Selective Inhibition of Matrix Metalloproteinase-13 Increases Collagen Content of Established Mouse Atherosclerosis

Thibaut Quillard, Yevgenia Tesmenitsky, Kevin Croce, Richard Travers, Eugenia Shvartz, Konstantinos C. Koskinas, Galina K. Sukhova, Elena Aikawa, Masanori Aikawa, Peter Libby

Objective—Evidence has linked collagen loss with the onset of acute coronary events. This study tested the hypothesis that selective matrix metalloproteinase-13 (MMP-13) collagenase inhibition increases collagen content in already established and nascent mouse atheromas.

Methods and Results—In vitro and in situ experiments documented the selectivity and efficacy of an orally available MMP-13 inhibitor (MMP13i-A). In vivo observations monitored macrophage accumulation and MMP-13 activity using molecular imaging. After 10 weeks of MMP13i-A treatment, apolipoprotein E−/− deficient mice with evolving or established lesions exhibited reduced MMP-13 activity without affecting macrophage content, measured either by intravital microscopy or fluorescence reflectance imaging. Histological analysis indicated that MMP13-iA did not affect plaque size or macrophage or smooth muscle cell accumulation. Administration of MMP13i-A to mice with evolving or established atheromas substantially increased plaque interstitial collagen content in the intima and locally in the fibrous cap, compared with vehicle-treated controls. Analysis of collagen revealed thicker collagen fibers within the plaques of treated groups.

Conclusion—Pharmacological MMP-13 inhibition yields collagen accumulation in plaques (a feature associated in humans with resistance to rupture), even in established plaques. This study, of considerable clinical relevance, furnishes new mechanistic insight into regulation of the plaque’s extracellular matrix and validates molecular imaging for studying plaque biology. (Arterioscler Thromb Vasc Biol. 2011;31:2464-2472.)

Key Words: atherosclerosis ■ collagen ■ inhibitor ■ MMP-13 ■ molecular imaging

Atherosclerosis and its thrombotic complications represent a major and still growing cause of death worldwide. Extensive studies have shown that most fatal coronary arterial thrombi result from physical disruption of atherosclerotic plaques. Atherosclerotic lesions that have ruptured and caused fatal acute myocardial infarction characteristically contain abundant macrophages underlying a thin and collagen-poor fibrous cap.1 Extracellular matrix macromolecules, especially fibrillar interstitial collagens, confer tensile strength on the plaque’s fibrous cap.2 We hypothesized that imbalance of collagen synthesis by smooth muscle cells (SMCs) and its degradation by matrix-degrading enzymes, particularly matrix metalloproteinase (MMP) collagenases, regulates plaque collagen content.1,3,4 We previously have provided evidence in vivo supporting the contribution of collagenolysis to the control of collagen content in plaques. We reported the overexpression of several interstitial collagenases, and localized intermediates in collagen breakdown in human atheromas using both biochemical and in situ morphological approaches.5–8 Plaques in atherosclerosis-susceptible, apolipoprotein E−/− (apoE−/−) mice crossed with genetically altered mice that express collagenase-resistant collagen I contained more interstitial collagen than those in apoE−/− control mice.8 Aortas of apoE−/− mice expressing collagenase-resistant collagen I showed increased tensile strength in a biomechanical assay, supporting a role for collagen content in determining the mechanical properties of atheromatous arteries. Among the MMP collagenases, MMP-1, MMP-8, and MMP-13 can cleave fibrillar collagen at neutral pH.9,10 In apoE−/− mice, genetically determined absence of MMP-13—a major interstitial collagenase in this species—yielded increased plaque collagen content with thicker, more aligned, and more organized collagen fibers than in MMP-13 wild-type controls.5 Compensatory changes in protease expression in these mice with congenital collagenase lack, and the focus on the initial phase of lesion development, limit the interpretation and generalizability of these results. These observations also raised the translational hypothesis that inhibition of MMP-13 reinforces the collagen content of the fibrous cap of established or evolving atherosclerotic plaques, in addition to nascent lesions. As nonspecific MMP inhibitors can produce unwanted effects that have consistently limited...
their utility clinically and as mechanistic probes, we administered a highly selective MMP-13 inhibitor (MMP13i-A) orally to test this hypothesis.11–13

The development of molecular imaging strategies to visualize plaque biology and progression represents a major opportunity for cardiovascular medicine, especially because many acute thrombotic events remain unpredictable and occur despite current optimum therapy. We and others have reported that imaging probes and modalities can visualize protease activity and macrophage content ex vivo and in vivo.14–19 To monitor plaque biology and actual MMP-13 inhibition in situ, this study used an activatable probe that is preferentially cleaved by MMP-13 and macrophage avid fluorescent iron nanoparticles, combined with intravitreal confocal microscopy and fluorescence reflectance imaging (FRI).

Materials and Methods
Detailed methods are given in the supplemental materials, available online at http://atvb.ahajournals.org.

