Objective—Substantial evidence implicates interstitial collagenases of the matrix metalloproteinase (MMP) family in plaque rupture and fatal thrombosis. Understanding the compensatory mechanisms that may influence the expression of these enzymes and their functions, therefore, has important clinical implications. This study assessed in mice the unknown relative effect of the 2 principal collagenases on collagen content and other plaque characteristics.

Approach and Results—Apolipoprotein E−deficient (apoE−/−) mice, MMP-13−/− apoE−/−, MMP-8−/− apoE−/− double knockout mice, and MMP-13−/− MMP-8−/− apoE−/− triple knockout mice consumed a high-cholesterol diet for 10 and 24 weeks. Both double knockout and triple knockout mice showed comparable atherosclerotic lesion formation compared with apoE−/− controls. Analysis of aortic root sections indicated that lesions of MMP-8/MMP-13–deficient and MMP-13–deficient mice accumulate more fibrillar collagen than apoE−/− controls and MMP-8−/− apoE−/− double knockout. We further tested the relative effect of MMPs on plaque collagenolysis using in situ zymography. MMP-13 deletion alone abrogated collagenolytic activity in lesions, indicating a predominant role for MMP-13 in this process. MMP-13 and MMP-13/MMP-8 deficiency did not alter macrophage content but associated with reduced accumulation of smooth muscle cells.

Conclusions—These results show that among MMP interstitial collagenases in mice, MMP-13 prevails over MMP-8 in collagen degradation in atheroma. These findings provide a rationale for the identification and selective targeting a predominant collagenase for modulating key aspects of plaque structure considered critical in clinical complications, although they do not translate directly to human lesions, which also contain MMP-1.

Key Words: atherosclerosis ▪ collagen ▪ matrix metalloproteinases

Atherosclerosis remains the major cause of death and premature disability worldwide.1 Rupture of plaques with a thin fibrous cap causes most fatal myocardial infarctions.2,3 Because interstitial collagen confers tensile strength on the fibrous cap, collagenolysis in atheroma participates critically in plaque disruption. Previous work has implicated interstitial collagenases of the matrix metalloproteinase (MMP) family in the regulation of aspects of the structure of atherosclerotic plaques that associate with rupture and fatal thrombosis in humans.4,5 These data collectively suggest that therapeutic targeting of interstitial collagenases might reduce characteristics of plaques associated with rupture and thus limit thrombotic complications.

Yet, broad-spectrum inhibitors of MMPs cause unwanted effects that preclude their clinical use.6–11 and pilot clinical studies failed to show beneficial effects of MMP inhibitors8 because of lack of specificity. Understanding the potential redundancy of these enzymes in the regulation of plaque collagen content, therefore, has not only mechanistic interest but also important implications for the design of targeted strategies for reducing complications of atherosclerosis. In particular, our prior work showed that mice bearing a mutant form of interstitial collagen that resists breakdown by MMPs accumulate plaque collagen.12 Subsequent studies showed that selective inhibition of MMP-13 can also increase the content of plaque collagen.8,13 In addition, these studies presented evidence that MMP-13 predominates as an interstitial collagenase in mouse atheromata. Yet, to date no data directly compare the contribution of combined deficiency of MMP-13 and the other principal interstitial collagenase in mouse lesions, MMP-8, on collagen accumulation and other aspects of plaque structure and cellular composition.1,14

Mouse atherosclerotic plaques contain 2 principal interstitial collagenases, whereas human lesions contain 3 MMPs of this class.3,13 Therefore, the mouse offers a defined approach for testing the redundancy of interstitial collagenase actions in atherosclerotic plaques.
during atherogenesis. The present study tested the novel hypothesis that loss of MMP-13 function alone achieves a similar increase in collagen content in experimental atheromata as that caused by deficiency of both MMP-13 and MMP-8. Our study examined both early and established atheromata to assess the role of collagenases in both formation and progression of lesions. The findings furnish new mechanistic insight into the biological roles of interstitial collagenases in experimental atherosclerosis and support the selective targeting of a single collagenase to influence aspects of plaque structure deemed critical in clinical complications decisively.

