Macrophage Metalloelastase Accelerates the Progression of Atherosclerosis in Transgenic Rabbits

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Background—Macrophage metalloelastase (matrix metalloproteinase [MMP]-12) is upregulated in atherosclerotic lesions and aneurysm; thus, increased MMP-12 activity may play an important role in the pathogenesis of atherosclerosis. However, the pathological roles of MMP-12 in the initiation and progression of atherosclerosis have not been defined.

Methods and Results—We compared the susceptibility of MMP-12 transgenic (Tg) rabbits to cholesterol-rich diet–induced atherosclerosis with that of non-Tg littermate rabbits. The rabbits were maintained at either relatively lower levels of hypercholesterolemia for shorter periods or higher levels of hypercholesterolemia for longer periods through a diet containing different amounts of cholesterol. We found no significant difference in the aortic atherosclerotic lesion size or quality between Tg and non-Tg rabbits at lower hypercholesterolemia. At higher hypercholesterolemia for longer periods, however, Tg rabbits developed more extensive atherosclerosis in the aortas and coronary arteries than did non-Tg rabbits. Histological examinations revealed that atherosclerotic lesions of Tg rabbits contained prominent macrophage infiltration associated with marked disruption of the elastic lamina in the tunica media with occasional formation of aneurysm-like lesions. Furthermore, increased expression of MMP-12 derived from macrophages was associated with elevated expression of MMP-3, suggesting that MMP-12 may play a pivotal role in the cascade activation of other MMPs, thereby exacerbating extracellular matrix degradation during the progression of atherosclerosis.

Conclusions—Overexpression of MMP-12 causes accelerated atherosclerosis in Tg rabbits. These results suggest that macrophage-derived MMP-12 participates in the progression of atherosclerosis. (Circulation. 2006;113:1993-2001.)

Key Words: animals, genetically modified • atherosclerosis • inflammation • metalloproteinases

The accumulation of macrophage-derived foam cells under the intima of large arteries is a hallmark of human and experimental animal atherosclerosis.1 Macrophages secrete a variety of proteinases that are thought to participate in vascular remodeling of the extracellular matrix (ECM) associated with all stages of atherosclerosis.2,3 Increased proteolysis of ECM molecules is probably involved in the initial mononuclear leukocyte emigration from the vascular lumen through the basement membrane into the subendothelial space, the migration and proliferation of medial smooth muscle cells (SMCs) through the elastic laminae in the intima, plaque destabilization, and rupture. Among the various types of proteinases, matrix metalloproteinases (MMPs) are particularly important in the pathogenesis of atherosclerosis and plaque rupture.4,5 Thus far, several MMPs have been reported to be expressed in atherosclerotic lesions6–10; however, the precise role of each of these MMPs in terms of lesion formation and progression has not been fully defined. In apolipoprotein E knockout mice, MMP-3 deficiency increases lesion size,11 whereas MMP-1 overexpression12 or MMP-913 deficiency reduces the lesion growth.

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MMP-12, also called macrophage metalloelastase, was first identified as a potent elastolytic metalloproteinase specifically secreted by macrophages,14,15 and increased activity of MMP-12 from inflammatory macrophages is associated...
MMP-12 also is able to degrade a broad spectrum of ECM protein-1 (MCP-1), and CD40 ligands; (2) MMP-12 elaborates high levels of MMP-12 in response to proinflammatory mediators such as GM-CSF, monocyte chemotactic protein-1 (MCP-1), and CD40 ligands; (3) MMP-12 undergoes self-activation through autolytic processing; and (4) recombinant rabbit MMP-12 has been shown to activate other MMPs such as MMP-2 and -3, suggesting that once MMP-12 is upregulated, there is a cascade of activation of other MMPs that leads to ECM degradation. Therefore, it is very likely that in the arterial wall, MMP-12 not only digests elastin but also degrades the basement membrane, which enables monocyte/macrophages to penetrate into arterial walls during the pathogenesis of atherosclerosis, although this hypothesis has not been tested.