Animal Preparation
All experiments conformed to a protocol approved by the Standing Committee on Animals of Harvard Medical School. We studied the impact of MMP-13 inhibition on atherosclerosis-susceptible apoE−/− mice with congenic c57bl/6 background. Male mice 8 to 10 weeks of age (n = 12) consumed an atherogenic diet (semipurified chow containing 1.25% cholesterol and 0% cholate, Research Diets, New Brunswick, NJ) for 10 weeks, together with oral administration of indicated doses of the MMP-13 inhibitor MMP13i-A (Amgen, Thousand Oaks, CA). To test the effects of MMP-13 inhibition on already established atheromas, mice were fed an atherogenic diet for 10 weeks before starting the 10-week treatment (n = 12) (Supplemental Figure I). For both studies, MMP13i-A and corresponding vehicle were administered by gavage twice a day, spaced by 10 to 12 hours. Controls were age-matched and treated with vehicle (0.5% methylcellulose, Convergent Bioscience, Toronto, Ontario, Canada). Age-matched MMP13−/−apoE−/− deficient mice (C57BL/6) consumed a high-fat diet for 10 weeks and were used to assess imaging agent specificity (n = 3). All mice were maintained in animal facilities at Harvard Medical School. Animal care and procedures were reviewed and approved by the institutional animal care and use committees and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Human Tissues
Endarterectomy specimens of human carotid plaques (n = 5) were obtained by protocols approved by the Human Investigation Review Committee at Brigham and Women’s Hospital. Surgical tissue samples were embedded in OCT compound and stored at −80°C until use. Serial cryosections were cut, air-dried onto microscope slides, and used in parallel for in situ zymography and immunohistochemistry.

Molecular Imaging

Molecular Imaging Agents
We used fluorescent nanoparticles based on an iron oxide core (Ex753/Em773) for the detection of macrophage accumulation. MMPsense (Ex680/Em700) fluorescence reflected MMP-13 activity.

Intravitreal Confocal Microscopy
The carotid artery was exposed at the level of the external and internal carotid bifurcation. Anesthetized mice were then placed under the confocal microscope to detect the fluorescence emitted by the probes in plaques (Olympus FV1000, Tokyo, Japan). Image collection of different channels was done sequentially to avoid cross-talk between channels, as we described previously.20

Ex Vivo FRI
After euthanasia, aortas were imaged and fluorescence was quantified using an FRI system equipped with multichannel filter sets (Carestream, Rochester, NY). The sum of the fluorescence levels in the aortic root and arch region was subtracted to the background level, calculated in a similar area size with no aorta exposed.

Cell Culture
Monocytes were cultured and differentiated into macrophages after 10 days in RPMI containing 5% human serum and 1% penicillin-streptomycin. Incubation with recombinant tumor necrosis factor-α and interleukin-1β elicited macrophage activation.

MMP Activity Assay
MMP-13 activity was measured using a fluorescent MMP-13 substrate, as previously described.11–13 Selectivity assays were performed using catalytic domains of human MMPs and omni-MMP fluorescent peptide (Enzo Life Sciences) as substrate.

In Situ Zymography
To estimate MMP collagenase activity in situ, we incubated 6- to 8-μm sections with DQ-Collagen (Invitrogen), in the presence of MMP13i-A (10 μmol/L), the broad-spectrum MMP-inhibitor ilomastat (10 μmol/L), or phenanthroline (0.5 mmol/L), for 48 hours.

Histological Assays
For histological evaluations of the aortic root, we used the method of Paigen et al.22 Plaque size and composition were characterized with anti-α-smooth muscle actin (Sigma-Aldrich) and anti-Mac3 (BD Pharmingen) antibodies. Quantification was performed on ImagePro Plus 5.1 (Media Cybernetics).8,23–25

Collagen Fiber Morphology and Quantification
Detection of interstitial collagen used picrosirius red staining with linear polarized light, as described previously.7,23,26,27 As fiber thickness increases, the color shifts from green to red. To discriminate the green and red birefringent fibers, green and red optic filters (HQ535/50m, D605/55m, Chroma) were disposed under polarized light. Images were analyzed using image analysis software (NIS-Elements, Nikon). The relative amount of each fiber color was expressed as a percentage of the total amount of collagen in the region.

Statistical Analysis
Differences between the treated and untreated groups were determined with the Mann-Whitney U test.