Material and Methods
Materials and Methods are available in the online-only Supplement.

Results
Characteristics of Mice
To examine the relative contribution of MMP-8 and MMP-13 to atherosclerosis, apolipoprotein E–deficient (apoE−/−) mice, MMP-13−/− apoE−/−, MMP-8−/− apoE−/− double knockout (DKO) mice, and MMP-13−/− MMP-8−/− apoE−/− triple knockout (TKO) mice consumed an atherogenic diet, either for 10 weeks to provoke early lesions (n=12 per group) or for 24 weeks for advanced atheroma (n=15). Total cholesterol and triglycerides did not differ between apoE−/− and MMP-8−/− apoE−/− mice after 10 weeks of the atherogenic diet (Table). TKO mice, however, had higher body weight (**P ≤ 0.01) than apoE−/− and DKO mice (***P ≤ 0.01) at this time point. After 24 weeks, TKO mice consistently exhibited increased body weight compared with DKO mice, with higher plasma cholesterol levels compared with controls (**P ≤ 0.01).

Lack of MMP-8 and MMP-13 Activity Does Not Affect the Development of Atherosclerosis in ApoE−/− Mice
Oil red O en face staining determined the lipid content of atherosclerotic lesions in the descending aorta. Overall, TKO mice exhibited similar plaque size after 10 and 24 weeks on an atherogenic diet compared with apoE−/− mice and DKO animals (Figure 1A–1C). In the aortic root and the brachiocephalic artery, where plaques form earlier, histological analysis of plaque size showed a similar result. Genetic inactivation or pharmacological inhibition of MMP-13 associated with comparable extent of atheroma compared with apoE−/− controls (Figure 1D and 1E), in accordance with the results observed in our previous studies. MMP-8 deletion combined with a lack of MMP-13 had no significant additional effect on plaque size, suggesting that MMP-8 and MMP-13 play a minor role in plaque lipid accumulation.

MMP-13 Deficiency Yields Increased More Intraplaque Collagen Accumulation Compared With MMP-8–Deficient Mice
We further investigated collagen content by analyzing aortic root sections stained by picrosirius red under polarized light. After 10 weeks on an atherogenic diet (Figure 2A), the intima of aortic lesions from MMP-13−/− apoE−/− mice accumulated more interstitial collagen than apoE−/− mice, affirming the critical role of MMP-13 in plaque collagenolysis. MMP-8−/− apoE−/− exhibited a slight and statistically insignificant increase in plaque collagen. MMP-13 deletion associated with a 71% increase in collagen accumulation compared with MMP-8–deficient mice, indicating a predominant role of MMP-13 in collagen degradation in murine atheromata. Combined deletion of MMP-8 and MMP-13 in TKO mice did not correlate with any significant additional increase of collagen accumulation compared with DKO mice, further suggesting that MMP-8 contributes little to this process (Figure 2B). Established plaques (Figure 2C) showed a 66.2% increase in the fibrillar collagen content in MMP-13 apoE DKO mice compared with apoE−/− controls (Figure 2D). Strikingly, lesions from TKO mice showed less collagen accumulation compared with those from DKO mice and did not differ significantly from apoE−/− controls or MMP-8−/− apoE−/− animals. Analysis of picrosirius red–stained collagen fibers under filtered polarized light allowed us to assess the effect of MMP-13 and MMP-8 on this index of collagen fiber thickness. This approach revealed an increased proportion of mature, thick collagen fibers in TKO mice (observed under polarized light with a red filter) in early and advanced atherosclerotic lesions compared with apoE−/− mice. When associated with MMP-13 deficiency, lack of MMP-8 did not enrich early or advanced atherosclerotic lesions with thick collagen fibrils.

