Lipid Lowering Promotes Accumulation of Mature Smooth Muscle Cells Expressing Smooth Muscle Myosin Heavy Chain Isoforms in Rabbit Atheroma

Masanori Aikawa, Elena Rabkin, Sami J. Voglic, Helen Shing, Ryozo Nagai, Frederick J. Schoen, Peter Libby

Abstract—Smooth muscle cells (SMCs) in the atherosclerotic intima characteristically differ from those in the arterial media, for example, by reduced expression of SMC differentiation/maturation markers such as smooth muscle myosin heavy chain isoforms (SM1 and SM2). This study tested the hypothesis that lipid lowering promotes maturation of intimal SMCs in 33 rabbits subjected to balloon injury and cholesterol feeding (0.3%) for 4 months (Baseline group, n=15); some of which then were switched to a low-cholesterol diet for 8 months (Low group at 8 months, n=3) or 16 months (Low group at 16 months, n=10). The remaining rabbits continued to consume a high-cholesterol diet for 16 months (High group, n=5). We monitored SMC phenotype by expression of immunoreactive α-smooth muscle actin, SM1, and SM2. α-Actin is an early marker, and SM1 and SM2 are late markers for SMC differentiation/maturation. Only fully differentiated or mature SMCs express SM2. Data are reported as the percentage of the α-actin-positive intimal area occupied by smooth muscle myosin–positive SMCs determined by color image analysis of immunostained sections. Levels of SM1 and SM2, highly expressed by SMCs in the normal aortic media (n=5) decreased in the aortic intima of the Baseline and High groups, indicating a less mature phenotype. In contrast, SM1 and SM2 increased in the Low (16 months) group, indicating that intimal SMCs exhibit a more mature phenotype after lipid lowering. Electron microscopy also showed the presence of mature intimal SMCs with abundant myofilaments. Furthermore, lipid lowering reduced levels of platelet-derived growth factor-B in the arterial intima, a factor known to suppress smooth muscle myosin expression. These data demonstrate that lipid lowering favors accumulation of mature SMCs in the atherosclerotic intima in association with reduced levels of platelet-derived growth factor-B expression. Intimal SMCs in the Low group also displayed reduced expression of matrix metalloproteinases-3 and -9 compared with the Baseline and High groups. These findings shed new light on the effects of lipid lowering at the level of the vascular wall, which may influence the biology of the atheroma. (Circ Res. 1998;83:1015-1026.)

Key Words: atherosclerosis ■ smooth muscle cell ■ differentiation ■ hypercholesterolemia ■ lipid lowering

Smooth muscle cells (SMCs) in the normal aortic media express a series of genes encoding contractile proteins such as α-smooth muscle actin, SM-22, caldesmon, and 2 isoforms of smooth muscle myosin heavy chain (SM1 and SM2). The expression of those proteins serves as a marker for SMC differentiation and maturation. α-Actin appears at an earlier stage of vascular development than other contractile proteins and provides an early marker for SMC differentiation/maturation. Markers of late stages of SMC differentiation/maturation include 2 isoforms of smooth muscle myosin isoforms. Expression of smooth muscle myosin isoforms is restricted highly to SMCs. SM1 is first detected in SMCs at the late fetal stage, and SM2 is expressed only after birth. In particular, SM2 is a useful marker for fully differentiated or mature SMCs. Thus, the combined assessment of α-actin, SM1, and SM2 expression permits monitoring of SMC phenotypes.

In human coronary arteries and aortae, intimal SMCs have decreased expression of smooth muscle myosin isoforms during aging and the progression of atherosclerosis, first of
SM2 and then of SM1, whereas the expression of α-actin is maintained, indicating that intimal SMCs undergo phenotypic modulation toward an immature state. The molecular and cellular mechanisms underlying this phenotypic shift remain unknown. Platelet-derived growth factor (PDGF)-B chain can suppress smooth muscle myosin expression on cultured SMCs, but the potential role of this factor in vivo is speculative.