Results
Highly Selective MMP-13 Inhibition by MMP13i-A
Preclinical trials with broad MMP inhibitors have persistently failed because of toxicity or limited efficacy.11–13 We therefore conducted this series of experiments with a novel putatively selective MMP-13 inhibitor (MMP13i-A) (Supplemental Figure II). To test the selectivity of the nonhydroxamic acid–based MMP13i-A compound against MMP-13, various MMP catalytic domains were incubated with a broad MMP fluorogenic substrate in the presence of MMP13i-A at pH 7.5, in the optimum range for MMPs. MMP13i-A inhibited with nanomolar potency (50% inhibition concentration [IC50]) recombinant active MMP-13, but not MMP-1, MMP-2, MMP-7, MMP-9, MMP-12, or MMP-14 (Figure 1A). MMP13i-A reduced MMP-13 activity by 80% in a concentration-dependent manner using a specific MMP-13 cleavable substrate (Figure 1B). Moreover, we
tested whether MMP13i-A could also block MMP-13 released by activated macrophages, the most abundant producers of this MMP in atherosclerotic plaques. Incubation of macrophage supernatants with MMP13i-A reduced MMP-13 activity by 53% (human, \( P < 0.001 \)) and 76% (mouse, \( P < 0.001 \)) (Figure 1C and 1D). In vitro incubation of this substrate with various MMP catalytic domains affirmed the selectivity of this substrate for MMP-13 (Figure 1E).

MMP-13 Inhibition Decreased Collagenolysis in Plaques
To further test the efficacy of MMP13i-A and the overall impact of MMP-13 inhibition on collagenolysis in plaques, we performed in situ zymography for collagenolysis using a fluorescent substrate. MMP13i-A blocked collagenolysis in murine and human atheromas using a collagen I–based fluorescent substrate assay (Figure 2A and 2B and Supplemental Figure III). The nonspecific metalloenzyme inhibitor orthophenanthroline and the general MMP inhibitor ilomastat served as controls. MMP13i-A blocked this activity to a greater extent in mouse than in human lesions. As adult mice do not express MMP-1, these data suggest that MMP-13 plays a prominent role in type I collagen degradation in this species and that this process depends less on MMP-8 and MMP-14 in mice.

Fluorescent Nanoparticles and MMP-13 Activatable Probes Allowed Imaging of Plaque Burden and MMP-13 Activity in Atherosclerotic Mice
Molecular imaging in vivo has considerable potential in diagnostics and in directing therapy, but it also may help to probe pathophysiological mechanisms. To image atherosclerotic plaques, we used near-infrared fluorescent nanoparticles based on an iron oxide core. We tested selective uptake by macrophages—a prominent cellular component of atheromas—by incubating nanoparticles for 4 hours with either murine macrophages or primary murine SMCs (Figure 3A). Macrophages internalized nanoparticles in a dose-dependent manner, but we observed no uptake by SMCs. Fluorescent-labeled low-density lipoprotein served as a positive control for selective macrophage uptake.

We used this imaging strategy primarily to follow and verify in vivo the ability of MMP13i-A to inhibit MMP-13 activity, using a quenched fluorescent activatable probe that increases in fluorescence by several logs when cleaved by active MMP-13. We tested the selectivity of this MMP-13 probe by comparing ex vivo FRI of aortas from \( \text{apoE}^{-/-} \) and \( \text{apoE}^{-/-} \) \( \text{MMP-13}^{-/-} \) atherosclerotic mice. After 10 weeks on an atherogenic diet, mice received an MMP-13 probe intravenously 48 hours before euthanasia and tissue collection. Although FRI detected the fluorescent signal in the aortic root or arch and in the abdominal portion of aortas of \( \text{apoE}^{-/-} \) mice, the signal intensity decreased substantially in \( \text{apoE}^{-/-} \) mice crossed with MMP-13–deficient mice, indicating the selectivity of the proteinase probe for this enzyme (Figure 3B).5 Tissue from \( \text{apoE}^{-/-} \) mice that did not receive the substrate showed no fluorescence. To estimate how much MMP-13 activity increases and macrophages accumulate in atherosclerotic plaques versus healthy tissue, we coadministered the MMP-13 probe and the macrophage-targeted nanoparticles to \( \text{apoE}^{-/-} \) mice on an atherogenic diet and to wild-type mice on regular chow. Moreover, administration of the MMP-13 probe and the macrophage-targeted nanoparticles to \( \text{apoE}^{-/-} \) (atherogenic diet) mice and wild-type (chow) mice revealed substantial increases of both MMP-13 activity and macrophage accumulation in atherosclerotic plaques versus healthy tissue (Figure 3C and 3D). Quantification of FRI showed a 407% increase in MMP-13 signal and...
a 344% increase in macrophage phagocytic activity in the arteries of the hypercholesterolemic mice (P<0.05).

