Hypercholesterolemia Causes Mechanical Weakening of Rabbit Atheroma
Local Collagen Loss as a Prerequisite of Plaque Rupture

Mark D. Rekhter, Gary W. Hicks, David W. Brammer, Hussein Hallak, Erick Kindt, Jing Chen, Wendy S. Rosebury, Maureen K. Anderson, Paul J. Kuipers, Michael J. Ryan

Abstract—Hypercholesterolemia may render atherosclerotic plaques prone to rupture. To test this hypothesis, catheters with matrix-covered balloons were implanted into the aorta of rabbits fed standard or 0.5% cholesterol chow (n = 70). In 1 month, fibrous plaques developed around the balloon. Time-dependent accumulation of cholesteryl esters and free cholesterol was detected in the plaques of the cholesterol-fed group only. The pressure needed to rupture the plaque by balloon inflation was used as an index of plaque strength. Three months after the catheter implantation, the breaking pressure was 2.1 times lower (P < 0.05) in cholesterol-fed rabbits. It was accompanied by collagen loss, as measured by plaque hydroxyproline content, but not with deficiency of collagen cross-linking. Sirius red staining showed preservation of collagen originally covering the balloon and accumulation of nascent collagen in the lesions of standard chow-fed rabbits. In the cholesterol-fed group, both mature and new collagen underwent degradation predominantly in the plaque shoulders. Collagen breakdown was associated with local accumulation of foamy macrophages. Gel zymography demonstrated relative enhancement of gelatinolytic activity at 92 and 72 kDa, as well as caseinolytic activity at 57, 45, and 19 kDa in the lipid-laden plaques. Lipid accumulation in the plaque was also associated with a loss of smooth muscle cells, the cellular source of the collagen fibers. The remaining smooth muscle cells showed increased collagen synthesis, although it was insufficient to counterbalance collagen degradation and cell loss. Thus, we have obtained direct evidence that hypercholesterolemia is accompanied by enhanced local collagen degradation, which is potentially responsible for plaque weakening. (Circ Res. 2000;86:101-108.)

Key Words: arteriosclerosis ■ cholesterol ■ collagen ■ macrophage ■ plaque rupture

Physical disruption of atherosclerotic plaque is a major cause of thrombosis and subsequent acute clinical manifestations of atherosclerosis, ie, unstable angina, myocardial infarct, and stroke.1 Several lines of evidence suggest a mechanistic association between lipids and plaque rupture. Hypercholesterolemia is the strongest predictor of acute coronary events.2 Pathological studies have demonstrated that ruptured human plaques usually contain large lipid pools.2 Clinical trials have shown that cholesterol-lowering therapy markedly reduces cardiovascular mortality.3 However, it is not completely understood how hypercholesterolemia leads to plaque rupture.

The following mechanism has been proposed4: hypercholesterolemia induces macrophage accumulation and activation in the atheroma; macrophages synthesize and secrete proteolytic enzymes, matrix metalloproteinases (MMPs); MMPs destroy collagen, thereby weakening the plaque. Although this mechanism looks very plausible, several key questions remain. First, the changes in plaque mechanical properties have long been assumed, although never directly demonstrated. Second, the most convincing evidence of the link between lipids and collagen was obtained in reverse sequence, when lipid lowering led to collagen accumulation in rabbit atheroma.5 It still remains to be seen whether lipid accumulation induces collagen breakdown. Third, collagen content is a net result of its degradation and synthesis. The role of collagen synthesis in plaque destabilization has not yet been addressed. Fourth, tissue mechanical properties depend not only on collagen content, but also on its cross-linking and distribution.6 These factors have not been studied in the context of plaque rupture.

We have recently described an animal model of atherosclerosis in which a plaque, formed around an inflatable balloon (Figure 1), can be ruptured at will.7 In the current study, rupturing pressure was used to measure plaque mechanical strength. We have tested the hypothesis that hypercholesterolemia induces local collagen loss and subsequent plaque mechanical destabilization. We report hypercholesterolemia-induced plaque weakening associated with changes in collagen content, architecture, and turnover. These data provide
the first direct measurement of reduced mechanical strength in lipid-rich plaques and suggest a mechanism for their destabilization that leads to acute coronary events.

