Loss of Flow-Mediated Endothelium-Dependent Dilation Occurs Early in the Development of Atherosclerosis

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Background. Healthy arteries exhibit endothelium-dependent dilation in response to both local acetylcholine and increased blood flow. In humans, clinically overt coronary artery disease is characterized by loss of dilation to both acetylcholine and blood flow. The temporal relation, however, between functional abnormalities of the endothelium and the development of atherosclerosis has not been established.

Methods and Results. We examined endothelial vasodilator function in vivo at an early stage of the development of atherosclerosis. Two groups of seven Macaca fascicularis monkeys were studied; one group was fed a high cholesterol diet (0.73–1.0 mg cholesterol per calorie) for 11 months. Cholesterol feeding was associated with increased plasma cholesterol levels and with intimal thickening of the iliac arteries but with no reduction in luminal diameter. Endothelium-dependent vasomotor responses of the iliac arteries were then examined in vivo by quantitative contrast angiography. Acetylcholine produced significant dilation in the controls but paradoxical constriction in the group with early atherosclerosis (+9.0±3.2% versus −5.3±5.4%, p<0.05). In response to a twofold increase in blood flow achieved by administering adenosine distal to the arterial segment under examination, the controls again dilated, whereas the atherosclerotic group failed to dilate (+11.6±2.1% versus +0.5±2.4%, p<0.05). Both groups, however, were able to dilate, and dilated equally, to the nonendothelium-dependent agent nitroglycerin (+13.7±4.8% versus +19.1±4.3%, NS).

Conclusions. Endothelium-dependent vasodilation in response to both acetylcholine and increased blood flow may be lost early in the course of developing atherosclerosis before the appearance of stenosing and occlusive disease. (Circulation 1991;84:1273–1278)

Normal arteries exhibit endothelium-dependent dilation in response to both local acetylcholine1–3 and to increased blood flow.4,5 In humans, advanced coronary artery disease, as defined angiographically by the appearance of intimal irregularities and stenoses, is characterized by loss of the normal dilator responses to both increased flow6 and acetylcholine.7 The temporal relations, however, between functional abnormalities of the endothelial layer and the development of atherosclerotic vessel wall disease have not been established because the onset and histological extent of disease cannot be determined in the clinical setting.

The aim of this study was to investigate endothelial vasodilator function early in the development of experimental atherosclerosis before the appearance of stenosing and occlusive disease by examining the development of different forms of endothelial vasodilator dysfunction including loss of receptor-mediated endothelium-dependent dilation (to acetylcholine) and loss of nonreceptor (flow-mediated) endothelium-dependent dilation.

Methods

Study Groups

Two groups of seven Macaca fascicularis monkeys matched for body weight and estimated age were studied. One group was fed a standard diet containing virtually no cholesterol for a period of 7–10 months before study (controls) while the other group

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was fed a diet containing 0.73-1.0 mg cholesterol per calorie for a period of 11 months (atherosclerotics). Preliminary studies suggested that this regime would produce histological changes similar to those found in early atherosclerosis in humans but would not produce luminal encroachment. Baseline data for the two groups are summarized in Table 1.

Experimental Design

After anesthetizing the animal with ketamine hydrochloride (12 mg/kg i.m.) and butorphanol tartrate (0.05 mg/kg i.m.), the right common carotid artery was exposed. A 5F arterial sheath (Cordis) was then passed via the carotid artery to the distal abdominal aorta, using a 5F femoral-visceral B (“shepherd hook”) catheter (Cordis) and a 0.035-in. J-shaped guide wire to negotiate the aortic arch. The catheter and guide wire were then removed and a 2.5F doppler/infusion catheter (Millar) was passed through the sheath over 0.014-in. angioplasty guide wire to the left external iliac artery. Aortic pressure was measured continuously using a pressure transducer (Gould Instruments) connected to the side-arm of the arterial sheath. Heart rate, arterial pressure, and Doppler shift (mean and phasic) were recorded throughout the study on a four-channel DC-driven polygraph (Grass Instruments, Quincy, Mass.). At this point, the left femoral artery was exposed and a ligature placed loosely around the artery.