MMP-13 Activity Colocalized With Macrophages In Vivo

Various cell types can produce MMP-13, but macrophages likely are the major source of MMP-13 in human atherosclerotic lesions. To test this hypothesis, we coinjected apoE<sup>-/-</sup> mice consuming an atherogenic diet for 10 weeks with the MMP-13 probe and phagocytosable nanoparticles. After 48 hours, anesthetized mice underwent in vivo confocal microscopy of carotid arteries. Sequential fluorescence imaging established that macrophages and MMP-13 activity colocalized in the carotid plaques. As expected, MMP-13–dependent fluorescence appeared more diffuse than the macrophage signal in vivo because MMP-13 is a secreted enzyme.
Moreover, both signals colocalized with plaques visible under white light (Supplemental Figure IV A). To characterize better the spatial correlation between MMP-13 activity and macrophages in the plaque, we collected aortic roots and arches after in vivo confocal microscopy to detect fluorescence precisely on cryosections of plaques. Fluorescent nanoparticles colocalized with a specific macrophage marker (mac3), indicating their selective uptake by this cell type (Supplemental Figure IV B). In addition, MMP-13 activity colocalized tightly with macrophages, further suggesting that this cell type contributes most of the MMP-13 activity in mouse atheromas.28

**MMP13i-A Treatment Experimental Design**

We used in vivo molecular imaging with the MMP-13 activatable probe to determine the sufficient dosage of MMP13i-A to block the fluorescence reflecting MMP-13 activity in plaques. A preliminary dose-response study allowed us to select 40 mg/kg per day as the minimal dosage needed to reduce MMP-13 activity in vivo (Supplemental Figure IX). Pharmacokinetic analysis of the compound in rodents estimates a peak circulating concentration of ~20 μmol/L that effectively inhibits MMP-13 (79%), while only modestly affecting the activity of other MMPs (<IC50) in vitro (Figure 1 and Supplemental Figures VIII and XI). To assess the impact of MMP-13 inhibition on plaque biology, age-matched atherosclerosis-susceptible apoE−/− mice (males) were treated for 10 weeks with MMP13i-A while consuming an atherogenic diet. We also administered the MMP-13 inhibitor for 10 weeks to another group of apoE−/− male mice that had already consumed the atherogenic diet for 10 weeks, to assess the effect of MMP13i-A on established lesions—a more clinically relevant situation (Supplemental Figure I). In neither study did MMP13i-A treatment at 40 mg/kg per day induce visible toxicity or alter body weight (Supplemental Table I). Previous preclinical studies with MMP inhibitors often failed because of painful musculoskeletal syndrome toxicity,11–13 which associated with an accumulation of collagen in and around joints. MMP13i-A–treated mice did not exhibit any apparent joint swelling, and all mice appeared normal without apparent pain or distress.

**MMP13i-A Treatment Decreased MMP-13 Activity But Did Not Alter Macrophage Accumulation**

After 10 weeks, MMP-13 inhibitor–treated mice had markedly less MMP-13 activity in carotid plaques than did control mice, as determined by intravitral confocal microscopy (Figure 4A). In contrast, the treatment did not modulate macrophage accumulation in plaques. Ex vivo FRI of the aortic root/arch indicated a 35.6% (P=0.0048) decrease in MMP-13 activity in the aortic arch of treated mice compared with control mice. In contrast, macrophage accumulation did not change significantly (Figure 4B and 4C). These results demonstrate that oral administration of the MMP13i-A compound inhibits MMP-13 activity in atherosclerotic plaques in vivo without substantial change in the cellular composition of lesions.
Because collagen accumulation at the base of atheroma might not improve plaque stability, we tested whether collagen content increased more particularly in the critical fibrous cap area. Fibrous caps contained 26% more collagen in MMP13i-A–treated mice with established lesions versus respective controls (Figure 6A and 6B). In addition to the increase in collagen in fibrous caps, inhibition of MMP-13 also yielded significantly larger and thicker fibrous caps—a feature associated with human plaques that have not ruptured (Figure 6C and 6D). Thus, the plaques of MMP13i-A–treated mice exhibit characteristics associated with stable human plaques.

MMP13i-A treatment did not affect the expression of mRNAs that encode other known collagenolytic enzymes, such as MMP-8, MMP-14, MMP-12, or cathepsin K in lesions, indicating that increased plaque collagen with MMP-13 blockade in vivo arose without compensatory alterations in these collagenases in vivo (Supplemental Figure VII).

Discussion

The thrombotic complications of atherosclerosis remain major causes of death worldwide, highlighting the need to unravel the underlying pathophysiology and develop new therapeutic approaches that can address the residual burden of events that occur in at-risk patients despite the current standard of care. Fracture of the fibrous caps of plaques causes most fatal acute myocardial infarctions. Human atheromas that have ruptured and caused such events characteristic have a thin, collagen-poor fibrous cap and a macrophage-rich lipid core, and bear other hallmarks of local inflammatory activation.30,31 We and others have furnished evidence supporting active regulation of collagen breakdown in plaque in humans and mice using in vitro and in situ analyses and experiments using genetically altered mice.7 Notably, our prior mouse experiments have established increased collagen content of experimental atheromas in animals with collagen substrate mutated to resist breakdown by MMP collagenases and in those with germline inactivation of MMP-13, a major interstitial collagenase in mice.5,8 These previous experiments proved that collagenolysis participates in the regulation of the collagen content of plaques, but they also raised several new important issues. First, unmeasured compensatory changes in the substrate or enzymes in animals with congenital defects might unwittingly confound their interpretation. Second, germline modification of a collagenase does not determine whether the enzyme participates in early or later stages of lesion development. Third, and highly clinically relevant, any efforts to develop or test strategies to inhibit collagenolysis therapeutically require demonstration that intervention on established lesion initiation can influence collagen content and plaque structure. The present study aimed to address these key outstanding questions that arose from our prior work using novel tools: an orally active, selective MMP-13 inhibitor, and molecular imaging to ascertain MMP-13 inhibition in vivo at the doses tested.

