bar represents unique experimental set of animals (n=5).

**Figure 4.** Histogram of fibrovascular growth in disks implanted in normal animals. Fibrovascular growth is doubled in bFGF-treated disks. bFGF-induced angiogenesis is impaired with local administration of ADMA. This impairment in angiogenesis can be reversed with oral LA supplementation. In this figure, the vehicle-treated groups are the same as those in Figure 3 (note, however, that scale has changed). With this exception, all bars represent unique experimental group of animals (n=5).
We found that plasma ADMA levels were increased in apoE-deficient, hypercholesterolemic mice. Plasma ADMA levels are also elevated in hypercholesterolemic rabbits, and administration of LA in these animals reverses endothelial vasodilator dysfunction. In hypercholesterolemic humans, intravenous or oral administration of LA improves endothelium-dependent vasodilation and enhances urinary nitrate excretion. Because ADMA levels are not altered with LA supplementation, we speculate that supplemental LA competes with ADMA for NOS. A vasodilator effect of intravenous LA has also been observed in patients with severe peripheral arterial occlusive disease. These observations indicate that exogenous LA may reverse the effects of elevated levels of the endogenous NOS inhibitor ADMA. We hypothesized that supplementation of dietary LA would thereby restore angiogenesis in hypercholesterolemic animals. Indeed, we observed that LA reversed the antiangiogenic effects of locally administered or systemically elevated ADMA. This finding is consistent with 2 decades of research in the wound-healing literature, demonstrating that LA can accelerate wound healing. Although this effect may be due in part to effects of LA on immune function and collagen formation, the enhancement by LA is also mediated by its metabolism to NO, as pharmacological or genetic antagonism of NO synthesis impairs wound healing. To conclude, ADMA is an endogenous inhibitor of NOS and is elevated in hypercholesterolemia.

We find that ADMA impairs basal and growth factor–induced angiogenesis. The local or systemic effects of ADMA can be reversed by administration of the NO precursor LA. Elevations of plasma ADMA occur in individuals with atherosclerosis or risk factors for atherosclerosis. The individual heterogeneity in collateral development may be determined in part by the coexistence of these risk factors and may have significant clinical implications. Therapeutic angiogenesis for peripheral or coronary artery disease is conducted in patients who uniformly have underlying conditions (hypertension, diabetes mellitus, hypercholesterolemia, homocysteinemia, aging, tobacco use or congestive heart failure) that are also associated with elevations in plasma ADMA. Our study raises the concern that by disturbing the NOS pathway, these common comorbid conditions may interfere with angiogenic therapies.

References
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