Interventions

Control solution (5% dextrose) was infused via the Doppler catheter at a rate of 0.8 ml/min. After 2.5 minutes, the first of nine cineangiograms was performed using 6 ml nonionic contrast medium (Omnipaque 350, Winthrop-Breon Laboratories) injected via a power injector (Cordis). The angiogram was recorded on 35-mm cine film for subsequent analysis. An infusion of phenylephrine (1 µg/min) was then commenced via a peripheral vein and was continued for the remainder of the study. The purpose of the phenylephrine was to induce some preconstriction because preliminary experiments had suggested that the iliac arteries exhibited little basal tone. Eight further infusions, each lasting 2.5 minutes and at a rate of 0.8 ml/min were then administered via the Doppler catheter. After a second control infusion, there were two infusions of adenosine calculated to achieve intra-arterial concentrations of approximately 10^{-12} M and 10^{-11} M, respectively, assuming an iliac blood flow of 50 ml/min. After the second adenosine infusion, the Doppler catheter was withdrawn to the proximal common iliac artery. A repeat control was followed by two infusions of acetylcholine calculated to achieve concentrations of 10^{-6} M and 10^{-5} M, respectively, repeat control, and, finally, nitroglycerin 60 µg. During the infusions of acetylcholine, blood velocity was kept constant by adjusting the ligature around the femoral artery in response to the velocity signal detected by the Doppler catheter. In practice, the ligature was adjusted to keep flow velocity just below the control level and any tendency for velocity to increase was countered by tightening the ligature further. The catheters were then removed, incisions closed, and the monkeys were returned to their cages.

Blood Flow

Change in blood flow (i.e., percent change from control) was determined after each intervention by calculating the product of the mean blood velocity and the cross-sectional area of the iliac artery at the Doppler tip as previously described.6,8,9

Quantitative Angiography

The iliac artery segment of interest was digitized at 20–40 µm per pixel using a videocamera (Cohu Inc., San Diego, Calif.), video interface (Recognition Concepts Inc.), and a Microvax II computer (Digital Equipment Corp., Maynard, Mass.). Four cine frames were scanned and averaged; two fixed anatomic features served as references to ensure accurate alignment. Sixteen video images of each cine frame were summed to reduce video noise, and two-line profile averaging was used to minimize anatomical noise.6,8,10,11 The two fixed anatomic features were also used to ensure accurate registration between different infusions, and, therefore, to allow assessment of serial changes in the same arterial segment. Using an edge detection algorithm, a series of measurements of diameter along the length of the arterial segment was derived for each pixel line and mean diameter of the arterial segment calculated for each infusion.

To calculate caliber change in response to increased blood flow, the segment proximal to the tip of the Doppler catheter was examined. This segment, therefore, was exposed to increased blood flow but not to the direct effects of adenosine. To control for any confounding effects of recirculating adenosine or changes in blood pressure that occurred during the infusion of adenosine, the contralateral iliac artery was also examined. The results expressed represent the difference between the iliac artery under study (left) and the control iliac artery (right).
Histological Studies
Within 2 months of the functional experiments, monkeys were reanesthetized and the left common iliac artery was isolated and removed for histological studies. The incision was closed and the monkeys were returned to their cages.

Iliac arteries were immersion-fixed in 10% neutral buffered formalin for 48 hours. Three serial tissue blocks were cut at approximately 5-mm intervals perpendicular to the long axis of the artery. Sections were stained with Verhoff-van Gieson’s stain. The sections were then projected and the cross-sectional area of plaque lesion was expressed as the mean cross-sectional area of the intima in millimeters squared.

Statistical Analyses
All data are expressed as mean±SEM. Differences between groups were calculated using a two-tailed t test for unpaired data. Significance was assumed if the null hypothesis could be rejected at the level of 0.05.

Results

Plasma Lipids
Cholesterol feeding was associated with a marked elevation of mean total plasma cholesterol (120±24 mg/100 ml versus 581±44 mg/100 ml, p<0.001) and with a significantly lower HDL-cholesterol (24.0±8.1 mg/100 ml versus 51.9±4.4 mg/100 ml, p<0.001) (Table 1).

Morphology and Morphometry
Intimal plaque area was 0.188±0.065 mm² in the atherosclerotic group compared with 0.017±0.008 mm² in the controls (p<0.05). Despite the formation of intimal plaques, however, the arteries of the atherosclerotic animals showed no areas of focal stenosis and the resting iliac artery diameter was not significantly different between the control and atherosclerotic groups (2.673±0.438 mm versus 2.623±0.392 mm, respectively; NS).

Blood Pressure Response
The blood pressure responses to the various agents administered are summarized in Figure 1. In response to the preconstricting agent phenylephrine, blood pressure rose in both groups. During infusion of adenosine, blood pressure fell equally in the two groups and then returned to baseline values during the second control infusion. Blood pressure then remained constant during the two infusions of acetylcholine but fell during administration of nitroglycerin. Throughout the various drug infusions, there were no significant differences in the responses of the control and atherosclerotic groups.