After validating the efficacy and selectivity of the MMP13i-A compound for MMP-13, we colocalized MMP-13 activity with macrophages in plaques, affirming in vivo the important roles of these inflammatory cells as sources of this interstitial collagenase and the relevance of therapeutic strategies that target macrophages. By exploiting
the fluorescent properties of MMP and macrophage imaging probes, we demonstrated directly through in vivo and ex vivo imaging the inhibition of MMP-13 activity under the conditions of the experiments by MMP13i-A without affecting macrophage content within lesions, as attested by immunohistochemical analysis. Quantitative and qualitative analysis of the plaque collagen demonstrated that oral administration of a selective MMP-13 inhibitor significantly increased the overall content of collagen and yielded thicker fibers, to a similar extent as observed previously in early lesion formation in mice with congenital loss of MMP-13 due to germline manipulation. As MMP13i-A administration did not significantly increase the content of either SMCs or collagen Iα1 transcript levels (data not shown) in lesions, the collagen accumulation in plaque likely results from reduced degradation (MMP-13 being the prominent collagenase in mouse plaques), rather than from an increase in collagen production. These alterations in the extracellular matrix of a plaque’s fibrous cap would likely lessen the chance of rupture in human lesions.

Although adding collagen to the fibrous cap of atherosclerotic lesions should render them more resistant to rupture by increasing thickness or strength, changing plaque matrix composition might have multiple effects and could even render lesions more fragile. Adding organized fibrillar collagen in the fibrous cap would add tensile strength to that region, but adding collagen nearby—but not within the actual rupture location—could increase stress in the rupture region. An analogous double-edged sword may apply to plaque calcifications. Although increased calcium may strengthen plaques, very local microcalcifications may prove destabilizing. Increasing fibrillar collagen only at the base of the atheroma, near the arterial media, without strengthening the fibrous cap, might also not improve overall stability and could increase local stresses. Indeed, we have shown that genetically-driven increases in adventitial collagen enhance aneurysm formation in atherosclerotic aortas in mice. We further showed with formal biomechanical testing that these aortas can exhibit increased susceptibility to fracture. It therefore was particularly important that mice receiving the MMP-13 inhibitor accumulated collagen in the fibrous cap. MMP13i-A administration also significantly increased fibrous cap area and thickness in plaques from MMP13i-A–treated mice compared with those from vehicle-treated control mice. These observations further indicate that plaques from mice treated with a selective MMP-13 inhibitor show reinforcement of the characteristics of human plaques thought to confer resistance to rupture.

In mice, the interstitial collagenases MMP-8 and MMP-13 can cleave helical collagen fibers at the neutral pH that prevails in the extracellular space. The present results affirm a major role for MMP-13 in collagen degradation in mouse atherosclerosis. Using an in situ assay for collagenolysis, selective inhibition of MMP-13 substantially limited collagenolysis in plaques to an extent similar to that produced by broad-spectrum MMP inhibitors. This result also indicates that in mice, MMP-8 contributes less to plaque collagenolysis than does MMP-13.

Several studies in humans and animals have associated high local and systemic levels of MMP-2, MMP-9, or both with atherothrombotic events, suggesting a role in plaque destabilization. MMP-2 and MMP-9 are important in matrix remodeling, particularly through the further degradation of fragmented collagen molecules following primary cleavage by MMP collagenases that possess the rare ability to cleave intact interstitial fibrillar collagen. Our results presented here therefore imply that blockade of MMP-13 collagenase, and consecutive blockade or decrease in primary cleavage of interstitial fibrillar collagen, would prevent or delay its further degradation by gelatinases—even at high levels—as found in plaques considered prone to rupture.

Major differences between mouse and human atherogenesis limit the ready extrapolation of this and other studies. Mature mice do not express MMP-1, considered a major interstitial collagenase in humans, which we and others have previously localized in human plaques. Moreover, mouse atheromas seldom rupture and cause thrombotic complications, except under extreme conditions or after major manipulations. Thus, these experiments cannot prove that MMP-13 inhibition stabilizes plaques or prevents thrombotic complications, but they provide novel mechanistic insight into the regulation of the lesional extracellular matrix.

