Delayed Arteriogenesis in Hypercholesterolemic Mice

Daniela Tirziu, PhD; Karen L. Moodie, DVM; Zhen W. Zhuang, MD; Katie Singer, RALAT; Armin Helisch, MD; Jeff F. Dunn, PhD; Weiming Li, PhD; Jaipal Singh, PhD; Michael Simons, MD

Background—Hypercholesterolemia has been reported to inhibit ischemia-induced angiogenesis. To address its effects on arteriogenesis, we investigated arterial growth in hypercholesterolemic low-density lipoprotein receptor−/−/ApoB-48−/− (HCE) mice.

Methods and Results—The extent and the time course of arteriogenesis after femoral artery ligation was evaluated in HCE and strain-matched control mice. Distal limb perfusion was measured by laser Doppler imaging, whereas MRI was used to visualize arterial flow and micro-computed tomography to assess vascular growth. After femoral artery ligation, serial laser Doppler imaging demonstrated significantly delayed restoration of perfusion in untreated HCE compared with control mice (day 3, 0.09 versus 0.19, P<0.05). Treatment with Ad-PR39 in control mice led to a significant restoration of arterial blood flow and tissue perfusion at day 3, whereas in HCE mice, hindlimb perfusion began increasing only by day 7. Micro-CT analysis confirmed increased growth of smaller arterioles (16 to 63 μm in diameter) in the Ad-PR39–treated control compared with HCE mice. The delay in arteriogenesis in HCE mice correlated with delayed tissue appearance of F4/80⁺ cells. Analysis of gene expression after Ad-PR39 treatment demonstrated that HCE mice had significantly reduced expression of FGF receptor 1, hypoxia-inducible factor-1α, vascular cell adhesion molecule-1, macrophage scavenger receptor-1, and cyclophilin A compared with controls 3 days after arterial ligation that equalized by day 7, mimicking relative changes in arteriogenesis and tissue perfusion.

Conclusions—Hypercholesterolemia results in delayed native arteriogenesis because of reduced early monocyte/macrophage influx and delayed and impaired arterial growth response to growth factor therapy. (Circulation. 2005;112:2501-2509.)

Key Words: arteries ■ atherosclerosis ■ genes ■ gene therapy ■ arteriogenesis

The development of progressive occlusions in arterial conduits initiates a series of events that seek to restore the tissue perfusion by increasing the size of the arterial tree (arteriogenesis) and the distal capillary bed in the ischemic tissue (angiogenesis). Although such restoration of flow is usually complete in settings of mechanical injury in healthy young patients or in animal models of ischemia in young and healthy animals, it is far less successful in aged individuals and in certain disease settings, including atherosclerosis. Furthermore, not only is native neovascularization impaired, but the response to various therapeutic angiogenic agents is also markedly decreased, as demonstrated by numerous failures of clinical trials that used agents and delivery techniques effective in healthy animal models.1

Clinical Perspective p 2509

Atherosclerosis appears to be one of the key variables associated with impaired neovascularization.2–4 However, the precise nature of this abnormality has not been established. The present study was undertaken to investigate the effect of hypercholesterolemia on the growth and development of the arterial tree and arterial blood flow in both nonischemic and ischemic territories as well as the ability of hypercholesterolemic animals to respond to an angiogenic agent. To this end, we used mice deficient in low-density lipoprotein receptor (LDLR) and ApoB-48 (LDLR−/−/ApoB-48−/−) and thereby expressing only ApoB-100. In this model, ApoB-48 deficiency provides higher levels of LDL cholesterol and a more human disease–like profile of lipid abnormalities compared with the commonly used Apo E−/− mice.5

As a therapeutic angiogenic agent, we chose PR39 because of its ability to stimulate both vascular endothelial growth factor (VEGF)– and fibroblast growth factor (FGF)–dependent signaling pathways, inducing an extensive angiogenic response.6–8 Laser Doppler and time of flight (TOF) MRI were used to track perfusion changes and the development of vascular conduits carrying fast arterial blood flow, whereas micro-CT was used to visualize and quantify the arterial vasculature. Finally, analysis of gene expression was performed to gain insights into molecular abnormalities associ-
ated with impaired angiogenesis in hypercholesterolemic mice.