Intimal SMCs of injured rabbit arteries and human coronary arteries after angioplasty also have reduced expression of smooth muscle myosin isoforms. Interestingly, these intimal SMCs appear to regain a more mature phenotype with time after injury as determined by increased smooth muscle myosin expression.

Arterial SMCs also contain at least 2 types of nonmuscle myosin heavy chain isoforms. Kuro-o et al isolated cDNA clones encoding rabbit and human nonmuscle myosin (SMemb), which are predominantly expressed by embryonic SMCs. SMemb is identical to myosin heavy chain (MHC)-B. SMemb/MHC-B expression increases in neointimal SMCs after vascular injury in both animals and humans as well as in fibroblasts in culture, so-called myofibroblasts in rejected rat and monkey hearts after transplantation, and activated mesangial cells in rat models of glomerular disease. The other nonmuscle myosin expressed in SMCs is MHC-A. However, expression of this isoform does not change substantially during vascular development compared with SMemb/MHC-B (M.A. and R.N., unpublished observations, 1993).

Intimal SMCs can overexpress certain genes that probably contribute to arterial lesion formation and coronary events. Expression of matrix metalloproteinases (MMPs) including MMP-1, MMP-3, and MMP-9 increases in the fibrous cap of unstable atheromatous plaques of human arteries, indicating a role for matrix-degrading proteinases in the pathogenesis of plaque vulnerability and rupture, resulting in acute coronary events.

Recent clinical trials have shown that lipid lowering reduces coronary events and mortality. These studies suggest that lipid lowering stabilizes the vulnerable plaque. Several animal studies have demonstrated decreased macrophage accumulation and lipid content and increased extracellular matrix content during lipid lowering. However, these previous studies have not addressed the molecular and cellular mechanisms underlying the stabilization of atheromatous plaque. We recently have demonstrated that dietary lipid lowering reduces macrophage accumulation and protease expression and activity and increases collagen content within the aortic lesions of hypercholesterolemic rabbits. However, the effects of lipid lowering on SMC phenotype have not been evaluated.

The present study investigated the hypothesis that lipid lowering can promote maturation of intimal SMCs in atherosclerotic rabbits. We report here that dietary lipid lowering promotes the accumulation of mature SMCs in the intima of aortic lesions in atherosclerotic rabbits as assessed by immunohistochemistry for smooth muscle and nonmuscle myosin isoforms and by transmission electron microscopy. Furthermore, we demonstrate reduced expression of PDGF-B chain in the arterial intima during lipid lowering, providing a potential mechanism of increased expression of smooth muscle myosin isoforms. To determine functional significance of maturation of intimal SMCs, we also examined expression of MMPs by and proliferative activity of SMCs.

**Materials and Methods**

**Animal Experimental Protocol and Diet**

Thirty-six New Zealand White male rabbits (2.5 to 3 kg; Millbrook Farm, Amherst, Mass) were housed individually in stainless-steel cages. All experiments were performed in accordance with a protocol approved by the Standing Committee on Animals of Harvard Medical School. Thirty-three animals consumed an atherogenic diet (certified Purina Rabbit Chow, 5322, 95% with 0.3% cholesterol and 4.7% coconut oil, Research Diets) for 4 months to induce atheroma formation. Fifteen animals killed at 4 months comprised the Baseline Group. Five animals continued the atherogenic diet for an additional 16 months (High Group). The remaining animals consumed a chow diet with no added cholesterol or fat for 8 months (Low Group [8 mos]) or 16 months (Low Group [16 mos]).