Broad-spectrum MMP inhibitors have failed consistently in clinical trials due to lack of efficacy (eg, in metastatic cancer) and deleterious side effects (most prominently, musculoskeletal toxicity). Some MMP-13 inhibitors appear to limit such toxicity and show beneficial clinical outcomes in arthritic diseases, which may also involve MMP-13. In our study, the highly selective MMP13i-A compound did not induce any apparent musculoskeletal toxicity at 40 mg/kg per day, an issue that warrants further in-depth study. As humans express 3 MMP interstitial collagenases (MMP-1, MMP-8, and MMP-13), selective MMP-13 inhibition might obviate the undesired effects encountered in the clinic with broad-spectrum MMP inhibitors that have impeded their use in humans. Our preclinical results obtained in mice or human tissues not only furnish mechanistic insight into plaque biology, but also provide proof of concept that merits consideration for further clinical development.

Molecular imaging for atherosclerosis promises to offer new tools for testing pathophysiologic hypotheses in humans, for choosing effective doses of novel therapeutics to guide trials with clinical end points, and for obtaining early signals of in vivo efficacy in altering plaque biology. This study used molecular imaging in vivo and ex vivo to colocalize macrophages with MMP-13 activities in human and murine atheromas. In mice, intravital confocal microscopy imaged macrophages and MMP-13 in plaques and helped establish the proper dosage to achieve MMP-13 blockade in vivo (data not shown) and to monitor and validate MMP-13 inhibition during MMP13i-A treatment. These experimental findings illustrate the potential utility of imaging inflammation and protease activity in dose ranging in the design and conduct of clinical trials. The potential combination of imaging and targeting technologies with therapeutic compounds (eg, MMP-13 inhibitor) also raises the challenging prospect of “theranostic” strategies that could associate selective target-
ing and imaging-based assessment of biochemical efficacy in vivo.

In conclusion, this study advances the mechanistic conjecture that collagenases participate decisively in plaque biology in several important ways, and points a path toward clinical translation of this concept. The results establish that interstitial collagenase inhibition can alter the collagen content of evolving and established atheromas, a key unresolved issue in plaque biology and in consideration of this strategy for therapeutic intervention. We further demonstrate that a highly selective, orally administered interstitial collagenase inhibitor might have efficacy in this regard, avoiding the adverse effects encountered with broad-spectrum MMP inhibitors in the clinic. Finally, the results of this study illustrate how molecular imaging approaches might aid clinical development of MMP inhibitors by permitting dose ranging and providing biochemical evidence of efficacy in situ, as a prelude to trials of clinical efficacy.

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Disclosures

Dr M. Aikawa received an unrestricted donation from Alantos Pharmaceuticals (Cambridge, MA).

References


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Supplemental Methods

Animal Preparation

All experiments conformed to a protocol approved by the Standing Committee on Animals of Harvard Medical School. We studied the impact of MMP-13 inhibition on atherosclerosis-susceptible apoE−/− mice with congenic c57bl/6 background. Male mice aged 8–10 weeks (n=12 per group) consumed an atherogenic diet (semi-purified chow containing 1.25% cholesterol and 0% cholate, Research Diets, New Brunswick, NJ) for 10 weeks, together with oral administration of indicated doses of the MMP-13 inhibitor MMP13i-A (Amgen, Thousand Oaks, CA). To test the effects of MMP-13 inhibition on already established atheromata, mice were fed an atherogenic diet for 10 weeks before starting the 10-week treatment (n=12 per group) (Supplemental Fig. I). For both studies, MMP13i-A and corresponding vehicle were administered by gavage twice a day, spaced by 10–12 hours. We used in vivo molecular imaging with the MMP-13 activatable probe to determine the sufficient dosage of MMP13i-A to limit the fluorescence generated by MMP-13 activity in plaques. A preliminary dose–response study allowed us to select 40 mg/kg/day as the minimal dosage needed to reduce MMP-13 activity in vivo (Supplemental Fig. IX). Controls were age-matched and treated with vehicle (methylcellulose 0.5%, Convergent Bioscience, Toronto, Canada). Age-matched MMP13−/− apoE−/− deficient mice (c57bl/6) consumed a high-fat diet for 10 weeks and were used to assess imaging agent specificity (n=3). All mice were maintained in animal facilities at Harvard Medical School. Animal care and procedures were reviewed and approved by the Institutional Animal Care and Use Committees and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Human Tissues

Endarterectomy specimens of human carotid plaques (n=5) were obtained by protocols approved by the Human Investigation Review Committee at Brigham and Women’s Hospital. Surgical tissue samples were embedded in OCT compound and stored at -80°C until use. Serial cryo-sections were cut, air-dried on to microscope slides, and used in parallel for in situ zymography (ISZ) (8 µm) and immunohistochemistry (6 µm).
**Molecular Imaging**

*Molecular imaging agents*

We used fluorescent nanoparticles based on an iron oxide core (Ex753/Em773) for the detection of macrophage accumulation. MMPsense (Ex680/Em700) fluorescence reflected MMP-13 activity. Probes (VisEn Medical, Inc., Woburn, MA) were coinjected in the tail vein 48 hours before imaging at 15 mg of Fe and 4 nmol per kg of body weight, respectively. Selectivity of the MMP-13 probe was affirmed by in vitro incubation with various recombinant active domains of human MMPs (Supplemental Fig. X).