Materials and Methods
The experimental strategy was to (1) develop lipid-poor and lipid-rich lesions around inflatable balloons, (2) determine rupturing pressure by inflation of intraplaque balloons, and (3) evaluate relationships between rupturing pressure and plaque architecture.

Our model of plaque rupture has been recently described. Catheters with matrix-covered balloons (Figure 1) were implanted into the aorta of rabbits fed standard or 0.5% cholesterol chow (n=70). Balloon coverage facilitated plaque formation and served as a substrate to evaluate in vivo collagenolytic activities. Animals were euthanized 1, 2, and 3 months after catheter implantation. The pressure needed to rupture the plaque by balloon inflation was used as an index of plaque strength. All procedures that used animals were conducted in compliance with state and federal laws, as well as guidelines established by the Parke-Davis Animal Care and Use Committee.

Lesion Formation
Catheters with matrix-covered balloons (Figure 1) were implanted into the aorta of rabbits fed standard or 0.5% cholesterol chow (n=70). Balloon coverage facilitated plaque formation and served as a substrate to evaluate in vivo collagenolytic activities. Animals were euthanized 1, 2, and 3 months after catheter implantation. The pressure needed to rupture the plaque by balloon inflation was used as an index of plaque strength. All procedures that used animals were conducted in compliance with state and federal laws, as well as guidelines established by the Parke-Davis Animal Care and Use Committee.

Results
Plasma Cholesterol, Lipid Accumulation, and Lesion Formation
At all time points, plasma cholesterol was significantly higher in cholesterol-fed rabbits compared with standard chow–fed rabbits. In cholesterol-fed rabbits, plasma cholesterol increased in a time-dependent manner and reached a plateau at 2 month after surgery. Plasma cholesterol levels in standard chow–fed rabbits were 40±4, 40±3, and 100±74 mg/dL, whereas in the cholesterol-fed group they reached 426±171, 1974±295, and 1740±400 mg/dL at 1, 2, and 3 months after catheter implantation, respectively.

The collagen-covered balloons were firmly attached to the aortic wall and were covered with fibrotic tissue in all animals, regardless of the diet. However, balloon-associated lesions were bigger in cholesterol-fed animals (Table 1). Free cholesterol and cholesterol esters accumulated in the plaques of cholesterol-fed rabbits in a time-dependent manner and significantly exceeded respective concentrations in the lesions of chow-fed animals (Table 2). Phospholipid content was also higher in hypercholesterolemic rabbits, although it did not significantly change over time. Triglyceride accumulation did not differ among the experimental groups (Table 2).

Rupturing Pressure
Balloon-associated plaques were ruptured ex vivo by inflation of intraplaque balloons. Representative pressure-volume curves obtained from 3-month-old plaque-covered balloons are illustrated in Figure 2. A notable inflection in the trace coincided with the balloon opening, and plaque fracture was observed visually. Plaque-rupturing pressure did not significantly change over time in chow-fed rabbits, whereas it gradually decreased in cholesterol-fed rabbits (Figure 3). At 3 months, the rupturing pressure in cholesterol-fed rabbits was 40% less than in the standard chow-fed rabbits. Thus, the cholesterol diet reduced plaque mechanical strength in a time-dependent manner.

Analysis of video images revealed that the weakening of the plaque was associated with changes in the location of the fissure. In chow-fed rabbits, the fissure site averaged 2.5 to 3 arbitrary units at each of the 3 time points (Figure 4). This indicates that on average plaque fisses occurred midway between the plaque cap and base region. Plaque fisses also occurred at the midpoint in 1- and 2-month-old plaques from high-cholesterol rabbits. However, by the third month, the fissure site had shifted toward the base, i.e., shoulder region, as indicated by the increase to 4.6 arbitrary units. The shift in the fissure location to the base of the plaque was coincident with a reduction in rupturing pressure. This strongly suggests the local nature of plaque weakening.

