Genetically Determined Resistance to Collagenase Action Augments Interstitial Collagen Accumulation in Atherosclerotic Plaques

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Background—We hypothesized that collagenolytic activity produced by activated macrophages contributes to collagen loss and the subsequent instability of atheromatous lesions, a common trigger of acute coronary syndromes. However, no direct in vivo evidence links collagenases with the regulation of collagen content in atherosclerotic plaques.

Methods and Results—To test the hypothesis that collagenases influence the structure of atheroma, we examined collagen accumulation in atherosclerotic lesions of apolipoprotein E–deficient mice (apoE−/−) that express collagenase-resistant collagen-I (ColR/R/apoE−/−, n = 12) or wild-type collagen-expressing mice (Col+/+/apoE−/−, n = 12). Aortic atheromata of both groups had similar sizes and numbers of macrophages, a major source of collagenases. However, aortic intimas from ColR/R/apoE−/− mice contained fewer smooth muscle cells, a source of collagen, probably because of decreased migration or proliferation or increased cell death. Despite reduced numbers of smooth muscle cells, atheromata of ColR/R/apoE−/− mice contained significantly more intimal collagen than did those of Col+/+/apoE−/− mice.

Conclusion—These results establish that collagenase action regulates plaque collagen turnover and smooth muscle cell accumulation. (Circulation. 2004;110:1953-1959.)

Degradation of extracellular matrix by matrix metalloproteinases (MMPs) may play various critical roles in the progression and complications of atherosclerotic plaques. Atherosclerotic lesions prone to the onset of acute coronary syndromes usually contain abundant macrophages underlying a thin and collagen-poor fibrous cap. Extracellular matrix macromolecules, notably fibrillar interstitial collagens, confer tensile strength on the plaque’s fibrous cap. Direct evidence suggests that macrophage expression of MMPs in atheromata weakens the plaque’s protective fibrous cap and promotes disruption and subsequent thrombosis. In particular, collagenases of the MMP family (MMP-1/collagenase-1, MMP-8/collagenase-2, and MMP-13/collagenase-3) break down fibrillar collagens. However, no direct in vivo evidence has determined that MMP-family collagenases regulate the interstitial collagen content of atheroma. Indeed, plaques also contain non-MMP collagenases, including cathepsin K, a potent cysteine proteinase that can degrade interstitial collagen. The relative contributions of MMP and non-MMP collagenases to collagen metabolism in the plaque remain unknown.

Smooth muscle cells (SMCs) participate in the progression of atherosclerosis. Migration from the tunica media and the balance between proliferation and cell death determine SMC content in the intima. Although some studies suggest that MMPs may mediate SMC migration through the collagenous matrix of plaques, the in vivo role of collagenases in this context remains unproven.

Interstitial collagenases of the MMP family initiate degradation of type I collagen by cleavage at a single highly conserved site between Gly775 and Ile776 of the α1(I) chain. The present study used mice with a targeted mutation in both alleles of Col1a1 (Col1a1tm 129 or ColR/R) that yields amino acid substitutions around the collagenase cleavage site in the α1(I) chains that render collagen completely resistant to attack by MMP collagenases. We crossed ColR/R mice and apoE-deficient strain (apoE−/−) mice to test the specific biological hypothesis in vivo that MMP collagenases regulate collagen accumulation in intimal plaques, an aspect of plaque structure critical to the progression and complications of atherosclerosis.

Methods

Animal Preparation
ColR/R mice (C57BL/6×129) were backcrossed 7 generations into C57BL/6 mice and then crossed into apoE-deficient mice (apoE−/−).
C57BL/6) to render ColR/R mice atherosclerosis susceptible. The procedure yielded ColR/R/apoE−/− and Col+/−/apoE−/− mice, as demonstrated by polymerase chain reaction (PCR)–based genotyping. ColR/R/apoE−/− (n=12) and Col+/−/apoE−/− (n=12) littermates consumed an atherogenic diet (semipurified chow containing 1.25% cholesterol and 0% cholate) for 10 weeks. All experiments conformed to a protocol approved by the Standing Committee on Animals of Harvard Medical School.