Figure 1. Experimental protocol. Atheroma was created in 33 rabbits by feeding of the atherogenic diet for 4 months and balloon injury on the thoracic aorta 1 week after initiation of the diet. Fifteen animals killed at 4 months comprised the Baseline Group. Five animals continued the atherogenic diet for an additional 16 months (High Group). The remaining animals consumed a chow diet with no added cholesterol or fat for 8 months (Low Group [8 mos]) or 16 months (Low Group [16 mos]).
the atherogenic diet. In 1 group (n=13), we switched from the atherogenic diet to purified chow with no added cholesterol and fat to reduce blood lipid levels. Three of these rabbits were killed after an additional 8 months (Low group at 8 months) and 10 were killed 16 months after changing diet (Low group at 16 months). The remaining rabbits continued to consume a cholesterol- and lipid-supplemented diet (0.05% to 0.2% cholesterol and 4.95% to 4.8% coconut oil). The amount of dietary cholesterol supplementation in this group was adjusted during this period based on serial lipid determinations to avoid levels of cholesterolemia (>1000 mg/dL) that would produce manifestations of liver disease. All of these rabbits on a continued hypercholesterolemic diet were killed 16 months after assignment to the dietary condition (High group, n=5). The collagen content, expression of MMPs, and characterization of cell populations in these animals is the subject of a separate report.30

Figure 2. Reduced expression of SM1 and SM2 in plaque's fibrous cap overlying macrophage accumulation in the Baseline lesion. SM1-positive SMCs were less numerous than α-actin-positive cells, and SM2 was nearly undetectable in the intima, whereas medial SMCs stained positive for both SM1 and SM2. Arrowhead indicates the internal elastic lamina. Bar=200 μm. Original magnification ×100.

Figure 3. Continued suppression of SM1 and SM2 expression in the intima after 16 months of continued hypercholesterolemia. The number of SM1-positive SMCs in the intima was lower than that of α-actin-positive cells. SM2 was not detected in the intima. Bar=200 μm. Original magnification ×100.
Low Cholesterol, 8 months

![Image showing low cholesterol, 8 months](image)

**Figure 4.** After 8 months of lipid lowering, few macrophages localized in the intima. SM1 expression increased in the intima compared with that in the Baseline and High groups. SM2 expression was still suppressed, although some intimal cells stained positively. Most medial SMCs stained positively for SM1 and SM2. Arrowhead indicates the internal elastic lamina. Bar=200 μm. Original magnification ×100.

Plasma Cholesterol and Triglyceride Levels
Peripheral blood was collected from the ear artery under local anesthesia for measurement of plasma cholesterol and triglyceride concentrations by enzymatic assays (Sigma Diagnostics).

Low Cholesterol, 16 months

![Image showing low cholesterol, 16 months](image)

**Figure 5.** After 16 months of lipid lowering, many intimal SMCs stained positively for both SM1 and SM2, indicating that intimal SMCs exhibit a mature phenotype similar to medial SMCs. Arrowhead indicates the internal elastic lamina. Bar=200 μm. Original magnification ×100.

Tissue Preparation
Rabbits were killed by intravenous sodium pentobarbital (120 mg/kg). Heparin (100 U/kg) was injected simultaneously to avoid blood clotting. The aortae were excised and rinsed briefly with...
Figure 6. SM1 and SM2 expression in SMCs. Quantitative data for SM1 and SM2 expression in the intima of rabbit atheroma and in the media of the aorta of normal rabbits. The data were reported as the percentage of the α-actin-positive area occupied by SM1- or SM2-positive cells (myosin:α-actin ratio [%]) quantified by computer-assisted color image analysis. Expression of SM1 and SM2 was significantly reduced in the intima of the Baseline and High groups compared with medial SMCs of normal aorta. Lipid lowering produced statistically significant increase in SM1 and SM2 expression at 16 months (Low 16 mos) whereas both SM1 and SM2 were suppressed after 16 months of continued hypercholesterolemia (High 16 mos). Control indicates normal aorta (n = 3); Baseline, Baseline group (n = 15); High, High group at 16 months (n = 5); Low 8 mos, Low group at 8 months (n = 3); Low 16 mos, Low group at 16 months (n = 10); and bars, SEM.