*Intravital confocal microscopy (IVM)*

We performed multichannel fluorescence imaging on anesthetized mice (ketamine, 80 mg/kg, and xylazine, 5 mg/kg). Briefly, the carotid artery was exposed at the level of the external and internal carotid bifurcation. After removal of connective tissue, anesthetized mice were placed under the confocal microscope to detect the fluorescence emitted by the probes in plaques (Olympus FV1000, Tokyo, Japan). Image collection of the different channels was done sequentially to avoid crosstalk between channels, as we described previously.²

*Ex vivo fluorescence reflectance imaging (FRI)*

After euthanasia, aortas were perfused with saline, dissected, and imaged using a FRI system equipped with multichannel filter sets (Carestream, Rochester, NY). The sum of the fluorescence levels in the aortic root and arch region was subtracted to the background level calculated in a similar area size with no aorta exposed. Quantitative data were extracted from the scanned pictures using Kodak Molecular Imaging Software®.

**Cell Culture**

*Human primary monocyte-derived macrophages*

We used lymphocyte separation medium gradient centrifugation to isolate human peripheral blood mononuclear cells from the peripheral buffy-coat blood, as previously described.³ Monocytes were cultured and differentiated into macrophages after 10 days in RPMI containing 5% human serum (Gemini Bio-Products, West Sacramento, CA) and 1% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO). Incubation for 24 hours with recombinant tumor necrosis factor-α (TNFα) (20 ng/ml, R&D Systems, Minneapolis, MN) and interleukin-1β (IL1β) (20 ng/ml, R&D Systems) elicited macrophage activation.
Murine primary macrophages and smooth-muscle cells (SMCs)

Three days after intraperitoneal injection of 4.0% thioglycollate, we harvested peritoneal macrophages from mice and cultured them. After 48 hours of incubation with DMEM containing 10% FCS, culture media of adherent cells was refreshed with DMEM and incubated for an additional 24 hours with TNFα (10 ng/ml) and IL1β (10 ng/ml). To test the uptake of nanoparticles by macrophages versus SMCs, RAW264.7 cells and primary aortic SMCs were cultured as previously described.4

MMP Activity Assay

MMP-13 activity was measured using MMP-13 fluorescent substrate (30 mg/L): MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2 [Cha = L-cyclohexylalanine; Dpa = 3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Nva = L-norvaline] (EMD Biosciences, San Diego, CA), as previously described.5 Selectivity assays were performed using recombinant catalytic domains of human MMPs (50 nM) and omniMMP fluorescent peptide (50 µM): Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2.AcOH [Mca=(7-methoxycoumarin-4-yl) acetyl; Dpa=N-3-(2,4-dinitrophenyl)-L-a,ß-diaminopropionyl] (Enzo Life Sciences, Farmingdale, NY) as substrate in Buffer M (50 mM Tris pH7.5, 300 mM NaCl, 5 mM CaCl2, 0.05% Brij-35, and 20 µM ZnCl2).

In situ Zymography (ISZ)

To estimate MMP collagenase activity in situ, we used in situ zymography assay (ISZ), incubating 6–8 µm sections with DQ-Collagen (Invitrogen) or MMPsense (Visen) in Buffer M, agarose 1%, in presence of MMP13i-A (10 µM), Ilomastat (10 µM), ortho-phenanthroline (0.5 mM), or EDTA (10 mM) for 48 hours.

Histological Assays

For histological evaluations of the aortic root, we used the method of Paigen et al.6 Hearts were embedded in tissue-tek OCT, and sectioned at 6 µm. Plaque size and composition were characterized with anti-α-smooth-muscle actin (Sigma-Aldrich) and anti-Mac3 (BD Pharmingen, San Diego, CA) antibodies. Quantification was performed on ImagePro Plus 5.1 (Media Cybernetics, Bethesda, MD).4, 7-9
Collagen Fiber Morphology and Quantification

Detection of interstitial collagen used picrosirius red staining with linear polarized light, as described previously.\textsuperscript{7, 10-12} We used the optical properties of picrosirius red–stained collagen fibers to analyze the fiber color.\textsuperscript{1, 13-15} As fiber thickness increases, the color shifts from green to red. To discriminate the green and red birefringent fibers, green and red optic filters (HQ535/50m, D605/55m, Chroma, Bellows Falls, VT) were disposed under polarized light using a Nikon Eclipse 80i polarizing microscope. Images were recorded by a digital camera (DS-U2, Nikon, Tokyo, Japan), and analyzed using image analysis software (NIS-Elements, Nikon). We assessed the amount and spatial distribution of each color fiber.\textsuperscript{15} The relative amount of each fiber color was expressed as a percentage of the total amount of collagen in the region.