Tissue Preparation
Mice were anesthetized and perfused with phosphate-buffered saline (pH 7.4) through a 23-gauge cannula in the left ventricle. The hearts were subsequently immersed in saline for 1 hour and then with 4% phosphate-buffered paraformaldehyde (pH 7.4) for an additional 48 hours. Hearts dissected in the region of the proximal aorta23 were embedded in paraffin, and 5-μm serial sections were cut. Histological analyses used sections ~50 μm above the beginning of the aortic sinuses. The aortas for RNA extraction were harvested, and the adventitia was removed while being viewed under a microscope.

Histological Assays
Immunohistochemistry studies included use of a mouse monoclonal antibody against human α-SM actin (Dako) with a kit for mouse antibodies on mouse tissue (InnoGenex), rat monoclonal antibody against mouse macrophages (Mac3, PharMingen), rabbit polyclonal antibody against MMP-13/collagenase-3 (a gift from Dr C. William Wu, University of Tennessee, Memphis),24 rabbit polyclonal anti-mouse cathepsin K (Santa Cruz), or nonimmune rabbit IgG as a negative control. The peptide at the linker region between propeptide and catalytic domains (PNPKHPKPTPEK) was chosen from the amino acid sequence of mouse MMP-13/collagenase-3 to raise the specific antibody. The sequence of this peptide significantly differs from all other proteins including MMPs (E values >4.7, NCBI Blast). A mouse proliferating cell nuclear antigen staining kit (Zymed) and ApopTaq Plus (Intergen) were used to determine cell proliferation and death. We analyzed picrosirius red staining under polarized light to detect interstitial collagen.

Peritoneal Macrophage Culture
Four days after an intraperitoneal injection of 4.1% thioglycollate, primary peritoneal macrophages were harvested from mice and cultured with Dulbecco’s modified Eagle’s medium (DMEM). After incubation with DMEM containing 10% fetal calf serum for 24 hours, the culture media of adherent cells were changed to fresh DMEM with or without 10% fetal calf serum and incubated for 72 hours.

RT-PCR and Western Blotting
Total RNA was extracted from peritoneal macrophages (n=3 per group) and mouse aortas (pooled, n=3 per group) and reverse-
transcribed (RT). Real-time PCR used SYBR Green PCR master mix and the ABI PRISM 5600 sequence detection system (Applied Biosystems). Oligonucleotide primer pairs used to recognize mouse mRNAs included MMP-8, 5'-CAA-CCT-ATT-TCT-CGT-GGC-TG-3' and 5'-TGC-AGG-TCA-TAG-CCA-CTT-AG-3'; MMP-13, 5'-TCC-CTT-GAT-GCC-ATT-ACC-AGT-C-3' and 5'-AAA-AAG-AGC-TCA-GCC-TCA-ACC-TG; 1 procollagen-I, 5'-AAG-GTG-CTG-ATG-GTT-CTC-C-3' and 5'-TCT-TTC-TCC-CTG-ACC-G-3'; and -actin (internal control), 5'-CAC-ACT-GTG-CCC-ATC-TAC-GA-3' and 5'-GTT-TCA-TGG-ATG-CCA-CAG-GA-3'.

Cell lysates for Western blotting from peritoneal macrophages were obtained with use of a buffer containing 125 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 4.6% sodium dodecyl sulfate, and 10% mercaptoethanol. Western blotting used the same rabbit polyclonal antibody against MMP-13/collagenase-3 as for immunohistochemistry.