Our laboratory previously demonstrated that the expression of MMP-12, compared with that of MMP-1, -2, -3, and -9, was prominently upregulated in atherosclerotic lesions of cholesterol-fed rabbits. We postulated that macrophage-derived MMP-12, in concert with other MMPs, may play a central role in the lesion formation because (1) macrophages elaborate high levels of MMP-12 in response to proinflammatory mediators such as GM-CSF, monocyte chemotactic protein-1 (MCP-1), and CD40 ligands; (2) MMP-12 undergoes self-activation through autolytic processing; and (3) recombinant rabbit MMP-12 has been shown to activate other MMPs such as MMP-2 and -3, suggesting that once MMP-12 is upregulated, there is a cascade of activation of other MMPs that leads to ECM degradation. Jornosjo and colleagues further reported a common polymorphism within the human MMP-12 gene promoter (an A-to-G substitution at position −82) that is associated with coronary artery luminal dimensions in diabetic patients. We hypothesized that excess MMP-12 production by macrophages plays important and distinct roles in both the initiation and progression of atherosclerosis. To examine this hypothesis, we generated MMP-12 transgenic (Tg) rabbits that specifically overexpress human MMP-12 in tissue macrophages and investigated the effect of MMP-12 on the development of cholesterol diet–induced atherosclerosis. Rabbits were used for this undertaking because they have several advantages as an animal model for the study of atherosclerosis. Rabbits have lipoprotein profiles similar to that of humans, and they are susceptible to the development of atherosclerosis, in which the lesions (from early stage to advanced stage) resemble those seen in humans. Furthermore, the relatively large size of the rabbit heart facilitates the investigation of coronary atherosclerosis. Our results obtained here demonstrated that overexpression of macrophage-derived MMP-12 in Tg rabbits accelerated both aortic and coronary atherosclerotic lesion progression, thus providing the first compelling evidence that MMP-12 upregulation affects the progression of atherosclerosis and enhances the degradation of the medial elastic laminae.

Methods

Experimental Design

Tg rabbits (kbtJW, Biotek, Saga, Japan) expressing human MMP-12 were generated in our laboratory as described previously. The Tg construct used was composed of human MMP-12 CDNA (catalytic domain sequence) under the control of the human scavenger receptor A enhancer/promoter, which directs the specific expression of the transgene in the macrophage lineage and foam cells of atherosclerotic lesions. Male Tg and non-Tg littermate rabbits 5 to 6 months of age were divided into 2 groups. The first group of rabbits (n = 14 for non-Tg, n = 13 for Tg) were fed a diet containing 0.2% cholesterol and 3% soybean oil by weight for 16 weeks, which makes rabbits develop mild hypercholesterolemia and supposedly early-stage lesions (mainly fatty streaks). The animals were fed each diet ad libitum, and plasma levels of total cholesterol were measured weekly. The second group (n = 17 for both non-Tg and Tg) was given a diet high in cholesterol (≈0.8%) for 28 weeks. We maintained the plasma total cholesterol in these rabbits at constant “higher” levels (compared with the first group) in an attempt to produce more advanced atherosclerosis (such as fibrous plaques and complicated lesions). To achieve this, we monitored plasma lipids weekly and adjusted the cholesterol content in the diet according to the change in the plasma cholesterol level of each individual animal. All animal experiments were performed with the approval of the Animal Research Committee of the University of Tsukuba (Japan). For the analysis of lipoprotein distribution, plasma lipoproteins were isolated by sequential ultracentrifugation as described.

Quantification of Aortic and Coronary Atherosclerosis

At the end of cholesterol diet feeding, all rabbits were killed by injection of an overdose of sodium pentobarbital solution. The aortas were en face stained with Sudan IV for evaluation of the gross atherosclerotic lesions as described previously. For the microscopic quantification of the lesion area, each segment of the aorta from all rabbits was cut into cross sections (8 to 10 for the aortic arch and 20 for the thoracic aorta as described previously. All sections were embedded in paraffin and stained with hematoxylin and eosin (H&E) and elastica van Gieson (EVG). For microscopic evaluation of cellular components and MMPs in the lesions, serial paraffin or frozen sections of the thoracic aorta were immunohistochemically stained with the panel of monoclonal antibodies (mAbs) shown in Table I of the online Data Supplement. To assess coronary atherosclerosis, rabbit hearts were sectioned into 7 blocks as shown in Figure 1 of the online Data Supplement, and the lesions were expressed as the intimal lesion area of the left and right coronary arteries. All sections (EVG and immunostained) for microscopic quantification were captured under an Olympus BX51 light microscope equipped with a DP70 digital camera (Olympus, Tokyo, Japan) and were measured with Lumina Vision V2.2 image analysis software (Mitani Co, Tokyo, Japan). We evaluated the grade of the elastic lamina destruction of the aorta using the following criteria: Erosion of the tunica media (designated grade 1) referred to the destruction from the internal elastic lamina to less than one third of the tunica media or <8 layers of the elastic laminae; fragmentation (grade 2) referred to the destruction of more than one third of the tunica media or 8 layers of the elastic laminae; and disappearance (grade 3) referred to those lesions in which the elastic lamina was totally degraded or disappeared. Grading and measurements were performed independently by 2 blinded observers.

