Lipid Lowering by Diet Reduces Matrix Metalloproteinase Activity and Increases Collagen Content of Rabbit Atheroma
A Potential Mechanism of Lesion Stabilization

Masanori Aikawa, MD, PhD; Elena Rabkin, MD, PhD; Yoshikatsu Okada, MD, PhD; Sami J. Voglic; Steven K. Clinton, MD, PhD; Constance E. Brinckerhoff, PhD; Galina K. Sukhova, PhD; Peter Libby, MD

Background—Proteolytic enzyme activity in lipid-rich atheroma may promote plaque rupture and precipitate acute coronary syndromes. This study tested the hypothesis that lipid lowering stabilizes plaques by reducing proteolytic activity.

Methods and Results—We produced experimental atheroma in 33 rabbits by balloon injury and an atherogenic diet (0.3% cholesterol and 4.7% coconut oil) for 4 months. At that time, 15 rabbits were killed (baseline group). The remaining animals were divided into two groups: a hyperlipemic group continued to consume a cholesterol-enriched diet (0.05% to 0.2%) for 16 more months (n=5) and a lipid-lowering group consumed a purified chow diet with no added cholesterol or fat for 8 (n=3) or 16 months (n=10). Macrophage accumulation and interstitial collagenase (matrix metalloproteinase-1, MMP-1) expression in the lesion were measured by quantitative image analysis of immunostained aortas. Baseline lesions expressed high levels of MMP-1 and contained many macrophages. These features of plaque instability persisted in the hyperlipemic group. However, the lipid-lowering group showed progressive reduction in both macrophage content and MMP-1 immunoreactivity with time. Aortic rings of the baseline and hyperlipemic groups elaborated gelatinolytic, caseinolytic, and elastinolytic activity attributable to MMP-2, MMP-3, or MMP-9, monitored by SDS-PAGE zymography. Proteolytic activity decreased markedly in the lipid-lowering group. Aortic content of interstitial collagen, determined by sirius red staining, increased in the lipid-lowering group compared with the baseline or continued hyperlipemic groups, indicating that lipid lowering reinforced the fibrous skeleton of the atheroma.

Conclusions—These results establish a mechanism by which lipid lowering may stabilize vulnerable plaques by reduced expression and activity of enzymes that degrade the arterial extracellular matrix and render atheroma less susceptible to disruption and thrombosis by favoring collagen accumulation in the fibrous cap.

Key Words: metalloproteinases ■ atherosclerosis ■ diet ■ hypercholesterolemia ■ collagen

Disruption of the atheromatous plaque participates in the pathogenesis of thrombus formation and consequent acute coronary syndromes such as unstable angina and acute myocardial infarction.1–3 Pathologic studies have distinguished several features of ruptured plaques.4 Lesions that have caused fatal coronary thrombus typically contain a large lipid core underlying a thin and collagen-poor fibrous cap. Rupture-prone lesions also usually have prominent macrophage accumulation.5 Macrophage-rich areas are frequently found in coronary plaques of patients with acute coronary syndromes.6

We and others have found that lesional macrophages produce proteolytic enzymes including members of the MMP family. Henney et al7 described stromelysin (MMP-3) expression by macrophages within atheromatous lesions by in situ hybridization for mRNA. We demonstrated the expression of at least three types of MMPs within human atherosclerotic lesions.8 Shah et al9 demonstrated that cultured macrophages can digest collagen obtained from the human fibrous cap. Our further studies described constitutive expression of MMPs by the macrophage foam cells within atheroma of hypercholesterolemic rabbits.10 These data suggest that macrophage-related proteolysis within atheroma may contribute to weakness of the protective fibrous cap of the plaque and hence promote the propensity of those plaques to rupture and trigger thrombosis.11

Recent clinical trials have shown repeatedly that lipid lowering can reduce coronary events and mortality rates.12–14