We find that both native arteriogenic response and the response to Ad-PR39 treatment are delayed in HCE mice. The primary defect lies in the reduced growth and development of small arteries (16 to 63 μm in diameter), correlates with a late appearance of blood-derived mononuclear cells in tissues, and is associated with decreased expression of genes involved in several growth factor signaling cascades, including that of FGF, VEGF, and transforming growth factor-β.

Methods

Hypercholesterolemic Mouse Model
LDLR- and ApoB-48−deficient mice were obtained from the Jackson Laboratory (Bar Harbor, Me). The colony was maintained by homozygous sibling mating and fed the normal chow diet. Male mice of similar genetic background (C57Bl/129S) and age were used as controls. All animal experiments were performed under a protocol approved by the institutional animal care and use committee of Dartmouth College. Total plasma cholesterol was determined enzymatically in the presence of cholesterol esterase (Raichem Co). Arterial lipid depositions were detected by Sudan IV staining.

Hindlimb Ischemia Model
The femoral artery was dissected and ligated at 2 positions spaced 5 mm apart, 1 just proximal to the caudal femoral artery and 1 distal to it and proximal to popliteal artery. The arterial segment between the ligatures was excised (Figure 1A). Treated mice received 1×10^6 pfu of Ad-PR39 or Ad-Null at the time of surgery by 2 injections (15 μL each), placed 2 to 3 mm deep, 1 in the adductor and 1 in the quadriceps muscle at the level of femoral artery ligation.

Monitoring of Hindlimb Blood Flow

Laser Doppler Imaging
The acquisition of flow images in the foot was performed using a Moor Infrared Laser Doppler Imager (LDT; Moor Instruments Ltd) at 37.4°C to 38.0°C under ketamine/xylazine (80/5 mg/kg) anesthesia. The data were analyzed with Moor LDI image processing software V3.09 and reported as the ratio of flow in the right/left (R/L) hindlimb after background subtraction (values for dead mice).

Magnetic Resonance Imaging
MRI was performed with a 7-T horizontal bore magnet with a Varian Unity console and a 2.5-cm quadrature birdcage coil. Measurements were based on a modification of a TOF method used previously. The image acquisition was performed with the mice under anesthesia with 1.5% isoflurane in oxygen at 37°C. A gradient echo MR sequence was used with TR/TE/α = 0.03/0.0035/90°, slice thickness 600 μm, 3×3-cm field of view, and 512×128 matrix size, resulting in a pixel resolution of 59×234×600 μm. For processing, a region of interest was selected in an area of the nonischemic leg that did not include any visible vessels. The image was windowed to include only pixels with the signal intensity that was above the signal mean of this region plus 6 SD. The number of bright spots (conductance vessels) was counted in the ischemic leg for both groups at different time points.

Volumetric Micro-CT
After the mice were euthanized, the vasculature was flushed with 0.9% normal saline containing heparin (1000 IU/L), papaverine (4 mg/L), and adenosine (1 g/L) for 3 minutes. For fixation, 2% paraformaldehyde was perfused for 5 minutes at 100 mm Hg pressure. Contrast agent containing bismuth (0.5 mL) (Sigma-Aldrich Inc) in 5% gelatin was injected over a period of 2 minutes with a syringe pump. The mice were then immediately chilled on ice and immersion-fixed in 2% paraformaldehyde overnight. The hindlimb vasculature was imaged with a high-resolution micro-CT imaging system (GE eXplore Locus SP), set to a 0.016-mm effective detector pixel size. Data were acquired in an axial mode, covering a volume of 2.0 cm in the z direction with a 1.04-cm field of view (covering a single hindlimb). Micro-CT was calibrated using standard wires of different sizes (10, 20, 30, 40, and 50 μm). During postprocessing of the image, a 40 000 gray-scale value was set as a threshold to eliminate bone with minimal sacrifice of vessel visualization. Microview software (GE Healthcare) was used to reconstruct three 2D maximum-intensity projection images (x, y, and z axes) from raw images. A volume of interest was reconstructed of the upper (500 pixels) and lower (250 pixels) hindlimb. Quantification was performed by use of a modified Image Pro-Plus 5.0 algorithm. The data are expressed as vascular segment number, representing total number of vessels, of specified diameter, counted in 500 z sections for thigh or 250 z sections for calf images.