DMEM (BioWhittaker) without serum. The proximal portion of the thoracic aorta (5 mm thick, 2 to 7 mm below the ligamentum arteriosus) was excised and snap-frozen with OCT Compound (Sakura Finetek, Inc) in isopentane prechilled with liquid nitrogen for fresh-frozen sections for PDGF-B, MMP-3, and MMP-9 staining. An adjacent portion of the aorta (7 to 12 mm below the ligamentum arteriosus) was fixed with 95% ethanol and 1% glacial acetic acid for immunohistochemistry for myosin isoforms, α-actin, and macromolecules. Paraffin-fixed tissues were embedded in paraffin by conventional procedures.

Immunohistochemistry
Paraffin-embedded and fresh-frozen tissues were sectioned in 6-μm slices from the proximal end. Sections were preincubated with 0.3% hydrogen peroxide and Protein Block Serum-Free (X0909, Dako Corp, Carpinteria, Calif). Mouse monoclonal antibodies against rabbit SM1, rabbit SM2, rabbit SMEm (E0354, Dako A/S, Glostrup, Denmark), human α-smooth muscle actin (1A4, Dako A/S, Glostrup, Denmark), human PDGF-B (PGF 007, a gift of Mochida Pharmaceutical Co, Tokyo, Japan), rabbit MMP-3 (148-I-A3, Oncogene Research Products, Cambridge, Mass), and human MMP-9 (7-11C, Oncogene Research Products) were applied and incubated for 60 minutes at room temperature. Sections were incubated with biotinylated anti-mouse immunoglobulins (E0354, Dako A/S; or BA-2000, Vector Laboratories, Burlingame, Calif) for 30 minutes and then incubated with horseradish peroxidase–labeled (for paraffin sections) or alkaline phosphatase–labeled (for frozen sections) streptavidin solution (Vectorstain Standard, PK-6100 or AK-5000, Vector Laboratories) for 30 minutes. Slides were rinsed in phosphate-buffered saline (pH 7.4) after each incubation step. Peroxidase activity was revealed by diaminobenzidine (Dako Corp), or Sigma Fast Red. Slides were counterstained with hematoxylin and mounted.

In Situ Hybridization for Histone mRNA
To determine replication of intimal SMCs, in situ hybridization for histone mRNA was performed using the Hyb-Probe Detection System (Shandon/Lipshaw) according to the protocol recommended by the provider. Briefly, fresh-frozen sections were fixed with 4% paraformaldehyde for 5 minutes and hybridized with FITC-labeled oligonucleotide cocktail for histone H2B, H3, and H4 mRNA (Shandon/Lipshaw) at 65°C for 10 minutes and then at 37°C for 2 hours. Sections were rinsed with Tris-buffered saline (pH 7.6) containing 0.1% Triton X-100 and then incubated with alkaline phosphatase–conjugated antibody against FITC at 37°C for 30 minutes. Alkaline phosphatase activity was revealed by incubation with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate chromogen at room temperature for 12 hours. Sections were washed in running water to stop the reaction. Then, immunohistochemistry for SMCs (1A4) was performed as mentioned above. Sections from the Low group were counterstained briefly with methyl green. Quality control was performed using FITC-labeled random oligomers (as a negative control probe) and a FITC-labeled poly d(T) probe (as a positive control). FITC-labeled oligonucleotide cocktail for histone mRNAs were applied for human cancer tissues as an additional positive control.

Quantitative Analysis for Histology and Statistics
Analysis of immunohistochemistry for α-actin, SM1, SM2, and PDGF-B was performed using a personal computer-based quantitative 24-bit (16.2 million unique combinations) color image analysis system32 by S.J.V., who did not know the background of each animal. Photographs were scanned into a 1 K×1 K image buffer of the Optimas 5.2 image analysis system (Optimas Co). A color threshold mask for immunostaining was defined to detect the red color by sampling, and the same threshold was applied to all specimens. The percentage of the α-actin–positive intimal area occupied by SM1– or SM2-positive SMCs for each section was recorded to determine phenotype of intimal SMCs. The percentage of the positive area of PDGF-B staining within the intima also was measured. Statistical testing used 1-way ANOVA. The differences between 3 groups were determined by post-hoc tests.