Received November 25, 1997; revision received December 29, 1997; accepted January 1, 1998.
From the Vascular Medicine and Atherosclerosis Unit, Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School (M.A., E.R., Y.O., S.J.V., G.K.S., P.L.), and the Department of Medicine, Dana-Farber Cancer Institute, Harvard Medical School (S.K.C.), Boston, Mass; and the Department of Medicine, Dartmouth Medical School (C.E.B.), Hanover, NH.
Correspondence to Dr Peter Libby, Vascular Medicine and Atherosclerosis Unit, Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, 221 Longwood Ave, LMRC 307, Boston, MA 02115.
E-mail plibby@rics.bwh.harvard.edu
© 1998 American Heart Association, Inc.
The substantial degree of clinical benefit appears out of proportion to the relatively modest improvement of the degree of stenosis produced by similar lipid-lowering regimens in angiographic studies. This disparity suggests that hypolipidemic treatment may somehow "stabilize" plaques in a qualitative manner independent of angiographically assessed lesion size itself. However, the precise molecular and cellular mechanisms that might produce such "stabilization" of atheroma remain conjectural. The classic works of Armstrong et al.16,17 and Small et al.18 as well as more recent investigations19,20 have suggested decreased macrophage number, decreased lipid content, and relative increase connective tissue during lipid lowering in animals. However, these previous studies have not generally addressed the biochemical and molecular aspects of the functions of lesional cells during lipid lowering.

This study tested in rabbits the hypothesis that lipid lowering stabilizes the atheromatous plaque by reducing the level and activity of proteinases that can degrade the key structural components of the arterial extracellular matrix and thereby reinforces the ability of the plaque to resist rupture. We report the changes produced by dietary lipid lowering in procoagulant activity, expression and activity of proteo-

clytic enzymes, and the amount of proteinases. Such lesions resemble the so-called "vulnerable" human atheroma more closely than the typical foam cell lesions in rabbits produced by hypercholesterolemia alone.20 Fifteen rabbits were killed 4 months after initiation of the atherogenic diet to evaluate the baseline lesions ("baseline group"). The remaining rabbits were divided into two groups with similar ranges of hypercholesterolemia to avoid bias caused by variations in individual responsiveness to the atherogenic diet. In one 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, 8m") and 10 were killed 16 months after changing the diet ("low group, 16m"). 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 on the basis of serial lipid determinations to avoid levels of cholesterol 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).

**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).

**Tissue Preparation**
Rabbits were killed by administration of intravenous sodium pentobarbital (120 mg/kg). Heparin (100 U/kg) was simultaneously injected to avoid blood clotting. The aortas were excised and rinsed briefly with Dulbecco’s Modified Eagle’s Medium (DMEM, BioWhittaker) without serum. The proximal portion of the thoracic aorta (2 mm below the ligamentum arteriosum) was excised and snap-frozen with OCT compound (Sakura Finetek Inc) in isopentane prechilled with liquid nitrogen for fresh-frozen sections for immunohistochemistry for TIMP-1 and sirius red staining. An adjacent portion of the aorta (7 mm below the ligamentum arteriosum) was fixed with 95% ethanol and 1% glacial acetic acid for immunohistochemistry for macrophages and MMP-1. Ethanol-fixed tissues were embedded in paraffin by conventional procedures. Another adjacent portion (12 mm below the ligamentum arteriosum) was excised for organoid culture.

**Immunohistochemistry**
Paraffin-embedded and fresh-frozen tissues were sectioned in 5-μm and 6-μm slices, respectively. Sections were preincubated with 0.3% hydrogen peroxide and Protein Block Serum-Free (X0909, Dako Corp). Mouse monoclonal antibodies against rabbit macrophages (RAM11, Dako Corp), rabbit MMP-1 (a gift of Dr Michael W. Lark, Merck Research Laboratories), human α-smooth muscle actin (1A4, Dako A/S), and human TIMP-1 (7 to 6C1, Oncogene Science, Inc) were applied and incubated for 60 minutes at room temperature. Sections were incubated with biotinylated anti-mouse rabbit immu-
noglobulins (E0354, Dako A/S) for 30 minutes and then incubated with horseradish peroxidase–labeled streptavidin solution (Vec-