Western Blot Analysis and Zymography

Aortic lesions were homogenized in ice-cold suspension buffer supplemented with a protease inhibitor cocktail (Sigma, St Louis, Mo) as described previously. Aliquots of the crude protein were fractionated by electrophoresis on 10% SDS-polyacrylamide gels, followed by immunoblotting with the panel of antibodies shown in Table I of the Data Supplement. Substrate gel zymography of the activity of MMPs was performed with the method reported elsewhere.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction Analysis

Total RNA from the aortic lesion area, along with alveolar macrophages, was isolated with Trizol reagent and then analyzed by real-time reverse-transcriptase (RT) polymerase chain reaction (PCR) (DNA Engine Opticon, MJ Research, Tokyo, Japan). mRNA expression levels of MMP-1,-2,-3,-9,-12, and -13; MT1-MMP; tissue inhibitor of metalloproteinase (TIMP)-1,-2, and -3; and MCP-1 were evaluated with DyNAmo SYBR Green qPCR kits.
The panel of specific primers used for analyzing the gene expression is shown in Table II of the Data Supplement.

**Macrophage Chemotaxis Study**

To evaluate the effect of MMP-12 on macrophage migration ability, we performed a chemotaxis assay using Biocoat cell culture inserts coated with laminin (Becton Dickinson Labware, Bedford, Mass). Half a milliliter of alveolar macrophages (2.5 × 10^5 cells/mL) in 1640 medium isolated from either Tg or non-Tg rabbits was plated in the upper wells. The lower compartments were loaded with the same medium containing recombinant human MCP-1 at 10 ng/mL (Pepro Tech, London, UK). After 48 hours of incubation (37°C, 5% CO2), the number of macrophages that had penetrated the gels was determined by counting 10 high-power fields at random from each well.

**Human Coronary Arteries**

Human coronary arteries were obtained from either autopsy cases (n = 29) or patients (n = 10) who underwent directional coronary atherectomy as shown in Tables III and IV of the Data Supplement. The lesions with diffuse intimal thickening, fatty streak, and fibrous plaques were selected for immunohistochemical staining of MMP-12 as described above.

**Statistical Analysis**

All values were expressed as mean ± SE. Statistical significance was determined with the Mann-Whitney U test for nonparametric analysis of the lesions. The Student t test was used to compare the results of other assays. In all cases, statistical significance was set at P < 0.05.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Effect of MMP-12 on the Initiation of Atherosclerosis**

In the first experiment, we examined whether increased MMP-12 affects the initiation of atherosclerosis (early-stage lesions) using rabbits that had “lower” hypercholesterolemia; the mean total cholesterol levels ranged from 384 to 530 mg/dL in non-Tg and 285 to 522 mg/dL in Tg (Figure IIA in the online Data Supplement). Quantitative analysis of the aortic sudanophilic area showed that Tg and non-Tg rabbits were similar in atherosclerotic lesions (P > 0.05) (Figure 1). Microscopic examination, coupled with immunohistochemical staining, revealed that the lesions from both Tg and non-Tg rabbits were composed of fatty streaks in which a small number of macrophages and SMCs were scattered. There was, however, no significant difference in cellular components or intimal lesion size between the 2 groups (Figure IIB and IIC in the Data Supplement).

**Effect of MMP-12 on the Progression of Atherosclerosis**

In the second experiment, we used rabbits that were maintained at higher hypercholesterolemia for 28 weeks to evaluate whether MMP-12 affects the progression of advanced lesions. Although plasma lipids (total cholesterol, Figure 2A; triglycerides and HDL cholesterol, not shown), lipoprotein profiles, and apolipoprotein were not significantly different between the 2 groups (Figure IIIA and IIIB in the Data Supplement), Tg rabbits developed more extensive and severe aortic atherosclerosis than non-Tg rabbits. To assess the severity of atherosclerosis, we characterized lesions by (1) the sudanophilic area of the aortic arch and thoracic aortas, (2) quantitative microscopic measurement of intimal size and types, (3) immunohistochemical staining, and (4) distribution of macrophages.
nohistochemical quantification of the lesional cellular components (macrophages versus SMCs), (4) histological grading and measurement of the elastic lamina destruction of the tunica media, and (5) microscopic analysis of coronary atherosclerosis.