Collagen Content and Cross-Linking
Tissue mechanical strength is dependent on collagen content and cross-links between collagen molecules. Plaque collagen content was expressed as hydroxyproline concentration, whereas collagen cross-linking was characterized by the ratio of pyridinoline to hydroxyproline. In the standard chow group, hydroxyproline concentration did not change over time. In the cholesterol-fed group, there was a significant, time-dependent decrease in hydroxyproline content (Figure 5A). The degree of collagen cross-linking did not differ between standard chow–fed and cholesterol-fed groups. However, in both groups, the pyridinoline/hydroxyproline ratio increased in a time-dependent manner. At 3 months, both lipid-poor and lipid-rich plaques had a significantly higher ratio than their respective 2-month-old lesions (Figure 5B). Progressive elevation of pyridinoline bonds most likely reflects collagen maturation and appears to be diet independent. Thus, plaque weakening was associated with an overall collagen loss, but not with a deficiency in the number of collagen cross-links.
Collagen Distribution
In our model, balloon-associated plaques contain 2 collagen pools (Figure 1): the first pool contains “old” collagen, which was originally wrapped around the balloon, and the second pool contains “new” collagen, a product of plaque smooth muscle cells (SMCs), that occupied the lesion periphery. In the standard diet group, old collagen was preserved over the 3-month period, and new collagen accumulated in a time-dependent fashion. In contrast, lipid-rich plaques exhibited time-dependent collagen loss in the shoulder region, whereas old collagen was preserved and new collagen accumulated in the fibrous cap (Figures 5C, 5D, and 6). The presence of old collagen is a unique feature of this model. It served as a substrate for accumulated in vivo collagenolytic activities, thereby enabling unequivocal interpretation of histological data (any hole in the old collagen layer was interpreted as a signature of local collagen breakdown). Local collagen loss in the shoulder area corroborates our data on preferential localization of plaque fissures.

Cellular Composition
Plaques from standard chow rabbits contained very few macrophages, whereas macrophage accumulation was a prominent feature of lipid-rich lesions (Table 1). In both groups, the number of macrophages did not significantly change over time (Table 1). In lipid-rich plaques, the vast majority of macrophages were located in the shoulder areas, whereas fibrous caps were virtually macrophage-free (Figure 6).

SMCs progressively accumulated in the lesions of standard chow–fed rabbits and reached plateau at 2 months after catheter implantation (Table 1). They evenly occupied both cap and shoulder regions (Figure 7). In the cholesterol-fed group, the number and distribution of SMCs did not differ from their standard chow counterparts at 1 month. However, at 2 months the number of SMCs in cholesterol-fed group did not increase and was significantly less than in standard chow group. At 3 months, the number of SMCs in the cholesterol-fed group dramatically dropped and became significantly smaller than both 2-month cholesterol-fed and 3-month standard chow–fed counterparts (Table 1). SMC loss took place almost exclusively in the plaque shoulders (Figure 7). It is likely that observed loss of actin-positive SMCs may be at least partially attributed to changes in SMC phenotype. How-