MMP-13 Deletion Alone Abrogates Collagenolysis in Atherosclerotic Lesions
To estimate further the relative contribution of MMP-13 and MMP-8 on collagenolysis in lesions, we performed in situ zymography in the aortic roots of mice after 24 weeks of atherogenic diet. For each group, we compared the mean fluorescence intensity derived from a cleavable collagen-based probe in the presence or absence of a broad-spectrum metalloproteinase inhibitor (ilomastat) as a control (Figure 3). MMP-13 deletion alone in DKO mice completely inhibited collagen degradation in lesions, as did ilomastat treatment (Figure 3A). MMP-8 deficiency did not significantly reduce collagenase activity compared with apoE−/− control. MMP-13 apoE DKO and TKO mice exhibited similar collagenase activity, indicating that compensatory changes in MMP-8 activity do not contribute to plaque collagenolytic capacity in mice with congenital absence of MMP-13 (Figure 3B and 3C).

MMP-13 Drives the Reduction of Smooth Muscle Cell Accumulation on Atheromata
Smooth muscle cells (SMCs) produce most of the interstitial collagen in atherosclerotic lesions. We assessed whether increased collagen content also could result, in part, from

Nonstandard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>apoE−/−</td>
<td>apolipoprotein E–deficient</td>
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<tr>
<td>DKO</td>
<td>double knockout</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<td>TKO</td>
<td>triple knockout</td>
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an increase in SMCs in atheromata in the absence of MMP collagenases. Histological analysis showed that SMC accumulation decreased in both MMP-13 apoE DKO and TKO mice compared with MMP-8−/− apoE−/− animals, which did not show significant difference with the apoE−/− controls. There was a nonsignificant trend for the early lesions of TKO mice to have fewer SMC (Figure 4B), as previously reported by Fukumoto et al12 in atheromata of mice with genetically induced collagenase resistance. In established lesions in mice with MMP-13 or with combined MMP-8/MMP-13 deficiencies, SMC content declined significantly (Figure 4C).12 DKO and TKO mice had similar SMC content. The number of SMCs per mm² of plaque did not vary significantly but still indicated the trend of the decrease in SMC content in collagenase mutant mice (Figure 4D and 4E). The ratio between intimal collagen accumulation and SMC number was higher within the DKO mice (2.6-fold compared with apoE−/−) and TKO mice (1.6-fold compared with apoE−/−) in established

Table. Characteristics of Knockout Mice After the Indicated Period of Atherogenic Diet

<table>
<thead>
<tr>
<th></th>
<th>ApoE−/−</th>
<th>ApoE−/− MMP-13−/−</th>
<th>ApoE−/− MMP-8−/−</th>
<th>ApoE−/− MMP-13−/− MMP-8−/−</th>
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<tr>
<td>10-wk atherogenic diet</td>
<td>(n=22)</td>
<td>(n=22)</td>
<td>(n=14)</td>
<td>(n=24)</td>
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<tr>
<td>Body weight, g</td>
<td>35.8±1.2</td>
<td>30.9±0.7</td>
<td>35.9±0.9</td>
<td>40.9±1.5</td>
<td>&lt;0.0001</td>
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<td>Total cholesterol, mg/dL</td>
<td>568.1±27.1</td>
<td>542.2±29.8</td>
<td>589.5±47.8</td>
<td>520.7±24.83</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>80.7±10.0</td>
<td>68.6±5.0</td>
<td>79.8±7.8</td>
<td>78.0±10.0</td>
<td>NS</td>
</tr>
<tr>
<td>24-wk atherogenic diet</td>
<td>(n=28)</td>
<td>(n=27)</td>
<td>(n=15)</td>
<td>(n=31)</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>33.2±0.6</td>
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<td>36.6±1.5</td>
<td>41.8±1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>660.1±92.0</td>
<td>844.8±70.5</td>
<td>704.6±58.3</td>
<td>972.4±88.2</td>
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<tr>
<td>Triglycerides, mg/dL</td>
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<td>121.3±7.8</td>
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</table>