Analysis of Gene Expression
Gene expression was performed by use of an angiogenesis gene array (SuperArray, Bioscience Co) using 1 μg tRNA. The data were extracted from raw images using ScanAlxse software (SuperArray, Bioscience Co) and reported as signal intensity relative units. The data analysis was performed after background extraction using

Figure 1. Hindlimb ischemia model and Ad-PR39 gene transfer. A, Schematic representation of the ischemia model used in this study. In the right hindlimb, the common femoral artery was ligated twice, proximal to the popliteal and the caudal femoral artery; the arterial fragment between the ligatures was cut and removed. B, Representative Sudano IV staining of the aorta from an HCE mouse on the chow diet. Note diffuse lipid-rich atherosclerotic lesions on the aortic arch, carotid arteries, and descending aorta. C, Reverse transcription–polymerase chain reaction analysis of PR39 gene expression in the hindlimbs 3 days after adenoviral transfer. Note expression in the right, but not left, hindlimb adductor muscle. Expression in HUVECs transduced with 100 MOI Ad-PR39 is shown as a control. The blot demonstrates a representative example of PR39 expression in the adductor at day 3 in normal treated mice. Similar levels of PR39 expression were detected in Ad-PR39–treated HCE mice at day 3 (not shown). D, Effect of PR39 expression on VEGF levels. Western blot analysis of VEGF expression in HUVECs transduced with Ad-PR39 or Ad-Null viruses after 48 hours. Note increased VEGF levels in Ad-PR39–transduced cells.
membranes were blocked with 5% nonfat dried milk in TBS, pH 7.4. SDS-PAGE. After transfer to Immobilon membranes (Millipore), the protein extract, determined by BCA assay (Pierce) was subjected to

$$-GGCCCTTCATAATATCCCCCA-3$$

/H11032

5/H11032

/H11032

After 2 minutes of denaturation at 14 000 rpm, 15 minutes at 4°C, and 35 µg protein extract, determined by BCA assay (Pierce) was subjected to SDS-PAGE. After transfer to Immobilon membranes (Millipore), the membranes were blocked with 5% nonfat dried milk in TBS, pH 7.4, containing 0.1% Tween 20 and immunoblotted with anti-VEGF antibody (Santa Cruz), anti–hypoxia-inducible factor (HIF)-1α (Novus Biologicals), or anti-F4/80 antigen (rat anti-mouse monoclonal antibody clone CI:A3-1 Abcam) for adductor samples. Immunoreactive bands were visualized by use of the horseradish peroxidase–conjugated secondary antibody and enhanced chemiluminescent substrate (Pierce).

**Statistical Analysis**

Data are presented as mean±SEM. Differences between groups were considered statistically significant at a value of $P<0.05$ as determined by a Student’s $t$ test, 2-tailed distribution.

**Results**

**Hypercholesterolemic Hindlimb Ischemia Model and Adenoviral Gene Transfer**

We used a hindlimb ischemia model in hypercholesterolemic LDLR$^{-/-}$/ApoB-48$^{-/-}$ mice (mean age, 6.93±1.17 months) and strain- and age-matched (mean, 6.57±1.16 months) control mice. The LDLR$^{-/-}$/ApoB-48$^{-/-}$ mice showed a significant increase in plasma cholesterol levels compared with normal mice (342.5±12.8 versus 89.2±6.8 mg/dL, $P<0.0001$, $n=14$ mice per group) and developed mild diffuse lipid-rich atherosclerotic lesions predominantly in the aortic arch, carotid arteries, and descending aorta (Figure 1B).

Some mice received injections of $1\times10^9$ pfu adenoviruses encoding PR39 or empty (Ad-Null) vectors into the ipsilateral adductor and quadriceps muscles. The PR39 gene expression was limited to the ligated right hindlimb, with no expression detectable in the nonligated left hindlimb (Figure 1C), and a similar level of expression was achieved in HCE and control mice. To demonstrate the biological activity of Ad-PR39, VEGF protein levels were assayed in Ad-Null and Ad-PR39–transduced HUVECs. There was a significant increase in VEGF expression after Ad-PR39 (2.87±0.8 compared with...
artery ligation, there was a substantial influx of F4/80 antigen in adductor muscle 3 and 7 days after ligation, detected by Western blotting. Color code as in Figure 2.