<table>
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<tr>
<th>Parameters by Diet</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Lesion size, mm²</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0.9±0.1</td>
<td>1.8±0.5</td>
<td>1.3±0.3</td>
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<td>2.7±0.5*</td>
<td>2.7±0.5*</td>
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<tr>
<td>Cell density (number of nuclei per mm²)</td>
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<tr>
<td>Standard</td>
<td>1133±112</td>
<td>1328±116</td>
<td>1284±73</td>
</tr>
<tr>
<td>Cholesterol-fed</td>
<td>1422±70</td>
<td>1138±73</td>
<td>856±32†</td>
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<td>Macrophages (RAM-11–positive cells per mm²)</td>
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<td></td>
</tr>
<tr>
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<td>48.4±20.6</td>
<td>47.6±16.5</td>
<td>18.6±5.7</td>
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<tr>
<td>Cholesterol-fed</td>
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<td>625.4±96.3*</td>
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<td>SMCs (HHF-35–positive cells per mm²)</td>
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<td>802.5±121.0†</td>
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<td>474.5±52.7</td>
<td>164.2±36.5</td>
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<td>76.4±42.4</td>
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<td>148.6±13.8*</td>
<td>266.0±87.8*</td>
<td>148.4±58.4*</td>
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<td>Microvessel profiles per mm²</td>
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<tr>
<td>Standard</td>
<td>10.5±2.8</td>
<td>11.5±2.1</td>
<td>9.4±1.5</td>
</tr>
<tr>
<td>Cholesterol-fed</td>
<td>10.5±1.8</td>
<td>6.3±1.1</td>
<td>9.4±2.2</td>
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</tbody>
</table>

Values are mean±SEM; n=5 per group.
*Statistically significant (P<0.05) differences between cholesterol-fed and chow-fed groups regardless of time, combined with no significant effect (P>0.05) of diet-time interaction by 2-way ANOVA.
†Statistically significant (P<0.05) differences between cholesterol-fed and standard chow–fed groups at individual time points by Student t test, combined with statistically significant (P<0.05) effect of diet-time interaction by 2-way ANOVA.
ever, the simultaneous decrease in the number of immunostainable SMCs, increase in the number of immunostainable macrophages, and decrease in total cell number (Table 1) are indicative of true SMC loss.

We did not find a statistically significant difference in microvessel density between standard and cholesterol-fed rabbits at any time point (Table 1). Hemosiderin depositions were found in both types of lesions with relatively equal frequency. These facts most likely reflect the specifics of our animal model and do not necessarily uncover the role of plaque microvessels and associated hemorrhages in human plaque rupture.

Thus, local collagen loss in lipid-rich plaques was spatially and temporally associated with macrophage accumulation and SMC depletion, whereas in this animal model it did not appear to be microvessel-dependent.

**Collagen Synthesis**

One month after catheter implantation, lesions from both groups contained similar numbers of procollagen-positive cells (Table 1). Plaques from standard chow–fed rabbits contained fewer collagen-producing cells at 2 months and exhibited almost no synthetic activity at 3 months (Table 1, Figure 7). In contrast, the number of procollagen-positive cells dramatically increased in 2-month-old, lipid-rich plaques. At 3 months, lipid-rich lesions were still characterized by a significant number of collagen-synthesizing cells, although the number of positive cells was lower than that at 2 months (Table 1, Figure 7). Both plaque caps and shoulders displayed procollagen positivity. The relative decline in collagen production between 2 and 3 months was associated with local (in the shoulder area) loss of SMCs, a predominant source of collagen in atherosclerotic plaques. Thus, lipid accumulation in the plaque was associated with a severe loss of SMCs. The remaining SMCs showed increased collagen synthesis, although it was insufficient to counterbalance collagen degradation and SMC loss.

**MMP Activity**

Because the most dramatic collagen loss in lipid-rich plaques was detected at 3 months, gel zymography studies were limited to the 3-month group. Negative control samples (normal rabbit aorta) exhibited gelatinolytic activity at 72 kDa (pro–MMP-2) only and no caseinolytic activity (Figure 8). In contrast with normal aorta, both types of plaques also

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**TABLE 2. Lipid Accumulation in Rabbit Balloon-Associated Plaques**

<table>
<thead>
<tr>
<th>Tissue Content</th>
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<td>Tissue by Diet</td>
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<td>Cholesterol</td>
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<td>Cholesterol-fed</td>
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<tr>
<td>Cholesteryl esters</td>
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<tr>
<td>Cholesterol-fed</td>
<td>193±17*</td>
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<tr>
<td>Triglycerides</td>
<td>Standard</td>
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<tr>
<td>Cholesterol-fed</td>
<td>58±38</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Standard</td>
</tr>
<tr>
<td>Cholesterol-fed</td>
<td>42±42*</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=6 per group. *Statistically significant (P<0.05) differences between cholesterol-fed and standard chow–fed groups regardless of time, combined with no significant effect (P>0.05) of diet-time interaction by 2-way ANOVA.