ApoE−/− indicates apolipoprotein E–deficient; MMP, matrix metalloproteinase; and NS, nonsignificant.
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Figure 3. In situ zymography for collagenolytic activity in mouse arteries. Representative sections of aortic roots of mutant mice after 24 weeks of atherogenic diet incubated with a fluorescent collagenase-activatable probe in presence or in absence of the broad matrix metalloproteinase (MMP) inhibitor ilomastat (A). Quantification of the mean fluorescence in the intima (B) and the mean fluorescence ratio compared with ilomastat-treated samples (C) are represented (n=5). Bars represent mean±SEM (*P<0.05, **P<0.01, ***P<0.001). ApoE−/− indicates apolipoprotein E–deficient.

Figure 2. Picrosirius red staining viewed under linearly polarized light to show fibrillar collagen in plaques after 10 (A) and 24 (C) weeks of atherogenic diet. Quantification of intimal collagen content (B and D). Bars represent means±SEM (*P<0.05, **P<0.01, ***P<0.001). ApoE−/− indicates apolipoprotein E–deficient; and MMP, matrix metalloproteinase.

atheromata (Figure 4F and 4G). Previous studies reported that MMP-13 collagenase does not affect the de novo collagen expression in lesions.6 Taken together, these results suggest that increased collagen content in MMP-deficient mice results from inhibition of collagen degradation and not from increased production by intimal cells.
Neither MMP-8 nor MMP-13 Influences Macrophage Accumulation

Because macrophages constitute a major source of matrix-degrading proteinases, particularly interstitial collagenases within human atheromata, we investigated their presence in atheromatous lesions lacking MMP-8 and MMP-13. Macrophage accumulation did not differ between the intima of aortic roots lacking MMP-13, MMP-8, or lacking both MMP-8 and MMP-13 after 10 and 24 weeks on an atherogenic diet (Figure 5A and 5B). These results indicate that MMP collagenases, while affecting SMC accumulation in advanced plaques, do not impede recruitment of inflammatory cells.

Lack of MMP-13 and MMP-8 Reduces Features Associated With Plaque Rupture

We further assessed features associated with plaque disruption in histological sections of lesions in brachiocephalic arteries, a site of lesions considered prone to rupture in atherosclerotic plaques. 

Figure 4. Representative α-smooth muscle cell (SMC) actin staining of aortic root sections of knockout mice after 10 and 24 weeks on an atherogenic diet (A). Quantification of smooth muscle cells number per lesion and per lesion area after 10 weeks (B and D) and 24 weeks of atherogenic diet (C and E). Intimal collagen content normalized by the number of SMCs in early (F) and advanced lesions (G). Bars represent mean±SEM (n≥10 per group, *P<0.05, **P<0.01, ***P<0.001). ApoE−/− indicates apolipoprotein E–deficient; and MMP, matrix metalloproteinase.
mice. We measured the size of the necrotic core area in the plaques and showed that lack of both MMP-8 and MMP-13 associated with a significant decrease in necrotic core size (Figure 5C and 5D).

Discussion

Degradation of the extracellular matrix in the plaque’s fibrous cap likely renders a lesion more prone to rupture in humans. Previous data from our group and others suggest that regulation of collagenolysis by MMP collagenases in conjunction with reduced interstitial collagen synthesis by SMCs in inflamed atheromata generates so-called vulnerable lesions. These studies have not only provided novel mechanistic insight into pathogenesis but have identified MMPs as an appealing target for clinical intervention to prevent the thrombotic complications of atherosclerosis. Three members of the MMP family, denoted interstitial collagenases (MMP-1, MMP-8, and MMP-13), can cleave triple helical fibrillar collagen and associate with inflamed plaques in humans. These enzymes catalyze the critical initial step in collagen catabolism, yielding fragments that can be degraded by other collagenases or MMPs. Initial attempts to inhibit MMPs in clinical trials have consistently failed because of lack of selectivity of the agents used and associated unwanted actions. In atherosclerosis, therefore, identifying which MMP collagenase(s) plays a major role in this process and assessing whether lack of function of one interstitial collagenase leads to compensatory changes in other family members have both mechanistic and therapeutic interest.