**Methods**

**Animal Experimental Protocol and the Diet**
Thirty-three male New Zealand White rabbits (2.5 to 3 kg) were individually housed in stainless steel cages. All experiments were performed in accordance with protocol approved by the Standing Committee on Animals of Harvard Medical School. Fig 1 schematizes the disposition of the animals. All 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.21 (Fig 1) One week after initiation of the atherogenic diet, we injured the thoracic aortas by withdrawal of a 4F Fogarty embolectomy catheter introduced through the left iliac artery. This procedure was performed under general anesthesia by intramuscular injection of ketamine (35 mg/kg)/xylazine (7 mg/kg) and local anesthesia of the inguinal region by lidocaine. The balloon injury accelerates atheroma formation, renders lesions more uniform in size and distribution, and produces plaques with a smooth muscle–rich fibrous cap overlying a layer of lipid-laden macrophages. Such lesions resemble the so-called “vulnerable” human atheroma more closely than the typical foam cell lesions in rabbits produced by hypercholesterolemia alone.20 Fifteen rabbits were killed 4 months after initiation of the atherogenic diet to evaluate the baseline lesions (“baseline group”). The remaining rabbits were divided into two groups with similar ranges of hypercholesterolemia to avoid bias caused by variations in individual responsiveness to the atherogenic diet. In one 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, 8m”) and 10 were killed 16 months after changing the diet (“low group, 16m”). 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 on the basis of serial lipid determinations to avoid levels of cholesterol 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).

**Figure 1.** Thirty-three New Zealand White rabbits were fed an atherogenic diet for 4 months to create the atheroma. The balloon injury on the thoracic aortas was performed 1 week after initiation of the atherogenic diet. Fifteen rabbits killed at 4 months comprised the baseline group. Five animals continued to consume the atherogenic diet for 16 more months (high group). The remaining animals consumed a chow diet with no added cholesterol and fat for 8 (low 8m group) or 16 months (low 16m group).
tastain Elite Standard, PK-6100, Vector Laboratories) for 30 minutes. Slides were rinsed in phosphate-buffered saline (pH 7.4) after each incubation step. Peroxidase activity was revealed by aminoethylcarbazole (AEC, K3464, Dako Corp). Slides were counterstained with hematoxylin and mounted.

Organoid Culture
Excised aortic rings were blotted, weighed, and rinsed with DMEM. Approximately 100 mg aortic rings were cut into three pieces and incubated with DMEM without serum at 37°C in humidified 5% CO₂ and 95% air for 48 hours. Conditioned media were collected and concentrated with Ultrafree-4 Centrifuge Filter Units (Millipore Corp).

SDS-PAGE Zymography
To detect gelatinolytic, caseinolytic, or elastinolytic activity in the conditioned media of aortic rings, zymographic analysis with a 7.5% acrylamide gel containing 0.2% gelatin or casein, or 0.12% α-elastin was performed. Briefly, samples for SDS-PAGE were not boiled before the electrophoresis under nonreducing conditions. After electrophoresis, the substrate gels were soaked twice with Triton-X-100 solution (2.5%) for 30 minutes each at room temperature to remove SDS. The gels then were incubated in 50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, 5 mmol/L CaCl₂, 0.02% NaN₃, and 0.05% Brij 35 for 24 hours at 37°C. The lysis of the substrates in the gels was visualized by staining with 2.5% Coomasie brilliant blue (Sigma Chemical Co).

Sirius Red Polarization Method for Collagen Staining
Sirius red polarization microscopy detects interstitial collagen. Birefringency under illumination with polarized light identifies collagen, including types I and III. Fresh-frozen sections (6 μm) were rinsed with distilled water and incubated with 0.1% sirius red F3BA (Polyscience Inc) in saturated picric acid for 90 minutes. Sections were rinsed with 0.01N HCl for 1 minute twice and then immersed with distilled water. After dehydration with 70% ethanol for 30 seconds, sections were coverslipped. The stained sections were observed under polarized light and photographed with the same exposure time for each section.