Vascular Responses
Acetylcholine. Infusion of acetylcholine in the control group (during constant flow) produced significant dilation of the iliac artery at both 10⁻⁸ M (8.3±2.5%) and 10⁻⁶ M (9.0±3.2%) (Figure 2). In contrast, iliac arteries from the cholesterol-fed group showed no significant vasomotor response to the low concentration of acetylcholine (+1.3±2.0%) and demonstrated paradoxical constriction to the high concentration of acetylcholine (-5.3±5.4%) (p<0.05 versus control group) (Figure 2).

Increased blood flow. Intra-arterial adenosine, delivered distal to the test segment, increased iliac blood flow by 251±34% in the controls (p<0.001 versus baseline) and 261±43% in the atherosclerotic group (p<0.001 versus baseline; NS versus control group). The increased flow, however, resulted in very different arterial responses in the two groups (Figures 3 and 4). In response to increased flow, the arteries of control animals demonstrated significant dilation of the order of 11.6±2.1% (Figure 3),

**FIGURE 1.** Graph showing arterial pressure responses to the various intra-arterial infusions. All interventions, with the exception of the first control study, were carried out in the presence of phenylephrine. 1, control solution (before phenylephrine); 2, control solution; 3, adenosine 10⁻⁵ M (delivered distal to test segment); 4, adenosine 10⁻⁴ M (delivered distal to test segment); 5, control solution; 6, acetylcholine 10⁻⁸ M; 7, acetylcholine 10⁻⁶ M; 8, control solution; 9, nitroglycerin 60 µg. There were no significant differences in blood pressure responses between the two groups.

**FIGURE 2.** Plot shows change in vessel caliber in response to acetylcholine. Early atherosclerosis was associated with loss of the normal dilator response to acetylcholine. *p<0.05, difference between normal and atherosclerotic groups.
whereas mean blood velocity in the control group increased only slightly (Figure 4). In contrast, the caliber of iliac arteries from the atherosclerotic group did not alter significantly (+0.5±2.4%) (Figure 3). Increased blood flow in this group, therefore, was accommodated principally by increased blood velocity (Figure 4).

Nitroglycerin. In response to nitroglycerin, iliac arteries from the control and atherosclerotic groups exhibited equal and significant dilation (Figure 5) (13.7±4.8% and 19.1±4.3%, respectively; p<0.01 versus baseline for both groups, p=NS between groups).

Flow-mediated versus acetylcholine-mediated dilation. Figure 6 shows the maximal response to increased flow and the maximal response to acetylcholine for both the atherosclerotic and control groups. The correlation between the vasomotor response to increased flow and the response to acetylcholine during constant flow was highly significant (r=0.87, p<0.001).

Discussion

This study demonstrated that the normal dilator response of large arteries to increased blood flow is lost early in the course of atherosclerosis before the development of stenosing and occlusive disease, and that loss of receptor-mediated dilation (to acetylcholine) and loss of nonreceptor-mediated dilation (to increased blood flow) are closely correlated. That this loss of flow-mediated dilation is a functional rather than structural abnormality is demonstrated by the retained ability of the atherosclerotic vessels to dilate to the nonendothelium-dependent agent nitroglycerin.

Furchgott et al.1,2 first demonstrated that arterial strips show endothelium-dependent dilation in response to acetylcholine. Although dilation of conduit arteries in response to increased blood flow was first described in 1933,12 only recently did Bassenge and colleagues4,5 demonstrate that intact endothelial cells are an essential mediator not only of receptor-mediated but also of flow-mediated dilation. Mechanical removal of endothelial cells from the canine femoral artery abolishes dilation in response to both increased blood flow and acetylcholine but not in response to nitroglycerin, a direct smooth muscle (i.e., endothelium-independent) relaxant.13

In the present study, cholesterol feeding for a period of 8–11 months resulted in intimal thickening but no reduction in lumen diameter and no impair-

FIGURE 3. Plot shows change in vessel caliber in response to increased blood flow. While the control group dilated in response to increased blood flow, this response was lost in the atherosclerotic group. *p<0.05, difference between normal and atherosclerotic groups.

FIGURE 4. Plot shows change in blood velocity (centimeters per second) during increased blood flow. Failure to dilate in the atherosclerotic group resulted in a marked increase in blood velocity during increased flow. *p<0.05, difference between normal and atherosclerotic groups.

FIGURE 5. Plot shows change in vessel caliber in response to nitroglycerin. Both groups showed equal and significant dilation to nitroglycerin.