Transmission Electron Microscopic Study
Aortic rings were obtained from the thoracic aortae (12 to 15 mm below the ligamentum arteriosum) of 3 animals from each group and divided into 2 pieces for electron microscopy and immunohistochemistry for SM2. Few, if any, SMCs in the intima of 6 animals from the Baseline and High groups stained positively for SM2, and the majority of intimal SMCs of the Low group were positive. Tissue processed for electron microscopy was fixed with Karnovsky’s fixative (2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer; pH 7.4) followed by postfixation in 2.0% osmium tetroxide. Tissue was dehydrated in ethanol, treated in propylene oxide, and embedded in Poly/Bed 812 medium (Polysciences). All specimens were rinsed and stained en bloc with uranyl acetate before ethanolic dehydration. Five to six thick sections per animal were examined. Thin sections were cut at 60 nm, stained with lead citrate and uranyl acetate, and examined with a JOEL-100CX transmission microscope (JOEL USA, Inc) at an accelerating voltage of 80 kV.

Results
Plasma Lipids
At the beginning of the experiment, the mean total cholesterol and triglyceride levels (in mg/dL) were 43±4 and 53±14, respectively and rose to 1562±123 and 244±49 after 4 months on the atherogenic diet (0.3% cholesterol and 4.7% coconut oil), as previously reported30 (see Table, page 1023). The total plasma cholesterol and triglycerides levels returned to baseline after 8 months on the control diet lacking supplemental lipids (Low group at 8 months, total cholesterol, 53±3, triglycerides, 53±14; Low group at 16 months, total cholesterol, 19±3, triglycerides, 58±10) but remained elevated in the High group (total cholesterol, 1108±158; triglycerides, 224±87).
Intimal SMCs in the Atheroma of Hypercholesterolemic Rabbits Have Reduced Expression of SM1 and SM2

The intimal lesions of the aortae after 4 months of atherogenic diet (Baseline lesions) contained a superficial SMC layer detected with anti-α-actin antibody (1A4). This layer subtended numerous macrophages identified with the RAM11 monoclonal antibody, resembling the “fibrous cap” of human coronary lesions (Figure 2, top). These intimal SMCs exhibited decreased expression of SM1 and SM2 compared with medial SMCs (Figure 2, bottom). In particular, expression of SM2, a marker for mature SMCs, was nearly undetectable in the intima. After 16 months of continued hypercholesterolemia, the intimal SMC layer overlying macrophage accumulation showed suppression of SM1 and SM2 expression (Figure 3).

Intimal SMCs of the Rabbit Atheroma Regained Smooth Muscle Myosin Expression During Lipid Lowering

After 8 months of dietary lipid lowering, the region of SM2-positive SMCs was much smaller than that of α-actin-positive cells (Figure 4). After 16 months, many intimal

Figure 7. Reduced expression of a non-muscle myosin (SMemb/MHC-B) in the intima during lipid lowering. Some α-actin–positive cells in the intima of the Baseline and High groups stained positively for SMemb/MHC-B. After 16 months of lipid lowering, SMemb/MHC-B was nearly undetectable. Arrowheads indicate the internal elastic lamina. Bar=200 μm. Original magnification ×100.
SMCs stained positively for both SM1 and SM2 (Figure 5). To quantitate these results, the percent of the α-actin-positive intimal area occupied by SM1- or SM2-positive SMCs for all animals studied was determined by computer-assisted color image analysis (Figure 6). SM1 and SM2 expression at 16 months compared with baseline lesions increased significantly (SM1, \(P<0.001\); SM2, \(P<0.001\)).

Nonmuscle Myosin Expression in the Atheroma Decreased During Lipid Lowering

In all animals in the Baseline and High groups, some of the α-actin–positive intimal SMCs stained positively for SMemb/MHC-B, whereas the tunica media contained few if any SMemb/MHC-B–positive SMCs (Figure 7, top and middle). Expression of SMemb/MHC-B was nearly undetectable in...
the intima of all animals in the Low group at 16 months (Figure 7, bottom).