We previously demonstrated that MMP-13 regulates collagen accumulation in vivo in murine atheromata. Mice express another interstitial collagenase, MMP-8, an enzyme also found in human plaques. In addition, high serum levels of MMP-8 associate with adverse cardiovascular outcomes. Laxton et al. reported that inactivating MMP-8 reduced the extent of atherosclerosis in apoE−/− mice and increased collagen content in developing atheromata (12 weeks on atherogenic diet).

In accordance with our prior studies, the present results demonstrate that MMP-13 controls the collagen content of both early and established plaques in mice. This study addressed the crucial unresolved issue of the relative contribution of MMP-8 and MMP-13 to these critical aspects of plaques. Concomitant lack of MMP-8 and MMP-13 did not augment collagen accumulation to a greater extent than MMP-13 deficiency alone. This result indicates a lack of mechanisms that compensate deficiency of MMP-8 or MMP-13 in regard to collagen metabolism in plaques, in contrast to the situation we observed in mouse myocardium with impaired collagenolysis. Our in situ zymographic data furnish strong evidence that MMP-13 predominates over MMP-8 as an interstitial collagenase in mouse atheromata. Although MMP-8 shares collagenolytic activity with MMP-13, it does not add to the action of MMP-13 on plaque collagenolysis. This novel finding supports our previous studies, in which genetically driven or pharmacologically driven selective inhibition of MMP-13 did not affect the expression of other potentially collagenolytic enzymes, such as MMP-8, MMP-14, MMP-12, or cathepsin K, in lesions. Although we cannot exclude that MMP-8 plays a role in plaque biology, as shown...
by Laxton et al., the present study did not recapitulate the results obtained previously. This discrepancy could result from differences in time points studied or other experimental variations.

The present study also demonstrates a broader effect of MMP collagenases on atherosclerosis biology. Our results show that MMP-13 or MMP-13/MMP-8 deficiency associate with reduced lesional SMCs, even if it does not alter plaque size significantly. Increased intimal collagen content and lack of collagenolytic capacity could impede SMC migration within the plaque, as reflected by the reduced number of intimal SMCs observed in DKO and TKO mice. This situation resembles that which we previously observed in collagenase-resistant mice that harbor a mutation in the substrate rather than the proteases.

Beyond their roles in extracellular matrix remodeling, MMPs could cleave other substrates. The differences in the lipid profile and body weight in the TKO observed here indicate that such noncanonical substrates might include factors that regulate metabolism. This possibility provides a potentially fruitful field for further investigation, beyond the scope of the present study that focused on effects of interstitial collagenases on the extracellular matrix.

MMP-13 and MMP-8 can cleave various membrane-associated and extracellular proteins, in addition to collagen fibers, that could account for the reduced plaque progression in knockout mice. MMP-8 plays a more important role in the cleavage of important molecular mediators, such as macrophage inflammatory protein-1 α, CXCL-9, CXCL-10, or angiotensin I. Previously reported results showing decreased atherosclerosis in MMP-8−/− apoE−/− mice most likely relate to the modulation of angiotensin production and its effect on blood pressure and vascular inflammation. MMP-13 can also affect inflammatory processes by cleaving chemokines (MCP-1 and MCP-2) and adhesion molecules (ICAM-1). Although the effect of MMP-13 and MMP-8 on plaque development reported in this study could result, in part, from regulation of various inflammatory mediators, the similar macrophage content in mice lacking MMP collagenases suggests that MMP-8 and MMP-13 activities do not alter the recruitment, proliferation, and apoptosis of inflammatory cells.

This work probed the mechanism of the regulation of collagen structure in atherosclerotic plaques and did not aim to model the human disease. Because mice do not express MMP-1, a collagenase expressed in human plaques, the results do not translate directly to human disease. Although MMP-1 colocalizes with MMP-13 in the shoulder regions of inflamed human atheromatous plaques and that specific MMP-1 haplotypes associate with plaque burden, its direct role in atherosclerosis remains unclear.