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**Figure 2.** Representative pressure-volume curves obtained from a plaque-covered balloon 3 months after catheter placement. A, Standard chow–fed rabbit. B, Cholesterol-fed rabbit. Degassed water was infused into the balloon at a constant rate (0.5 mL/min). The abrupt pressure fall coincided with the balloon opening and plaque fracture observed visually. Pressure needed to rupture the plaque by balloon inflation was used as an index of plaque strength.

**Figure 3.** Cholesterol diet reduces plaque mechanical strength in a time-dependent manner. Data are average±SEM; n=6 per group. *Statistically significant (P<0.05) differences between cholesterol-fed and chow-fed groups regardless of the time, combined with no significant effect (P>0.05) of diet-time interaction by 2-way ANOVA.

**Figure 4.** Location of the fissures in the different plaque regions (based on the analysis of video recordings). Images of ruptured plaques were analyzed in a blinded fashion by 2 independent observers, and location of the fissure was coded from 0 to 6, where 0 represented the plaque cap, 6 represented the plaque base, and 3 represented an intermediate site between the plaque cap and base. Data are average±SEM; n=6 per group. Graph demonstrates that at 1 and 2 months the site of disruption was randomly distributed between plaque caps and shoulders in both groups of plaques. However, at 3 months the majority of fissures in the plaques of chow-fed rabbits were located in the cap area, whereas the majority of fissures in the plaques of cholesterol-fed rabbits were located in the shoulder region.
Hypercholesterolemia is a risk factor for plaque rupture and myocardial infarct. Plaque rupture occurs when external mechanical forces exceed the tensile strength of vulnerable regions of the plaque. It is implied, although never directly shown, that vulnerable plaques are mechanically weak. Our data are the first to demonstrate a causal relationship between hypercholesterolemia and plaque mechanical destabilization. Moreover, we have shown that dietary manipulation can be used to engineer both “stable” and “unstable” plaques, and rupturing pressure may be a measure of plaque stability.

Mechanical strength of atherosclerotic plaques is primarily determined by fibrillar collagen, specifically by collagen content, cross-linking, and distribution. We demonstrated a time-dependent decrease of overall collagen content in lipid-rich plaques, whereas the level of cross-linking did not differ between high- and low-cholesterol groups. These results indicate that collagen loss, rather than defective assembling, is most likely responsible for plaque destabilization. It still needs to be determined why a relatively modest decrease in collagen content led to a significant impairment of plaque strength. The functional consequences of collagen loss may be highly dependent on the local nature of this phenomenon. We found that collagen loss occurred primarily in plaque shoulders. It is feasible that the interface between highly cross-linked (ie, hard) collagen in the cap and the virtually collagen-free (ie, soft) shoulder creates a local stress concentration that rendered plaques prone to rupture. Preferential location of the fissures in the shoulders of lipid-rich lesions, which has been demonstrated in human plaques and reproduced in our animal model, corroborates this hypothesis.

A mechanistic link between hypercholesterolemia and collagen loss is still hypothetical, although a critical role of macrophages, as the major source of MMPs and other proteolytic enzymes, has been strongly suggested. Although our data do not elucidate the definitive mechanism of collagen degradation, observed temporal and spatial associations between lipid deposition, macrophage accumulation, and collagen breakdown present strong circumstantial evidence that hypercholesterolemia leads to plaque weakening via macrophage-dependent collagen degradation. At the same time, our data suggest that MMPs may not be solely responsible for macrophage-driven collagen breakdown. Those proteolytic enzymes playing a major role in plaque collagen degradation have yet to be identified. As in the case of other phenomena associated with inflammatory infiltrates, increased lytic activity may be very focal. Although such localized phenomena could significantly change the dynamics of the local environment, the effect would be lost when averaged at the tissue scale. The mechanism by which hypercholesterolemia leads to expression and/or activation of relevant proteolytic enzymes is another unknown.