Intimal SMCs in the Aorta of Treated Animals Show Features of a Mature Phenotype With Abundant Myofilaments Detected by Transmission Electron Microscopy

In addition to SMC-specific protein expression, we performed transmission electron microscopy to examine SMC morphology. SMCs within the intima of the Baseline and High groups showed characteristics of modulated SMCs with well-developed rough endoplasmic reticulum and Golgi apparatus, and scant myofilaments (Figure 8, left and middle). In contrast, many intimal SMCs after 16 months of dietary lipid lowering contained numerous myofilaments with focal density (dense body) and paucity of synthetic organelles, characteristics of a well-differentiated phenotype resembling the medial SMCs of mature animals (Figure 8, right).

Lipid Lowering Reduced Lesional Expression of PDGF-B

Macrophages and SMCs within the lesions of the Baseline and the High groups expressed PDGF-B chain, which is known to suppress smooth muscle myosin expression in cultured SMCs (Figure 9, top). However, expression of PDGF-B decreased in the Low group after 16 months of dietary lipid lowering (Figure 9, bottom left). The double immunostaining for PDGF-B (blue) and macrophages (CD11b, red) demonstrates localization of immunopositive PDGF-B in both macrophages and SMCs in the intima of the Baseline animal. PDGF-B–expressing macrophages stained purple as a result of the mixture of blue and red. The blue spindle-shaped cells are PDGF-B–positive SMCs (Figure 9, bottom right). Quantification of the percentage of positive areas of PDGF-B immunostaining on all animals by computer-assisted color image analysis showed a statistically significant decrease with lipid lowering (Figure 10).

Expression of MMP-3 and MMP-9 by and Proliferation of Intimal SMCs Decreased During Lipid Lowering

To determine functional significance of maturation of intimal SMCs, immunohistochemistry for MMP-3 and MMP-9 and in situ hybridization for histone mRNA were performed. Intimal SMCs in both Baseline (Figure 11, top) and High (data not shown) groups with reduced SM2

Figure 10. Quantitative analysis of PDGF-B expression. Lipid lowering produced a statistically significant reduction in PDGF-B expression in the aortic intima. Data are reported as the percentage of positive areas in the intima for immunostaining for PDGF-B quantified by computer-assisted color image analysis. Baseline indicates Baseline group (n=15); High, High group at 16 months (n=5); Low 8 mos, Low group at 8 months (n=3); Low 16 mos, Low group at 16 months (n=10); and bars, SEM.

Figure 11. Immunohistochemistry for MMP-3 and MMP-9 expression by intimal SMCs in the Baseline and Low group animals. Top, SM2 expression was reduced in the intima of the Baseline lesion, whereas the medial SMCs stained positively. Such intimal SMCs with immature phenotype express MMP-3 and MMP-9. The double immunostaining for MMP-3 (red) or MMP-9 (red) and macrophages (CD11b, blue) displayed expression of MMP-3 and MMP-9 by intimal SMC. MMP-3– or MMP-9–positive macrophages stained purple as a result of mixture of red and blue. Red spindle-shaped cells are MMP-positive SMCs in the intima. Bottom, Intimal SMCs with SM2 expression in the intima of the Low group animals showed reduced expression of MMP-3 and MMP-9. Bar=50 μm. Original magnification ×400.
expression stained positively for anti–MMP-3 and anti–MMP-9 antibodies. However, in the Low (16 months) group, MMP-3 and MMP-9 were almost undetectable in SMCs, which displayed high levels of SM2 expression (Figure 11, bottom). Based on the expression of histone mRNA, which peaks in cells during the S phase of the cell cycle and disappears rapidly during the G2 phase,33,34 SMCs replicate in atheroma of the Baseline (Figure 12, top) and High (data not shown) groups. However, in the Low group, few if any intimal cells contained histone mRNA (Figure 12, bottom). Most cells stained intensely with a poly d (T) probe (positive control) and no signals were detected with random oligomers (negative control) (data not shown). In the sections of human lung cancer, almost all cells were strongly positive for histone mRNA probe (data not shown). The results regarding expression of MMPs and histone are typical of 5 animals from each group in the study.