Preparation of Mouse SMCs and Migration Assay
Isolation of mouse SMCs for migration assay was adapted from the method of Gunther et al.25 Aortas from C57BL/6 mice were digested with 1 mg/mL collagenase type II (Worthington). After the endothelium and adventitia were carefully removed, aortas were chopped and digested with type I collagenase (1 mg/mL, Worthington) and elastase III (0.125 mg/mL, Sigma). Cells were then cultured and passaged. SMC migration was evaluated with dual-chamber 24-well plates with 8-μm polycarbonate membranes (Costar) coated with 100 μg/mL collagen solution extracted from Col-/-/apoE-/-/apoE-/- or Col(+)/apoE-/- mice. The lower chambers were filled with DMEM with or without 1 ng/mL platelet-derived growth factor-BB, and 40,000 cells were added to the upper chambers. After incubation for 4 hours, cells that had migrated to the lower surface of the membrane were fixed, stained with use of the Protocol HEMA3 staining kit (Fisher), and counted in 4 × 100 fields per well.

Quantitative Analysis and Statistics
Quantitative analysis of histological assays used a digital imaging system as previously described.8,12 In brief, images were transferred into an ImagePro image analysis system (Media Cybernetics). A color threshold mask for immunostaining was defined by sampling the red color, and the same threshold was applied to all specimens. The percentage of the total area with positive color for each section was recorded. For picrosirius red staining, a negative background (black) was chosen for thresholding, and the positive area was calculated by subtraction. Two investigators performed these analyses independently and without knowledge of genotype. Interobserver
correlation was excellent (r=0.98). Differences between the 2 groups or among multiple groups were determined by the Mann-Whitney U test or 1-way ANOVA followed by Fisher test, respectively.

### Results

#### Plasma Lipids

On the atherogenic diet, plasma total cholesterol levels in ColR/R/apoE−/− mice (n=12, 1113±118 mg/dL) and Col+/+/apoE−/− mice (atherosclerotic wild-type, n=12, 1279±202 mg/dL) did not differ.

### Alterations in Endogenous Collagenase and Procollagen Expression

Previous animal studies demonstrated disparate results with regard to the role of MMPs on atheroma burden.26–30 In aortic atheroma from cholesterol-fed ColR/R/apoE−/− and Col+/+/apoE−/− mice, lesion areas were similar (Figures 1A–1C). Collagenase resistance also did not change macrophage areas and areas where cells expressed MMP-13/collagenase-3 (Figures 1A–1C). We furthermore observed no significant difference in areas immunopositive for cathepsin K, a major non-MMP collagenase (data not shown, P=0.78) We then evaluated possible alterations in endogenous collagenase expression in response to collagenase resistance. Levels of MMP-8/collagenase-2 and MMP-13/collagenase-3 mRNAs by real-time RT-PCR on thiglycollate-stimulated peritoneal macrophages were similar in both groups of mice (Figure 2A). However, macrophage expression of MMP-13/collagenase-3 appeared to be more abundant than that of MMP-8/collagenase-2 (Figure 2A). Protein expression of latent and active MMP-13/collagenase-3 in both groups was evaluated from Western blots was similar (pro-MMP-13, ~57 kDa; active MMP-13, ~45 kDa with subsequent autolytic cleavage to a 20-kDa form; Figure 2B). Absorption by the same

peptide that was used to raise the antibody (PNPKHPKT-PEK) diminished these bands. These results indicate that collagenase resistance did not affect the capacity of macrophages to express collagenases.

We further examined levels of mRNAs encoding collagenases in the aortas. Real-time RT-PCR showed an increase in
MMP-13/collagenase-3 mRNA levels and a decrease in α1 type I procollagen mRNA levels in aortas from ColR/R-apoE−/− mice compared with those from Col+/+/apoE−/− mice (n=3 each, pooled), whereas MMP-8/collagenase-2 mRNA levels did not change substantially (Figure 2C). Levels of mRNA encoding Mcol-A, a possible mouse orthologue of MMP-1/collagenase-1,31 were below the limits of detectability in these assays (data not shown).