Macrophage-specific transgenic mice expressing human MMP-1 had less advanced atherosclerosis, but overexpression of human tissue inhibitor of metalloproteinase-1 (an inhibitor of collagenases and other MMPs) showed a tendency to reduce atherosclerotic lesions in apoE−/− mice.

Beyond the novel mechanistic insight into the redundancy and roles of collagenases in the context of atherosclerosis, these results have therapeutic implications. Broad-spectrum MMP inhibition has proven intolerable in clinical studies. Our present findings indicate that selective inhibition of a single member of the MMP interstitial collagenase family can alter fundamental aspects of plaques limiting potential indiscriminate MMP inhibition. The weight gain in the MMP-13/MMP-8 mice observed here provides an example of an unpredicted and unwanted consequence of broader interference with MMP function. Thus, this study provides new insights on the respective importance of MMP collagenases in murine atheroma. Identification of the predominant MMP collagenase in human lesions could, therefore, lead to the basis for modulating key aspects of plaque structure considered critical in clinical complications.

Sources of Funding
This work was, in part, supported by grants from the National Heart, Lung, and Blood Institute (R01 HL080472 to P. Libby) and from the Donald W. Reynolds Foundation (to P. Libby).

Disclosures
None.

References
Collagenase could modulate key aspects of plaque structure. Of concept that MMP collagenases have differential effect on collagenolysis in atherosclerotic plaques and that selective targeting of single broad-spectrum inhibition of MMPs is not translatable into the clinic, as a result of detrimental side effects, our findings provide the proof in vivo that among MMP interstitial collagenases, MMP-13 predominates over MMP-8 in collagen degradation in murine atheromata. Because in plaque rupture and fatal thrombosis by degrading the collagen that confers the tensile strength on the fibrous cap. Our study shows in vivo that among MMP interstitial collagenases, MMP-13 predominates over MMP-8 in collagen degradation in murine atheromata. Because broad-spectrum inhibition of MMPs is not translatable into the clinic, as a result of detrimental side effects, our findings provide the proof of concept that MMP collagenases have differential effect on collagenolysis in atherosclerotic plaques and that selective targeting of single collagenase could modulate key aspects of plaque structure.

Significance

Atherosclerosis remains the major cause of death and premature disability worldwide. Rupture of plaques with a thin fibrous cap causes most fatal myocardial infarctions. Substantial evidence implicates interstitial collagenases of the matrix metalloproteinase (MMP) family in plaque rupture and fatal thrombosis by degrading the collagen that confers the tensile strength on the fibrous cap. Our study shows in vivo that among MMP interstitial collagenases, MMP-13 predominates over MMP-8 in collagen degradation in murine atheromata. Because broad-spectrum inhibition of MMPs is not translatable into the clinic, as a result of detrimental side effects, our findings provide the proof of concept that MMP collagenases have differential effect on collagenolysis in atherosclerotic plaques and that selective targeting of single collagenase could modulate key aspects of plaque structure.
Matrix Metalloproteinase-13 Predominates Over Matrix Metalloproteinase-8 as the Functional Interstitial Collagenase in Mouse Atheromata
Thibaut Quillard, Haniel Alves Araújo, Gregory Franck, Yevgenia Tesmenitsky and Peter Libby
MMP-13 predominates over MMP-8 as the functional interstitial collagenase in mouse atheromata
Thibaut Quillard, Ph.D., Haniel Alves Araújo, Gregory Franck, Ph.D, Yevgenia Tesmenitsky, Peter Libby, M.D.