Quantitative Analysis for Histology and Statistics
Analysis of immunohistochemistry for macrophages, MMP-1, and sirius red staining was performed with a personal computer-based quantitative 24-bit (16.2 million unique combinations) color image analysis system. 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 total area with positive color for each section was recorded. For sirius red staining, negative background (black) was chosen for thresholding and the positive area was calculated by subtraction. Statistical testing used one-way ANOVA.

| TABLE 1. Plasma Cholesterol and Triglyceride Levels |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Before Cholesterol Feeding | Baseline | High 16m | Low 8m | Low 16m |
| Cholesterol, mg/dL | 43±4 | 1562±123 | 1108±158 | 53±3 | 19±3 |
| Triglycerides, mg/dL | 52±14 | 244±49 | 224±87 | 53±14 | 58±10 |
| (23–86)           | (44–507) | (93–480) | (33–80) | (35–83) |

Values are mean±SEM; ranges are shown in parentheses.

Figure 2. Aortic lesions of the baseline group contain numerous macrophage foam cells expressing MMP-1. Left and middle, RAM11-positive macrophages accumulate in the intima beneath a fibrous cap composed of smooth muscle cells detected by anti–α-actin antibody; right, MMP-1 is predominantly expressed by macrophages. Arrowhead indicates the internal elastic lamina. Scale bar, 200 μm. Original magnification ×100.
Figure 3. Reduced expression of MMP-1 associated with decrease in number of lesional macrophages. Top, Macrophages within the lesion of the high group continue to express MMP-1 strongly; middle, expression of MMP-1 decreased in the intima of the low group at 8 months after initiation of low cholesterol diet, accompanied by reduction of macrophage accumulation; bottom, at 16 months after cessation of the atherogenic diet, MMP-1 and macrophages are almost undetectable. Arrowheads indicate the internal elastic lamina. High 16m indicates high group at 16 months; Low 8m, low group at 8 months; and Low 16m, low group at 16 months. Scale bar, 200 μm. Original magnification ×100.
Results

Plasma Lipids
At the beginning of the experiment, mean total cholesterol and triglycerides levels (mg/dL) were 43 and 52, respectively, and rose to 1562 and 244 after 4 months on the atherogenic diet (0.3% cholesterol and 4.7% coconut oil) (Table 1). The total plasma cholesterol level returned to baseline after 8 months on the control diet devoid of supplemental lipids (low group) but remained elevated in the high group.

Lipid Lowering Reduces Lesional Macrophage Accumulation and MMP-1 Protein Expression
At baseline, the aortic lesions contained numerous macrophages identified by staining with RAM11 monoclonal antibody. These foam cells of macrophage origin accumulated in the intimal lesions beneath a layer of smooth muscle cells identified by anti-α-actin antibody 1A4 (Fig 2). MMP-1 (interstitial collagenase), a key member of matrix metalloproteinase family in initiating collagen degradation, localized predominantly in macrophages in the lesions at baseline (Fig 2). However, expression of immunoreactive MMP-1 decreased within the intima of the low group at 8 months after cessation of the atherogenic diet, accompanied by a reduction in macrophage number (Fig 3, middle), as verified by quantitative, computer-assisted image analysis (Fig 4). After 16 months on the chow diet, MMP-1 expression was almost undetectable (Fig 3, bottom). In contrast, lesional macrophages in the high group continued expression of elevated levels of MMP-1 (Fig 3, top). Fig 5 provides examples of immunoreactivity of MMP-1 in two individual animals from the low group (16 months) with the highest and lowest plasma cholesterol level at baseline before randomization. MMP-1 was detected on few cells in the aortic intima and adventitia in either animal, despite the disparate initial lipid levels.

Lipid Lowering Reduces Proteolytic Activity Elaborated by Aortic Rings
After an initial limited proteolytic cleavage by MMP-1, gelatinases continue the degradation of interstitial collagens. Blood vessels, like many other tissues, constitutively express the inactive zymogen form of one gelatinase, MMP-2. Cells in atheromatous plaques contain in addition an inducible gelatinase, MMP-9. SDS-PAGE zymography documented release of proteolytic activity for gelatin, casein, and elastin from aortic rings from all three groups (Fig 6). Aortic tissue from the baseline and high groups elaborated gelatinolytic activity at 92 kD (pro–MMP-9), 72 kD (pro–MMP-2), and 68 kD (an activated form of MMP-2), caseinolytic activity migrating at 52 kD (pro–MMP-3), as well as elastinolytic activity by all of these MMPs. However, proteolytic activity ascribable to MMP-9 (92 kD), MMP-3 (52 kD), or the activated form of MMP-2 (68 kD) was not produced by specimens from the low group (16 months) except for elastinolytic activity at 68 kD. At least three independent experiments with several samples from each group yielded qualitatively similar results (Table 2).