Micro-CT analysis of arterial vasculature in untreated mice. A, Micro-CT reconstruction at 16-μm resolution of calf and thigh portions of mouse hindlimb 14 days after femoral artery ligation. B, Quantitative analysis of micro-CT images in the thigh presented as total number of vascular structures in 500 z-axis slices. C, Quantitative analysis of micro-CT images in the calf as total number of vascular structures in 250 z-axis slices. Data shown as mean±SEM vascular segments for n=5 mice/group, P=NS normal vs HCE. D, Presence of F4/80 antigen in adductor muscle 3 and 7 days after ligation, detected by Western blotting. Color code as in Figure 2.

Effect of Angiogenic Therapy
Both normal and HCE mice groups were injected with 10⁶ pfu of Ad-PR39 or Ad-NULL immediately after femoral artery ligation. Ad-PR39 treatment resulted in a much more pronounced increase in perfusion in control compared with LDLR⁻/⁻/ApoB-48⁻/⁻ mice at day 3 (Figure 4). The effect of Ad-PR39 treatment in HCE mice became evident only at day 7 and then “caught up” with the Ad-PR39–treated control group (Figure 4).

Micro-CT analysis of the vasculature at day 14 demonstrated a significant increase in the number of vascular segments in the 16- to 63-μm-diameter range in the thighs of Ad-PR39–treated control compared with hypercholesterolemic mice (Figure 5A and 5B). At the same time, there were no significant differences in the number of vascular segments in the calf between the 2 groups (Figure 5A and 5C).

Western blotting demonstrated an earlier appearance of F4/80⁺ cells in Ad-PR39–treated control compared with HCE mice and a greater extent in the amount of induced HIF-1α gene expression (Figure 5D). By day 7, when the extent of perfusion recovery was the same in both groups, the extent of tissue F4/80⁺ cell presence and HIF-1α expression were similar (Figure 5D).

TOF MRI was used to visualize and quantify arterial vessels with fast flow at different time points in both mouse groups. Imaging of the arterial flow in the thigh after femoral artery ligation demonstrated one prominent arterial blood vessel that corresponded to the common femoral artery (Figure 6A). Immediately after ligation, no proximal arterial conductance flow was detected in either control or LDLR⁻/⁻/ApoB-48⁻/⁻ mice. Three days after Ad-PR39 treatment, flow in arterial conductance vessels was detectable in the normocholesterolemic but not HCE mice (Figure 6A). By day 7, however, arterial conductance flow was detectable in both groups and remained the same at day 14 (Figure 6A and 6B).

Similarly, in the calf, the arterial conductance flow became undetectable in both groups immediately after femoral artery ligation, and the effect of Ad-PR39 treatment was detectable by day 3 in control but not LDLR⁻/⁻/ApoB-48⁻/⁻ mice (Figure 6C). Other than in the thigh, however, the differences in the arterial conductance flow persisted at day 7 and

baseline, P<0.05) but not Ad-Null (1.45±0.3, P=NS) in 3 independent experiments (Figure 1D).

**Tissue Perfusion and Arteriogenesis in Untreated Mice**
We first examined the time course of blood flow changes in the distal hindlimb of HCE and normal control mice after femoral artery ligation using laser Doppler perfusion imaging. Immediately after surgery, there was a similar decline in the distal hindlimb blood flow in both strains (Figure 2A). Control mice demonstrated significantly faster recovery of flow at day 3 compared with LDLR⁻/⁻/ApoB-48⁻/⁻ mice (LDL R/L ratio, 0.19±0.04 versus 0.09±0.02, P<0.05) (Figure 2B). By day 7, however, perfusion in the ischemic hindlimb was virtually the same in both groups and remained so at day 14.

Consistent with the results of laser Doppler perfusion imaging, neither visual inspection of micro-CT angiograms obtained at day 14 (Figure 3A) nor quantitative analysis (Figure 3B and 3C) demonstrated any difference in the extent of arterial growth in either the thigh or the calf between the 2 groups at this time point.