Discussion
This study demonstrates the presence of mature SMCs in the arterial intima of hypercholesterolemic rabbits after dietary lipid lowering as determined by increased expression of smooth muscle myosin isoforms and typical ultrastructure for mature SMCs. Furthermore, we show that reduced expression of PDGF-B chain provides a potential mechanism for accumulation of mature SMCs. Decreased expression of MMP-3 and MMP-9 by SMCs after lipid lowering additionally affirms the functional significance of accumulation of mature SMCs in atheroma.

Intimal SMCs in the Atheroma Showed Immature Phenotype Gauged by Decreased Expression of Smooth Muscle Myosin Isoforms
Reduced expression of smooth muscle myosin isoforms has demonstrated phenotypic modulation of SMCs toward an immature state in culture and in vascular hyperplastic lesions. Smooth muscle myosin expression decreases in cultured SMCs.1,35 Reduced myosin expression also occurs in the arterial SMCs after mechanical injury and cholesterol-feeding of rabbits,5,9,36 and in rat or monkey coronary arteries after heart transplantation.15 Aikawa et al6,10 further demonstrated reduced expression of human SM1 and SM2 within the intimal SMCs of the aortae and coronary arteries and within the neointimal SMCs of human coronary arteries after angioplasty.

In the present study, an SMC layer resembling the fibrous cap of human coronary lesions overlaid the macrophage-rich atheromatous core in the Baseline and High groups. The percentage of SM1- or SM2-positive cells among the α-actin–positive population in these atheroma was significantly lower compared with that of medial SMCs in normal control animals, indicating that many lesional SMCs (α-actin–positive and smooth muscle myosin–negative) display features of immature SMCs at early developmental stages. These findings regarding reduced expression of smooth muscle myosin within α-actin–positive SMCs in rabbit atheroma match observations on chronic atherosclerotic lesions of human coronary arteries.6

Continued Suppression of Smooth Muscle Myosin Isoforms in the Rabbit Atheroma by High-Cholesterol Feeding
Okamoto et al9 previously demonstrated that neointimal SMCs of the rabbit aorta created by balloon injury without high-cholesterol feeding regain SM2 expression within 2 months. Aikawa

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**Plasma Cholesterol and Triglyceride Levels**

<table>
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<tr>
<th></th>
<th>Precholesterol Feeding</th>
<th>Baseline</th>
<th>High, 16 Months</th>
<th>Low, 8 Months</th>
<th>Low, 16 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol, mg/dL</strong></td>
<td>43 ± 4 (31–53)</td>
<td>1562 ± 123 (854–2658)</td>
<td>1108 ± 158 (752–1697)</td>
<td>53 ± 3 (45–58)</td>
<td>19 ± 3 (15–30)</td>
</tr>
<tr>
<td><strong>Triglycerides, mg/dL</strong></td>
<td>52 ± 14 (23–86)</td>
<td>244 ± 49 (44–507)</td>
<td>224 ± 87 (93–480)</td>
<td>53 ± 14 (33–80)</td>
<td>58 ± 10 (35–83)</td>
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Values are mean ± SEM and range.
et al. recently reported that neointimal SMCs on human coronary arteries 6 months after angioplasty express both SM1 and SM2. However, in the present study, intimal SMCs in the rabbit atheroma of the High group continued to show features of immaturity even at 20 months after balloon injury, probably because of hypercholesterolemia.

PDGF-B chain is the only mediator currently known to suppress smooth muscle myosin expression. Holycross et al. demonstrated that PDGF-BB, but not PDGF-AA, suppresses smooth muscle myosin expression by cultured SMCs. The β-receptor for PDGF likely mediates this effect of PDGF-B chain, because the SMCs used in their study lack the α-receptor. Lesional macrophages and SMCs in both human and experimental atherosclerosis express PDGF-B detected immunohistochemically using the same monoclonal antibody (PGF007) employed here. We now report overexpression of PDGF-B in rabbit atheroma. This result provides a potential mechanism for the continued suppression of smooth muscle myosin protein expression in atheroma of hypercholesterolemic rabbits.