Collagenase-Resistant Mice Have Fewer Intimal SMCs

SMCs synthesize most arterial collagen. Atheromata of ColR/R-apoE−/− mice contained significantly fewer SMCs than did those of Col+/+/apoE−/− mice (Figures 3A and 3B). Proliferating cell nuclear antigen and terminal dUTP nick end-labeling staining indicated decreased proliferation and increased death of intimal SMCs in ColR/R-apoE−/− mice compared with Col+/+/apoE−/− mice (Figure 4A and 4B). SMCs enter the intima by migration from the tunica media or from the blood. To determine whether collagenase resistance influences the ability of SMCs to migrate, we performed in vitro migration assay with dual-chamber plates. Because the current study used compound-mutant mice for the substrate, not for enzymes, we measured the traversal of wild-type SMCs from C57BL6 mice through polycarbonate filters coated with a layer of interstitial collagen extracted from Col+/+/apoE−/− or ColR/R-apoE−/− mice. This assay showed a decreased ability of SMCs to migrate through collagenase-resistant collagen compared with wild-type collagen (Figure 4C and 4D).

Collagenase Resistance Increases Collagen Content in the Intima of Aortic Atheromata of ApoE−/− Mice

The tunica intima of aortic atheromata of Col+/+/apoE−/− mice exhibited patchy accumulation of interstitial collagen, as determined by picrosirius red staining analyzed by polarization (Figure 5A). However, the aortic intimas of ColR/R-apoE−/− mice contained substantially more interstitial collagen than did those of Col+/+/apoE−/− mice. Quantitative analyses of picrosirius red birefringence in intima indicate that ColR/R-apoE−/− mice (n=12) had more collagen than did Col+/+/apoE−/− mice (n=12) in terms of absolute areas, percent areas, and areas divided by SMC number. Bars represent mean±SEM. Abbreviations are as defined in text.

Discussion

We previously advanced the hypothesis that a highly regulated balance of synthesis and degradation determines collagen content in the fibrous cap of atherosclerotic plaques.1,2 In
turn, collagen levels critically influence the integrity of the plaque’s cap, a structure whose biomechanical failure may cause most myocardial infarctions. Earlier indirect evidence suggested that collagenases of the MMP family can regulate collagen content in the plaque.5–12 We initially demonstrated overexpression of the prototypical interstitial collagenase MMP-1 in human atheroma3 and later showed colocalization of MMP-1/collagenase-1 and MMP-13/collagenase-3 with degraded collagen in these lesions as detected by an antibody specific for the collagenase cleavage site of collagen.9 Recently, our group showed that human atheroma contain a third interstitial collagenase, MMP-8/collagenase-2,11 also present in mouse atheroma, as shown here (Figure 2C). Shah et al12 reported that conditioned media of cultured macrophages could digest collagen obtained from the human fibrous cap and that addition of a nonselective MMP inhibitor blocked this process in vitro. Moreover, we demonstrated that lipid lowering in hypercholesterolemic rabbits decreased collagenase expression and, in parallel, increased collagen accumulation in atheroma, suggesting a potential role for collagenases in collagen metabolism and stability in atheroma.3,12 Lemaître et al10 reported that macrophage-selective overexpression of human MMP-1/collagenase-1 in apoE-deficient mice decreased lesion size, although they did not provide quantitative analysis of collagen accumulation in the lesions. Our present study indicates that impaired collagen degradation due to introduction of collagenase resistance increased the content of this extracellular matrix component in the atherosclerotic intima, despite reduced numbers of SMCs and procollagen-I expression. These results thus demonstrate directly in vivo a critical role for collagenolysis in determining the collagen turnover of plaques. Moreover, they suggest that regulation of the levels of degradation (by MMP collagenases) outweighs the contribution of synthesis (by SMCs) to collagen accumulation in the atherosclerotic plaque.

The reduced SMC accumulation in atheroma was an unexpected phenotype of these compound-mutant mice. The mechanistic studies presented here suggest that a combination of decreased migration, decreased proliferation, and/or increased cell death contribute to the relative paucity of SMCs in plaques of Col(β09/apoE(−/−)) mice. These results agree with previous studies that showed that native, undegraded interstitial collagen can limit SMC proliferation and that SMC hyperplasia.

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