TIMP-1 Is Not Overexpressed in Atherosclerotic Lesions of Baseline and High Groups
If levels of the endogeneous inhibitors of MMP, the TIMPs, increased in tandem with the MMP-1 in atheroma, the net proteolytic balance could remain unchanged. To evaluate this possibility, we examined expression of TIMP-1 (Fig 7). The tunica media of all animals tested contained immunoreactive TIMP-1. The smooth muscle cell–rich fibrous cap of atheroma in all groups also displayed TIMP-1 expression. However, areas of increased MMP-1 expression by macrophages underlying the smooth muscle layer in baseline and “high” lesions (especially the lipid core) did not show high levels of TIMP-1, which indicated an excess of collagenolytic potential in these lesions.

Lipid Lowering Increases Interstitial Collagen Content of the Atherosclerotic Intima
We performed sirius red staining for collagen to test the hypothesis that lipid lowering, and concomitantly reduced
activity of enzymes that degrade collagen, would yield an increase in interstitial forms of collagen in the arterial intima with time. Sirius red staining under polarized light visualizes collagen, including types I and III. Aortas from the baseline group showed positive sirius red staining under polarized light in the media and adventitia only, demonstrating a low content of interstitial collagen in the intima of lesions at baseline (Fig 8, top). Lesions in the high group, subjected to continued hypercholesterolemia, exhibited some increase in aortic intimal collagen content with time (Fig 8, bottom left). However, the low group showed substantial accumulation of interstitial collagen in the intima of atherosclerotic lesions (Fig 8, bottom right). Quantitative color image analysis substantiated significant changes in intimal collagen content in the lipid-lowering group (Fig 9). In the baseline lesion, an inverse relation prevailed between regions of high level of MMP-1 expression and low collagen content (determined by sirius red polarization microscopy) (Fig 10, top). In contrast, the aortic intima of animals in the low group exhibited little or no MMP-1 expression and intense sirius red staining of interstitial collagen (Fig 10, bottom).

Discussion
This study demonstrates that lipid lowering by dietary manipulation significantly reduces proteolytic activity and increases collagen content of established atheroma in rabbits. As in previous studies, we documented that lipid lowering decreased numbers of macrophages in experimental atheroma. However, functional attributes of these and other lesional cells have not been investigated heretofore. The results presented here provide an experimental basis for understanding potential mechanisms for stabilization of atheromatous plaques.

A major finding of this study is an increased accumulation of interstitial collagen in the lesions of the lipid-lowering group at the end of the experiment compared with those at baseline and in animals with continuing hypercholesterolemia. Several mechanisms may account for this observation. Shah at al demonstrated that macrophages induce breakdown of collagen obtained from fibrous caps of human atherosclerotic plaques and that an MMP inhibitor partially blocked this process. This finding supports the concept that overexpression of MMPs in human atheroma can degrade collagen of the fibrous cap and promote plaque rupture. The decreased MMP activity during lipid lowering documented here should thus permit the accumulation of arterial extracellular matrix macromolecules such as interstitial collagen. Lipid lowering may decrease matrix-degrading protease expression by limiting the stimulus for gene transcription or by reducing activation or secretion of these enzymes. Previous studies have established that proinflammatory cytokines potently stimulate MMP expression in macrophages and other cells including smooth muscle. Lipid lowering may decrease the stimulus for cytokine gene expression in turn by permitting egress of lipids from the atherosclerotic intima and/or by reducing continued influx. Lipoproteins that accumulate in the intima of hypercholesterolemic animals may undergo...
modifications such as oxidation or glycation. Products associated with lipoproteins modified in this manner may augment local expression of proinflammatory cytokines. In the case of MMP-1, constituents of oxidatively modified lipoproteins that stimulate protein kinase C might link to the activator protein-1 (AP1)-mediated transcriptional regulation that plays a key role in controlling transcription of MMP-1. Gelatinase-B, also known as 92-kD gelatinase, or MMP-9, may contribute importantly to instability of human atherosclerotic plaques. The regulation of tran-