Intimal SMCs in the Intima of Hypercholesterolemic Rabbits Regain Mature Phenotype During Lipid Lowering

The present study demonstrates that intimal SMCs in atherosclerotic rabbits exhibit a mature phenotype during lipid lowering. A reduction in serum cholesterol levels increased the expression of smooth muscle myosin isoforms, first of SM1 and then of SM2, in SMCs of the plaque’s fibrous cap. Lipid lowering also decreased nonmuscle myosin (SMemb/MHC-B) expression, which is augmented in activated mesenchymal cells such as immature SMCs, proliferating SMCs, and myofibroblasts.

In addition to myosin expression, features of mature SMCs in treated animals were determined by transmission electron microscopy as well. Kim et al. and Kimura et al. previously demonstrated that SMCs with reduced expression of smooth muscle myosin show features of the so-called “synthetic” phenotype with abundant cytoplasmic organelles such as rough endoplasmic reticulum and Golgi apparatus, and few myofilaments detected by transmission electron microscopy, as shown in the present study. We confirmed maturation of intimal SMCs during lipid lowering via the presence of abundant myofilaments, features typical of the so-called “contractile” state of SMCs.

We recently demonstrated that long-term lipid lowering reduced macrophage accumulation in these rabbit atheroma. The present study shows a concomitant decrease in PDGF-B expression in the intima of the treated animals. This inverse relationship between smooth muscle myosin and PDGF-B expression suggests 1 possible mechanism of maturation of intimal SMCs in atheroma by lipid lowering. The recent isolation and characterization of the SM1/2 and SMemb/MHC-B genes should facilitate understanding the molecular mechanisms that regulate smooth muscle and nonmuscle myosin gene expression. Such studies may clarify the mechanisms not only of SMC differentiation/maturation but also of plaque stabilization.

The greater abundance of SMCs exhibiting a “mature” phenotype in the arterial intima after lipid lowering could result from several mechanisms. We favor the explanation that immature SMCs in the atherosclerotic rabbit intima originate from mature SMCs in the media and regain more mature phenotype by lipid lowering, by mechanisms discussed above. Alternatively, some intimal SMCs may originate from an immature subpopulation of medial SMCs and acquire mature phenotype during lipid lowering. The present data cannot distinguish between these and other possible origins of the population of mature SMCs in the intima after lipid lowering.

Maturation of SMCs in the Fibrous Cap of Rabbit Atheroma as a Potential Mechanism of Stabilization of Human Atherosclerotic Plaques

Acute coronary syndromes such as unstable angina or myocardial infarction often result from rupture of vulnerable plaques. “Vulnerable plaques” are characterized by a thin SMC-layer (fibrous cap) overlying macrophage accumulation in the intima. Intimal SMCs differ from medial SMCs with respect to gene expression, function, and morphology. For example, SMCs in the fibrous cap also overexpress matrix-degrading enzymes such as MMP-1, MMP-3, and MMP-9 that may weaken the fibrous cap and promote plaque rupture. In the present article, we demonstrated decreased expression of MMP-3 and MMP-9 in intimal SMCs after sustained lipid lowering. As in human atheroma, proliferation in a small population of intimal SMCs in rabbit atheroma was detected by in situ hybridization for histone mRNA. However, after lipid lowering, few if any intimal SMC displayed this sign of replication. These results are consistent with recent observations by Kockx et al. demonstrating reduced proliferation of vascular cells of hypercholesterolemic rabbits during lipid lowering.

Residence in the intima of “mature” SMCs, gauged by increased expression of smooth muscle myosin isoforms and morphology, indicates a restoration of a more normal environment by lipid lowering. Elucidation of such alterations in the biology of the plaque will help us gain a mechanistic understanding of the reduced acute coronary events and death achieved by lipid lowering in patients at risk.

Acknowledgments

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References


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