Recently, Aikawa et al demonstrated that lipid lowering reduced MMP activity and increased collagen content of the rabbit atheroma. Our findings complement these data, although MMP changes in our model were not as dramatic. Taken together, both studies show the same phenomenon from the perspective of collagen loss caused by lipid accumulation and by collagen preservation as a result of lipid lowering.

Collagen content is the net result of its synthesis and degradation. As discussed above, collagen degradation was prominent in lipid-rich lesions. Interestingly, we have demonstrated that collagen production was also increased in these lesions. We found type I procollagen-synthesizing cells in both fibrous caps and shoulders. However, collagen breakdown prevailed in the shoulders and led to an overall local collagen loss. Thus, even enhanced collagen production was insufficient to replenish its breakdown. Because virtually every SMC in the shoulders was procollagen-positive, but the

Discussion

Recently, we described an animal model of atherosclerosis, in which a plaque formed around an inflatable balloon can be ruptured at will. The current study, in which rupturing pressure was used to measure plaque mechanical strength, demonstrates in this model that hypercholesterolemia impaired lesion mechanical properties.

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Figure 6. Collagen loss is accompanied by macrophage accumulation in plaque shoulders. A through D, Plaques from standard chow-fed rabbits. E through H, Plaques from cholesterol-fed animals 3 months after catheter placement. A, B, E, and F, Sirius red staining, polarization microscopy. Interstitial collagen exhibits strong birefringence. C, D, G, and H, Macrophage-specific RAM-11 immunostaining (red product of immunoalkaline phosphatase reaction). Collagen is preserved in plaque shoulders (A) and caps (B) in standard chow-fed group. These lesions are virtually macrophage-free (C and D). In the cholesterol-fed group, collagen is lost (E) in plaque shoulders, infiltrated by macrophages (G), but preserved in the cap area (F), where macrophages were not accumulated. Magnification, ×20. Specimens are oriented as in Figure 5.
Figure 7. Type I collagen synthesis is enhanced in lipid-rich plaques but limited by SMC number. A through D, Plaques from standard chow–fed rabbits. E through H, Plaques from cholesterol-fed animals 3 months after catheter placement. A, B, E, and F, Type I procollagen SPI.D8 immunostaining (red product of immunoalkaline phosphatase reaction). C, D, G, and H, Muscle actin-specific HHF-35 immunostaining (red product of immunoalkaline phosphatase reaction). In the standard chow–fed group, plaque shoulders (C) and caps (D) are occupied by SMCs. These cells are procollagen-negative (A and B). In the cholesterol–fed group, significant numbers of cells in both shoulders (E) and caps (F) are procollagen-positive. However, collagen production in shoulders is limited, because large areas are devoid of SMCs (G). In contrast, fibrous caps are enriched by SMCs (H). Magnification, ×20. Specimens are oriented as in Figure 6.
number of SMCs decreased, it appears that this relative deficiency of collagen production was determined by local SMC depletion. At the same time, lack of collagen degradation in the cap area combined with increased synthesis led to overall collagen accumulation. These results show that in our model, (1) hypercholesterolemia simultaneously stimulated plaque growth and destabilization, and (2) destabilization was determined by collagen breakdown combined with local loss of cellular source of collagen synthesis, rather than by inhibition of collagen gene expression. It remains to be determined how hypercholesterolemia led to SMC loss, although induction of SMC apoptosis is likely. It was demonstrated that SMCs undergo apoptosis in rabbit atherosclerotic plaques and that cholesterol withdrawal inhibits apoptosis.10

Thus, we have demonstrated that hypercholesterolemia induced local collagen loss and plaque destabilization. Identification of molecular mechanisms of this phenomenon may generate new targets for pharmacological plaque stabilization and prevention of acute coronary events.

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