To explore why the control group demonstrated better initial recovery of blood flow, we used Western blotting to monitor the appearance of blood-derived mononuclear F4/80⁺ cells in the adductor muscle. Three days after femoral artery ligation, there was a substantial influx of F4/80⁺ cells in normal but not HCE mice (Figure 3D). Expression of F4/80 antigen was absent in the contralateral adductor tissue, and the presence of these cells on the ipsilateral side was reduced to near baseline level by day 7 (Figure 3D).
equalized only by day 14 (Figure 6C and 6D). Interestingly, in the thigh, the precise location of new conductance vessels detected by TOF MRI was not localized to any specific region and was somewhat different from mouse to mouse. Conversely, in the calf, arterial blood flow seemed to recover at the same location in all mice, corresponding to the position of the conductance vessel before the femoral artery ligation. We interpret these findings as suggesting that the somewhat variable collateralization process in the thigh leads to restoration of arterial inflow into preexisting calf blood vessels.

Arteriogenic Gene Expression Profiling

To gain further insight into the molecular nature of the delayed arteriogenesis observed in HCE mice, we used cDNA microarrays to study serial differences in gene expression in the Ad-PR39–treated mice. To focus on differences related specifically to arteriogenesis, tissue for gene expression analysis was collected from the adductor muscle.

At day 3, 5 genes (HIF-1α, FGF receptor 1 [FGFR-1], macrophage scavenger receptor 1 [SR-A], vascular cell adhesion molecule [VCAM]-1, and cyclophilin A) were expressed at significantly higher levels in Ad-PR39–treated control compared with HCE mice (Figure 7A, left). There were no genes with higher expression in HCE mice then in control mice. Analysis of expression of the same 5 genes at day 7, the time when the difference in Ad-PR39–induced arteriogenesis between the 2 mice groups largely disappeared, showed that only SR-A expression was still decreased in LDLR+/−/ApoB-48−/− mice (Figure 7A, right).
At day 7, 3 genes (VEGF-D, FGF-7, and endoglin) were expressed at significantly higher levels in control than HCE mice (Figure 7B, left). Repeat examination of gene expression at day 14 demonstrated that the expression of these genes remained significantly reduced (Figure 7B, right).

**Discussion**

This study demonstrates that hypercholesterolemia results in an impaired native arteriogenic response as well as impaired response to a therapeutic agent.

Chronically elevated cholesterol levels produce a number of vascular abnormalities, including impaired vasodilation, defective wound healing, and growth of atherosclerotic plaques, among others, as well as abnormal angiogenesis. Therapeutic angiogenesis trials, which invariably enroll patients with advanced atherosclerosis, have demonstrated markedly reduced responses in this patient cohort, compared with anticipated results from preclinical nonatherosclerotic models.

The recent evolution of therapeutic angiogenesis concepts has emphasized that the growth and remodeling of collateral vessels (arteriogenesis) and not the proliferation of capillaries (angiogenesis) is the key biological process responsible for restoration of blood flow to ischemic tissues in the setting of atherosclerotic vascular disease. To date, however, there has been little information regarding the effect of hypercholesterolemia on arteriogenesis. One study that used a Watanabe heritable hyperlipidemic rabbit model of hindlimb ischemia demonstrated attenuated collateral development compared with the New Zealand White rabbit. Another study showed reduced neovascularization in ApoE \(-/-\) 129 compared with C57Bl/6 mice. These results are difficult to interpret, however, given the marked differences in the extent of collateral development in different animal strains and because of reliance on x-ray angiography, a technique with a limited spatial resolution.

In the present study, we find, using strain-matched mice and advanced imaging techniques, that even mild hypercholesterolemia delays the native arteriogenic response, in addition to delaying and reducing the response to a growth factor therapy.

We chose an ischemic hindlimb model involving resection of the femoral artery. In contrast to the distal limb tissue, in which ischemia is the primary driver of angiogenesis, arteriogenesis in the thigh is thought to be driven primarily by increased shear stress and blood-derived monocyte adhesion in proximal arterial segments. This model, therefore, allows serial analysis of arterial growth as well as monitoring of the distal perfusion.