FIGURE 6. Plot shows correlation of percent change in vessel diameter in response to acetylcholine with response of the same arterial segment to increased blood flow.
ment of the ability of the vessel to dilate to nitroglycerin. The degree of intimal thickening in this study was much less than in other studies using longer periods of dietary challenge (1.5–6.5 years) in a different species of monkey.14 Because the interval between the functional study and subsequent morphometric analysis was up to 2 months, it is likely that intimal thickening was even less marked at the time of the functional experiments. Nevertheless, our findings are consistent with previous studies both in experimental atherosclerosis14 and in human coronary artery disease15 demonstrating that arteries undergo compensatory enlargement as atherosclerosis develops. Lumen area, therefore, is not reduced until atherosclerosis is advanced. At this early stage of vessel wall disease, however, endothelial vasodilator function in response to acetylcholine is already abnormal. Similar results have recently been reported by another group using different methodology16: Using a perfused hind limb preparation, Lopez examined vascular responsiveness assessed by perfusion pressure/flow relations to endothelium-dependent and endothelium-independent agents. As in our study, administration of an atherogenic diet for approximately 9 months produced early atheromatous lesions and was associated with either impaired vasodilation or augmented vasoconstriction to endothelium-dependent agents but with normal responses to endothelium-independent agents.

Our study demonstrated that experimental atherosclerosis is associated not only with loss of receptor-mediated endothelium-dependent dilation, but also with loss of the normal dilator response to increased blood flow. Increased blood flow through the iliac artery, therefore, can be achieved only with a marked increase in blood velocity.

We can speculate that loss of flow-mediated dilation might predispose to further vascular injury. Shear stress, defined as the frictional force acting per unit area on the vessel wall, is directly related to blood velocity and inversely related to the third power of the vessel diameter.17 Endothelial cells are sensitive to shear stress18 and may be damaged by exposure to turbulent shear stress.19 Thus, loss of endothelium-dependent flow-mediated dilation early in the development of atherosclerosis may expose the vessel wall to unphysiological flow patterns resulting in further endothelial damage and potentiation of vascular injury.

We have no data relating to the mechanism of the observed changes in vessel caliber. Other studies, using a bioassay technique, have demonstrated that in healthy arteries, the properties of the relaxing substance released by acetylcholine are similar to those of the relaxing factor released by increased blood flow.13 Our results demonstrating a relation between the vascular responses to acetylcholine and to increased blood flow (Figure 6) would be consistent with the hypothesis that these two stimuli to endothelium-dependent dilation are acting through the same mechanism. This relation clearly merits further investigation.

Limitations of the Study

Since we investigated at one time point only, we cannot differentiate between the effects of hypercholesterolemia per se and the induced structural change in the vessel wall. Although recent studies have suggested that oxidized LDL, which can be isolated from the arteries of rabbits with hypercholesterolemia,20 may inhibit endothelium-dependent relaxations,21 other studies suggest that only longer periods of hypercholesterolemia with the development of structural change in the vessel wall lead to inhibition of endothelium-dependent vasodilation. Thus, cholesterol feeding of dogs for 4–5 weeks results in significant hypercholesterolemia but a longer period of dietary challenge is required before endothelium-dependent relaxation becomes abnormal.22 Furthermore, the veins of primates with diet-induced atherosclerosis show no structural abnormalities and normal endothelium-dependent dilation despite prolonged exposure to hypercholesterolemia.22 Adenosine increased iliac blood flow in both the normal and atherosclerotic animals. It was not an object of the study to induce maximal vasodilation and, therefore, no comment can be made regarding resistance vessel function in either group. As shown in Figure 1, adenosine resulted in a fall in arterial pressure. We controlled for the effects of pressure change on vessel caliber by subtracting any change in caliber seen in the contralateral iliac artery because this artery was exposed to changes in systemic arterial pressure but not to increased blood flow. Furthermore, it seems unlikely that the observed differences between the control and atherosclerotic groups were attributable to blood pressure changes because the blood pressure responses in the two groups were similar despite very different vasomotor responses.

In the present study, endothelium-dependent relaxation of the iliac artery could only be demonstrated after preconstriction with phenylephrine. While the loss of endothelium-dependent dilation in the atherosclerotic group clearly represents a functional abnormality, the pathophysiological importance of this abnormality may be restricted to those vascular beds with intrinsic tone.

Summary

Our results indicate that endothelium-dependent dilation of preconstricted large arteries, in response to increased blood flow and local acetylcholine, is impaired at an early stage of atherosclerosis before the development of stenosing and occlusive lesions. If these findings are extended to vascular beds with intrinsic tone, then loss of flow-mediated dilation will result in exposure of the arterial wall to high blood velocities and abnormal shear stresses, which may be important in potentiating arterial disease.
References


Key Words • acetylcholine • quantitative angiography
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