Real-Time RT-PCR

Total RNA was extracted from abdominal aortas of treated and control animals (n=5 per group) using BioMasher (Investigen, Hercules, CA) and RNeasy extraction kit (Qiagen, Hilden, Germany), then reverse transcribed (Invitrogen, Carlsbad, CA). Real-time RT-PCR used SYBR green PCR Master Mix, QuantiTect Oligonucleotides for mouse MMP-8, MMP-12, MMP-13, MMP-14, cathepsin K, and GAPDH (Qiagen), and MyiQ Real-Time PCR Detection System (Biorad, Hercules, CA).

Statistical Analysis

Continuous variables are summarized as mean ± SEM; categorical variables are summarized as actual numbers and percentages. Differences between the treated and untreated groups were determined with the Mann-Whitney U test (Prism, GraphPad Software, La Jolla, CA). Differences were considered statistically significant at the p<0.05 level.

Bibliography


We studied the impact of MMP-13 inhibition on atherosclerosis-susceptible apoE\(^{-/-}\) mice. Mice aged 8–10 weeks (n=12 per group) consumed an atherogenic diet for 10 weeks, together with oral administration of the MMP-13 inhibitor MMP13i-A. To test the effects of MMP-13 inhibition on already established atheromata, mice were fed an atherogenic diet for 10 weeks before starting the 10-week treatment (n=12 per group). For both studies, MMP13i-A and corresponding vehicle (0.5% methyl cellulose solution) were administered by gavage twice a day, spaced by 10–12 hours.
Supplemental Figure II

Chemical structure of MMP-13 inhibitor (MMP13i-A).

Supplemental Figure III

Quantification of \textit{in situ} zymography for collagenolytic activity in human (A) and murine (B) atheromata. The broad MMP inhibitor Ilomastat was used as control.
Supplemental Figure IV

IVM on carotid plaques from apoE<sup>−/−</sup> mice coinjected with MMP-13 activatable probe (green) and fluorescent nanoparticles targeting macrophages (red) (arrows indicate plaque locations) (A). Fluorescent microscopy interrogated signals from both MMP-13 and macrophage probes on aortic root sections from coinjected animals. Staining of macrophages was performed on adjacent sections (B). Images are representative of n ≥ 4 animals.
Supplemental Figure V

Apoptosis analysis by TUNEL staining after 10 weeks’ administration of vehicle or MMP13i-A to apoE<sup>−/−</sup> mice with already established lesions. Means and SEM are represented. p values reflect statistical significance between the MMP13i-A–treated group and the control group.

Supplemental Figure VI

Impact of MMP-13 inhibition on established plaque calcification. A, Representative Von Kossa staining (counterstained with eosin 1%) of aortic roots of apoE<sup>−/−</sup> mice with established lesions treated with vehicle or MMP13i-A for 10 weeks. B, Quantification of calcified area as a percentage of total lesion size. C, Quantification of the percentage of plaques with calcification. Means and SEM are represented. p values reflect statistical significance between the MMP13i-A–treated group and the respective untreated group.
Supplemental Figure VII

mRNA was extracted from aortas from MMP13i-A–treated and untreated apoE<sup>−/−</sup> mice (n=5 per group). Expression of MMP collagenases and cathepsin K in developing or established plaques was analyzed by quantitative RT-PCR in response to MMP-13 inhibition. Bars represent mean ± SEM.
Pharmacokinetic/pharmacodynamic data on the MMP13i-A inhibitor. MMP13i-A compound has a 4–5 hour half-life in rats when administered orally (see graph and chart). One administration of the compound at 5 mg/kg resulted in a maximum concentration of ~5.8µM after 1 hour. We gave 20 mg/kg of the compound every 10–12 hours (40 mg/kg/day) so we could approximate a maximum concentration of ~23.2 µM in vivo.

A more recent pharmacokinetic analysis of a related compound in mice showed a half-life of about 12 hours. We treated the mice twice a day to maintain blood levels of the compound in vivo.
**Supplemental Figure IX**

Quantification of FRI of dissected aortas from apoE⁻/⁻ mice treated with vehicle or various dosages of MMP13i-A, 24 hours after injection of an MMP-13 activatable probe. Means and SEM are represented.

**Supplemental Figure X**

Selective cleavage of MMPsense (13.3 µM) by various recombinant catalytic domains of human MMPs (20 pmol). Neutral pH was used for MMP-2, MMP-7, MMP-8, MMP-9, and MMP-13. Basic pH (9.5) was used for optimal MMP-12 activity. Means and SEM are represented.
Supplemental Figure XI

Selectivity of MMP13i-A inhibitor at high concentrations. Activity of various recombinant catalytic domains of human MMPs (10 pmol) was assessed using a non-specific fluorogenic substrate for MMPs after incubation with MMP13i-A inhibitor. Means and SEM are represented.

*p reflects statistical significance between MMP13i-A and the no inhibitor condition.
Supplemental Table A - Characteristics of apoE<sup>−/−</sup> mice with or without MMP13i-A treatment.

Table A. Characteristics of apoE<sup>−/−</sup> mice with or without MMP13i-A treatment

<table>
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