Several complementary techniques were used to address this question. Distal hindlimb perfusion was assessed using LDI, which allows assessment of tissue perfusion to the depth of 2 to 3 mm using a blue laser. This was supplemented with the TOF MRI, which allows visualization and quantification of arterial conductance vessels. Finally, we performed micro-CT of perfusion-fixed hindlimbs to assess the overall status of the vasculature.

The effect of hypercholesterolemia on arteriogenesis was assessed in LDLR \(-/-\)/ApoB-48 \(-/-\) and age- and strain-matched control mice in terms of native neovascularization (untreated animals) and a response to therapy with an angiogenic growth factor. For the latter part of the study, we chose PR39, a potent angiogenic growth stimulator. The choice of PR39 was dictated by its ability to stimulate both...
VEGF, via stabilization of HIF-1α expression, and FGF, via stimulation of FGF receptor 1 and syndecan-4 expression signaling pathways.

The assessment of the “native” arteriogenic response demonstrated that in hypercholesterolemic mice, the recovery of perfusion was delayed compared with controls. This delay in blood flow recovery in Ad-PR39–treated and untreated HCE mice correlated with delayed appearance of blood-derived F4/80+ mononuclear cells, whereas the flow “catch-up” corresponded to the time point of equal appearance of F4/80+ cells in thigh tissues of both groups. The F4/80 marker was chosen because of its specificity for blood-derived monocyte/macrophages in mice, although it may potentially identify other mononuclear cell populations that were not known at the time of F4/80 description. However, studies with anti-Mac3 antibodies gave similar results (data not shown).

As in the case of native arteriogenesis, blood flow recovery was delayed in Ad-PR39–treated HCE compared with control mice. MR assessment of arterial conductance vessels in the thigh demonstrated the appearance of arteries carrying fast blood flow 3 days after femoral artery ligation in normal mice. However, this pattern of arterial growth did not appear in the LDLR+/−/ApoB-48−/− mice until day 7, consistent with the observed delay in the foot perfusion recovery. Subsequently, arterial structures in control mice remodeled into several larger vessels, whereas the same process was not observed in the LDLR+/−/ApoB-48−/− mice. Micro-CT analysis of the thigh vasculature 14 days after femoral artery resection demonstrated a significantly larger number of vascular segments in the 16- to 63-μm-diameter range in the control group. At the same time, there were no significant differences in the number of vascular segments in the calf between the groups. These observations suggest that delayed recovery of distal tissue perfusion in these animals is primarily a result of the reduced arteriogenesis in the nonischemic thigh and not of differences in arterial growth in the ischemic calf.

Figure 7. Microarray analysis of angiogenic gene expression in the adductor muscle after Ad-PR39 treatment. Relative gene expression (presented as signal intensity) analysis of angiogenic genes derived from 96 gene cDNA arrays. A, Left, Expression levels of 5 genes differentially increased in control compared with HCE mice 3 days after Ad-PR39 treatment. Expression of the other 91 genes was not different between the groups. A, Right, Expression of the same 5 genes as shown at left 7 days after treatment. Note that differences in expression have largely disappeared (except for SR-A). B, Left, Expression levels of 3 genes differentially increased in control compared with HCE mice 7 days after Ad-PR39 treatment. Expression of the other 93 genes was not different between the groups. B, Right, Expression of the same 3 genes as shown at left 14 days after treatment. Note that differences persist. Data shown as mean±SEM relative units (ru) of signal intensity. *P<0.05 for A, left, and B, left and right, n=4 mice per group for each time point. CyA indicates cyclophilin A.
The appearance of F4/80⁺ blood-derived mononuclear cells after Ad-PR39 treatment was enhanced in both groups compared with untreated mice. However, similar to untreated groups, the extent of the appearance of these cells was delayed in HCE mice. Furthermore, the HCE mice demonstrated less pronounced increase in HIF-1α expression, a target of PR39 therapy, compared with control mice.