METHODS
Animal Preparation
We studied the impact of MMP-13 and MMP-8 inhibition on atherosclerosis-susceptible apoE−/− mice with congenic c57bl/6 background (Jackson Laboratory, Bar Harbor, ME). MMP-13−/− and MMP-8−/− mice were generated by embryonic gene targeting and crossed with apoE−/− mice to generate respectively MMP-13−− apoE−/− and MMP-8−− apoE−/− double knockout (DKO) mice, as previously described1.2. Dr. Stephen Krane (Massachusetts General Hospital, Boston, MA) generously provided the double knockout MMP-8−−MMP-13−− mice. Crossing MMP-8−−MMP-13−− mice with apoE−/− animals allowed us to generate triple heterozygous, then MMP-13−−MMP-8−−apoE−/− triple knockout (TKO) mice, once bred together.

Lipid and weight measurements
Total body weight was measured weekly after the introduction of high-fat diet. We collected blood samples by cardiac puncture and isolated plasma after centrifugation. We measured triglycerides and cholesterol levels using kits (Thermo Scientific, Rockford, IL), according to the manufacturer’s specifications.

Characterization of Aortic Lesions
Mouse aortae from the aortic arch to the iliac bifurcation were dissected and cleaned free of connective and adipose tissues. The isolated aortas were placed in 10% neutral buffered formalin overnight. We further assessed the extent of aortic atherosclerotic lesions by performing en face staining with oil red O.

Histological Assays
For histological evaluations of the aortic root, we used the method of Paigen et al.3 Briefly, hearts were dissected in the region of the proximal aorta, and aortic roots were embedded in optimum cutting temperature compound (OCT, Sakura Finetek, Netherlands). We performed immunohistochemistry studies using rat anti-mouse monoclonal antibody for Mac3, a macrophage marker (BD PharMingen, San Diego, CA), and smooth-muscle cell (SMC) α-actin staining with primary antibody FITC-conjugated α-actin mouse monoclonal (Sigma-Aldrich, St. Louis, MO), followed by anti-FITC biotin–conjugated secondary antibody (Sigma-Aldrich), and counterstained with hematoxylin (Sigma-Aldrich). For quantification of histological assays, captured photomicrographs were analyzed with an image analysis system (ImagePro Plus 5.1, Media Cybernetics, Rockville, MD). Similarly, brachiocephalic arteries were embedded in OCT and 6-μm sections were stained for SMC α-actin and counterstained with hematoxylin. We investigated for the presence of buried fibrous caps, characterized by SMC-rich layers invested with elastin and usually overlying foam cells, as described by Jackson et al.4 We evaluated the necrotic core by measuring the area of hematoxylin-negative acellular areas in the intima.5,6
Collagen fiber characterization
We performed quantitative analysis of fibrillar collagen content using picrosirius red staining of sections viewed under polarized light. Qualitative analysis of fiber thickness was assessed using green and red optic filters (HQ535/50m, D605/55m, Chroma, Bellows Falls, VT) disposed under polarized light. Fiber color variation progresses from green to red proportionally to the increase of fiber thickness, such that red represents thicker, larger fibrils. The relative amount of each fiber color was expressed as a percentage of the total amount of collagen in the region. Images were recorded by a digital camera (DS-U2, Nikon, Tokyo, Japan) mounted on a polarizing microscope (Nikon Eclipse 80i), and analyzed using image analysis software (ImagePro Plus).

In situ zymography
To assess MMP collagenase activity in situ, we incubated 6-µm aortic root sections with DQ-Collagen substrate (Invitrogen, Carlsbad, CA) in agarose 1%, in the presence of the broad-spectrum MMP inhibitor Ilomastat (10 µM, EMD Millipore, Billerica, MA). Quantification of the fluorescence intensity in the intima (mean fluorescence intensity) reflected the collagenolytic activity.

Statistical Analyses
Continuous variables are summarized as mean ± SEM. Two investigators performed the analyses blindly. Data were analyzed by the Kruskal–Wallis one-way analysis of variance and the Bonferroni post test for each factor at individual times (Prism, GraphPad Software, La Jolla, CA). Differences were considered statistically significant at the p<0.05 level.

REFERENCES