<table>
<thead>
<tr>
<th>Sample</th>
<th>52 kD</th>
<th>72 kD</th>
<th>68 kD</th>
<th>92 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>…</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>High</td>
<td>…</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Low</td>
<td>…</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Gelatin zymography

<table>
<thead>
<tr>
<th>Sample</th>
<th>52 kD</th>
<th>72 kD</th>
<th>68 kD</th>
<th>92 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>+</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>High</td>
<td>+</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>Low</td>
<td>–</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
</tbody>
</table>

Casein zymography

<table>
<thead>
<tr>
<th>Sample</th>
<th>52 kD</th>
<th>72 kD</th>
<th>68 kD</th>
<th>92 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>High</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Low</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Elastin zymography

<table>
<thead>
<tr>
<th>Sample</th>
<th>52 kD</th>
<th>72 kD</th>
<th>68 kD</th>
<th>92 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>High</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Low</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

PAGE substrate zymography was performed on serum-free aliquots of medium conditioned by an equivalent wet weight of descending thoracic aortic explants as described in “Methods.” Each sample was obtained from a separate individual animal. The zymographic activity was graded on a scale from – to ++++, corresponding to no activity or low, intermediate, or high activity.
scription of MMP-9 depends in part on a nuclear factor kappa B (NFκB) element in its promoter sequence. This transcription factor is known to be regulated by “oxidative stress” and may link the accumulation of oxidized lipoproteins in the intima to the expression of this particular protease.33–35

The reduced activity of extracellular matrix–degrading proteases in the lipid-lowered group suggests that decreased catabolism contributes to the arterial collagen accumulation documented here. A change in the levels of the endogenous inhibitors of MMPs, the TIMPs, might also yield a change in net proteolytic activity.36,37 The results of TIMP-1 immuno-
localization indicate lack of augmented levels of this inhibitor in the atherosclerotic intima in regions of MMP-1 overexpression. These observations support the concept that the net collagenolytic potential increases in the intimal lesions of baseline and high group rabbits that contain less interstitial collagen than nonatherosclerotic arteries or the lesions in the low group. In addition to a decrease in degradative rates, an increase in synthesis of collagen might contribute to the increase in collagen documented in the atheromatous arteries of animals with lowered lipids. Our prior work showed that interferon-γ, a lymphokine derived from activated T cells, can limit collagen biosynthesis by vascular smooth muscle cells. The accumulation of collagen described here may result from a decreased antigenic stimulus to T cells consequent to reduced lipoprotein levels in the intima. Oxidatively modified lipoproteins evoke not only a humoral immune response but also cellular immunity. Decreased antigenic stimulation to T cells consequent to reduced intimal lipoprotein could lower interferon-γ production and result in an increase collagen synthesis by releasing the inhibition caused by this lymphokine.

The interpretation of this study must consider several limitations. Rabbit models of atherosclerosis mimic the human situation imperfectly. In particular, spontaneous plaque

Figure 8. Interstitial collagen content in the aortic intima detected by the sirius red polarization method. Top left, Sirius red staining on the aorta of the baseline group without polarized light shows the thickened intima of the aorta; top right, serial aortic section in the baseline group shows positive sirius red staining under polarized light in the media and adventitia only; bottom left, aortic lesion of the high group shows some increase in interstitial collagen content with time; bottom right, aorta of the low (16m) group contains abundant interstitial collagen within the intima. Baseline indicates baseline group; High, high group at 16 months; Low, low group at 16 months. Scale bar, 400 μm. Original magnification ×40.