A number of differences in Ad-PR39–induced angiogenic gene expression between wild-type and hypercholesterolemic mice were observed in this model that could shed some light on the delayed arteriogenic response in the latter group. Compared with control mice, LDLR⁻/⁻/Apob-48⁻/⁻ mice demonstrated reduced expression of HIF-1α, FGFR-1, VCAM-1, cyclophilin A, and SR-A 3 days after arterial ligation. HIF-1α regulates expression of a number of genes involved in neovascularization, including the VEGF family and angiopoietin-1, among others.²³ The reduced expression of this transcription factor is likely to result in decreased levels of a number of important proangiogenic growth factors. Similarly, reduced FGFR-1 expression can be expected to result in attenuated FGF signaling, which is regulated predominantly at the level of receptor expression.²⁵

VCAM-1 is an important adhesion molecule that may play a role in the accumulation of monocytes/macrophages and other cells around growing collateral vessels, a potentially important step in the regulation of arteriogenesis,¹⁹,²⁶–²⁸ This is supported by the observed reduction in expression of SR-A, a major scavenger receptor expressed almost exclusively by macrophages,²⁹ suggesting reduced monocyte-macrophage infiltration of tissues, as supported by the F4/80⁺ data. This reduced presence of mononuclear cells is probably responsible for the aforementioned decrease in HIF-1α expression.³⁰–³² Finally, cyclophilin A is involved in cell growth and affects endothelial cells and smooth muscle cells proliferation.³³ The reduction in its expression is in accord with the observed decrease in the arterial growth in these mice.

Interestingly, the expression of all of the above-mentioned genes with the exception of SR-A in hypercholesterolemic mice “caught up” to levels seen in control animals by day 7, the time when delayed arteriogenesis was taking place. However, another set of genes, including VEGF-D, FGF-7, the time-of-flight quantification, and involved in arteriogenesis, including the VEGF family and angiopoietin-1, among others.²³ The reduced expression of this transcription factor is likely to result in decreased levels of a number of important proangiogenic growth factors. Similarly, reduced FGFR-1 expression can be expected to result in attenuated FGF signaling, which is regulated predominantly at the level of receptor expression.²⁵

VCAM-1 is an important adhesion molecule that may play a role in the accumulation of monocytes/macrophages and other cells around growing collateral vessels, a potentially important step in the regulation of arteriogenesis,¹⁹,²⁶–²⁸ This is supported by the observed reduction in expression of SR-A, a major scavenger receptor expressed almost exclusively by macrophages,²⁹ suggesting reduced monocyte-macrophage infiltration of tissues, as supported by the F4/80⁺ data. This reduced presence of mononuclear cells is probably responsible for the aforementioned decrease in HIF-1α expression.³⁰–³² Finally, cyclophilin A is involved in cell growth and affects endothelial cells and smooth muscle cells proliferation.³³ The reduction in its expression is in accord with the observed decrease in the arterial growth in these mice.

Interestingly, the expression of all of the above-mentioned genes with the exception of SR-A in hypercholesterolemic mice “caught up” to levels seen in control animals by day 7, the time when delayed arteriogenesis was taking place. However, another set of genes, including VEGF-D, FGF-7, and endoglin, were significantly reduced in LDLR⁻/⁻/Apob-48⁻/⁻ mice compared with the control group, and their expression remained depressed at day 14. Although it is uncertain what effect these differences in expression had on the observed incomplete arteriogenic response in these mice, several factors should be considered. VEGF-D possesses the strongest angiogenic effects among other VEGF variants and may be particularly involved in arteriogenesis, given its ability to bind fli-1.³⁴ Endoglin is a transforming growth factor-β coreceptor expressed primarily on endothelial cells and involved in vascular remodeling, most likely a key process in collateral development.³⁵ FGF-7 function is largely unknown, although it has been shown to regulate endothelial cell migration.³⁶

In summary, the timing of arteriogenic response, but not its magnitude, is delayed in untreated hypercholesterolemic compared with strain- and age-matched control mice. The hypercholesterolemic mice further display delayed and reduced arteriogenic response to a growth factor therapy. In both cases, the delay in arteriogenesis correlates with delayed tissue appearance of F4/80⁺ mononuclear cells. We conclude that hypercholesterolemia impairs both the native arteriogenesis and the ability to respond to growth factor therapy by reducing tissue accumulation of blood-derived mononuclear cells.

Acknowledgments

This study was supported in part by National Institutes of Health grants HL-70247 and HL-53793 (Dr Simons). We would like to thank Amy Hall (Dartmouth Medical School [DMS]) for her help with mouse breeding, Nicholas Shworak (DMS) for helpful advice with array studies, and Ebo DeMuinck (DMS) for his help with the hindlimb ischemia model.

Disclosure

Dr Simons has received a significant research grant from the National Institutes of Health.

References

It has long been suspected that the presence of atherosclerosis not only leads to disease of the arterial circulation but also impairs compensatory adaptations to the progressive compromise of the arterial blood flow. One such adaptation is the development of collateral circulation. The growth of collaterals is a poorly understood process that involves either remodeling of the preexisting vasculature, de novo formation of the new arterial tree, or a combination of both processes. In any case, the presence of shear stress leading to accumulation of the circulating mononuclear cells is thought to be required for collateral formation. Studying a murine model of atherosclerosis that is less severe than is typically found in patients with atherosclerotic cardiovascular disease, we found that atherosclerotic mice developed collaterals significantly required for collateral formation. It has long been suspected that the presence of atherosclerosis not only leads to disease of the arterial circulation but also impairs compensatory adaptations to the progressive compromise of the arterial blood flow. One such adaptation is the development of collateral circulation. The growth of collaterals is a poorly understood process that involves either remodeling of the preexisting vasculature, de novo formation of the new arterial tree, or a combination of both processes. In any case, the presence of shear stress leading to accumulation of the circulating mononuclear cells is thought to be required for collateral formation. Studying a murine model of atherosclerosis that is less severe than is typically found in patients with atherosclerotic cardiovascular disease, we found that atherosclerotic mice developed collaterals significantly later than their strain-matched counterparts. Interestingly, this delay correlated with the late appearance of blood-derived mononuclear cells in the area of collateral growth. Furthermore, when treated with an angiogenic agent, hypercholesterolemic mice responded with collateral growth later and to a lesser extent than strain-matched controls. In this case too, the delay correlated with the reduced and delayed appearance of blood-derived mononuclear cells. Thus, the presence of atherosclerosis delays the native arteriogenic response as well as delaying and diminishing a response to a proangiogenic agent. These observations may explain why the proangiogenic therapies so effective in normal animals have largely failed in patients with advanced atherosclerosis. Furthermore, the molecular defect responsible for this failure may be related to reduced accumulation of mononuclear cells at the site of collateral development.

**CLINICAL PERSPECTIVE**

It has long been suspected that the presence of atherosclerosis not only leads to disease of the arterial circulation but also impairs compensatory adaptations to the progressive compromise of the arterial blood flow. One such adaptation is the development of collateral circulation. The growth of collaterals is a poorly understood process that involves either remodeling of the preexisting vasculature, de novo formation of the new arterial tree, or a combination of both processes. In any case, the presence of shear stress leading to accumulation of the circulating mononuclear cells is thought to be required for collateral formation. Studying a murine model of atherosclerosis that is less severe than is typically found in patients with atherosclerotic cardiovascular disease, we found that atherosclerotic mice developed collaterals significantly later than their strain-matched counterparts. Interestingly, this delay correlated with the late appearance of blood-derived mononuclear cells in the area of collateral growth. Furthermore, when treated with an angiogenic agent, hypercholesterolemic mice responded with collateral growth later and to a lesser extent than strain-matched controls. In this case too, the delay correlated with the reduced and delayed appearance of blood-derived mononuclear cells. Thus, the presence of atherosclerosis delays the native arteriogenic response as well as delaying and diminishing a response to a proangiogenic agent. These observations may explain why the proangiogenic therapies so effective in normal animals have largely failed in patients with advanced atherosclerosis. Furthermore, the molecular defect responsible for this failure may be related to reduced accumulation of mononuclear cells at the site of collateral development.
Delayed Arteriogenesis in Hypercholesterolemic Mice
Daniela Tirziu, Karen L. Moodie, Zhen W. Zhuang, Katie Singer, Armin Helisch, Jeff F. Dunn, Weiming Li, Jaipal Singh and Michael Simons

Circulation. 2005;112:2501-2509
doi: 10.1161/CIRCULATIONAHA.105.542829
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/16/2501

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/