Figure 9. Quantitative analysis of interstitial collagen content. Percent of area positive for sirius red staining within the intima was determined by computer-assisted image analysis. Baseline indicates baseline group; High 16m, high group at 16 months; Low 8m, low group at 8 months; Low 16m, low group at 16 months. Bars represent SEM.
rupture does not occur in these animals, although provocative “triggering” stimuli may provoke arterial thrombosis in cholesterol-fed rabbits. The combination of an initial balloon injury with concomitant hypercholesterolemia was chosen to produce lesions that resemble those in humans more closely than lesions produced by hypercholesterolemia alone. Rabbit lesions produced by injury plus lipid do form a fibrous cap populated by actin-positive smooth muscle cells. However, human atheroma that typically form over decades may have features not modeled in relatively short-term rabbit experiments. Certainly, the degree of hypercholesterolemia produced in this rabbit preparation exceeds that usually encoun-

Figure 10. Inverse relation between MMP-1 (interstitial collagen) expression and interstitial collagen content. Top, Aortic lesion of the baseline group with high level of MMP-1 expression shows low collagen content within the intima detected by sirius red staining; bottom, aortic intima of the low group (16m) exhibits little MMP-1 expression and high collagen content. Baseline indicates baseline group; Low 16 months, low group at 16 months. Scale bar, 200 μm. Original magnification ×100.
tered in human patients. Also, the hyperlipoproteinemia induced by the atherogenic diet used here increases very low-density lipoproteins as well low-density lipoproteins. This pattern may resemble postprandial hyperlipoproteinemia and diabetic dyslipidemia more closely than conditions that more purely elevate low-density lipoprotein cholesterol such as familial hypercholesterolemia. Highly exaggerated levels of hypercholesterolemia in rabbits consuming atherogenic diets can lead to a systemic cholesterol ester storage disease that caricatures rather than mimics human atherosclerosis. Therefore we adjusted the cholesterol level in the diet in the animals subjected to prolonged hypercholesterolemia to limit this undesired aspect of experimental rabbit atherosclerosis. This rabbit model differs from human atherosclerosis in several respects and must therefore be extrapolated with caution to the clinical situation. Nonetheless, these experiments do establish unequivocally the novel principle that lipid lowering can influence qualitative aspects of lesions related to plaque stability.

Most of the clinical studies that inspired the present investigation used pharmacologic agents as a principle intervention to achieve lipid lowering. The recent large-scale clinical trials showing decreases in cardiovascular and total mortality used inhibitors of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase to this end.12-14 Accumulating evidence suggests that these agents may have direct effects on cells independent of systemic lipid lowering.42 For example, reductase inhibitors may inhibit the prenylation of intracellular molecules involved in signaling pathways.43,44 This study did not address these potential direct effects of reductase inhibitors at the level of the vessel wall. Because our experimental design examined hyperlipoproteinemia as an isolated variable, our results may actually underestimate potential effects on the end points measured that might pertain during therapy with HMG-CoA reductase inhibitors.

The results of these recent large-scale clinical studies have amply documented the decrease in cardiovascular events12 and total mortality accruing from lipid lowering.13,14 Yet angiographic studies performed with similar pharmacologic regimens show only modest improvement in luminal diameter.15 These results suggested to us that functional changes in the plaque at the cellular and biochemical level and alteration in the microanatomic attributes of the atheroma, poorly assessed by the angiogram, may explain the marked clinical benefits of lipid lowering. The results presented here demonstrate specific cellular and molecular alterations in plaques consequent to lipid lowering. We focused here on functions that may determine structural features of plaques related to the tendency to precipitate acute coronary syndromes. While other effects not studied here doubtless contribute, the present study illustrates how animal models may help unravel the underlying mechanisms of clinical benefits of therapies of hyperlipidemia. Identification of the cellular and molecular bases of the salutary effect of decreased lipid levels on the functions of atheroma may aid the identification of additional targets for therapeutic interventions in the future.

Acknowledgments
This work was supported in part by National Heart, Lung, and Blood Institute grant PO1 HL-48743 (to Dr Peter Libby). Dr Masanori Aikawa is an awardee of a Research Fellowship Grant from the Japan Heart Foundation. We acknowledge Eugenia Shvartz and Elissa Simon-Morrisey for their technical expertise. We also thank Dr Michael W. Lark for the anti–MMP-1 antibody, Dr Richard T. Lee for his help with computer analysis, and Kevin Mullally and his staff of the animal facility of Brigham and Women’s Hospital and Harvard Medical School for their excellent management of experimental animals.

References
5. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.