Analysis of en face preparations of the aortic arch and thoracic aortas showed a significant increase in plaque area (1.2- and 2-fold increases in the aortic arch and thoracic aorta, respectively) in Tg rabbits compared with non-Tg rabbits (Figure 2B and 2C). Consistent with the gross observations, microscopic intimal lesion area also was increased in Tg rabbits: 1.5-fold increase in the aortic arch (P=0.07 versus non-Tg rabbits) and 1.6-fold increase in the thoracic aorta (P<0.05) (Figure 3A, left). Analysis of the lesion types (early-stage lesions [types I and II] versus advanced lesions [types III through V]) based on the criteria of the American Heart Association revealed that the increased intimal lesion size in Tg rabbits was due to the increase in advanced lesions in the aortic arch (2-fold increase; P<0.05) and of both early (1.6-fold increase; P<0.05) and advanced (1.5-fold increase; P<0.05) lesions in the thoracic aorta (Figure 3A, middle and right). Histological and immunohistochemical examinations showed that the lesions of Tg rabbits were characterized by 2 prominent changes compared with non-Tg rabbits: significantly increased accumulation of macrophages (P<0.01) and SMCs (P<0.01) (Figure 3B) in the intima and pronounced destruction of the elastic laminae of the tunica media. In some areas, the aortic atherosclerotic lesions were so prominent that they focally protruded outward and resulted in aneurysm-like lesion formation (Figure 3C). We further analyzed the medial lesions and quantified the elastic destruction in the lesions using the standard described in Methods and illustrated in Figure 4A. Compared with non-Tg rabbits, Tg rabbits showed a remarkable and significant increase in the medial lesions and a higher degree of all 3 grades of elastic lamina destruction (Figure 4B). In the areas where the medial elastic laminae were disrupted, there were a considerable number of infiltrating macrophages and few SMCs. Data are expressed as mean±SE (n=17 for non-Tg, n=14 for Tg group). Magnification ×4 (C). *P<0.05, **P<0.01 vs non-Tg rabbits.

Figure 3. Microscopic analysis of the intimal lesion size, type (A), and cellular components (B) and of aneurysm lesions (C). Intimal lesions on EVG-stained sections were quantified with an image analysis system. Lesions were classified into early lesions (types I and II) and advanced lesions (types III through V); representative micrographs are shown on the right (A). Serial paraffin cross sections of aortic lesions were stained with H&E and mAbs against macrophages (Mφ) and SMCs. Positive area of immunostained macrophages and SMCs is shown on the right (B). C, Typical aneurysm-like lesion in Tg rabbits. The lesions were composed of many infiltrating macrophages and few SMCs. Data are expressed as mean±SE (n=17 for non-Tg, n=14 for Tg group). Magnification ×4 (C). *P<0.05, **P<0.01 vs non-Tg rabbits.

Expression of MMPs and Their Inhibitors in Lesions

To examine the distribution patterns of MMP-12 and other MMPs in the lesions of Tg rabbits, we performed immunohistochemical staining using frozen sections. Figure 5 shows that MMP-12 immunoreactive proteins were colocalized with macrophages in the lesions, as clearly demonstrated by double immunostaining (Figure 5A). In contrast to MMP-12, MMP-1 was present mainly on the
superficial areas of the lesions, whereas MMP-2 was diffusely distributed in both intimal lesions and the medial SMCs (Figure V in the Data Supplement). TIMP-2 (Figure V in the Data Supplement) and MMP-9 (data not shown), however, were faintly stained in the lesions. Because the mere presence of MMP proteins does not establish their catalytic capacity (the zymogens lack activity), we examined the MMP enzymatic activity using substrate gel zymography. Enzymatic activity of MMPs in the lesions could be demonstrated by using either \( \beta\)-casein (Figure 5B) or gelatin (data not shown) as a substrate. We further quantified the content of each MMP protein in the lesions using Western blotting and showed that among these MMPs and TIMPs examined, MMP-12, MMP-3, and MT1-MMP in the lesions of Tg rabbits were significantly increased compared with the levels in non-Tg rabbits (\( P < 0.05 \)), whereas MMP-1, MMP-9, and TIMP-1, -2, and -3 were not significantly different (Figure 6A). It is noteworthy that active-type proteins of MMP-2 (66 kDa) were significantly increased, whereas pro-type MMP-2 (72 kDa) remained unchanged (Figure 6A). Real-time RT-PCR confirmed that the lesions of Tg rabbits showed higher mRNA expression of MMP-12, with specific expression of the human MMP-12 transgene (in both lesions and isolated macrophages), along with increased rabbit endogenous...
MMP-12 (1.7-fold increase over control; \( P < 0.05 \)) (Figure 6B, top). In addition to MMP-12, expression of MMP-13 (2.8-fold increase), MMP-1 (2.3-fold increase) followed by MT1-MMP (1.5-fold increase), MMP-9 (1.3-fold increase), and MCP-1 (1.8-fold increase) in the lesions of Tg rabbits was concomitantly increased, although these increases did not reach statistical significance (Figure 6B, middle and bottom). MMP-2 and -3 expression was relatively reduced, whereas TIMP-1, -2, and -3 expression was not significant between the 2 groups.

**Macrophage Migratory Activity**

To explore the possible mechanisms of enhanced macrophage accumulation in the lesions of Tg rabbits, we compared the capacity of alveolar macrophages isolated from cholesterol-fed Tg and non-Tg rabbits to invade an immobilized ECM in vitro. Without a chemoattractant, neither Tg nor control rabbit macrophages showed migratory activity (<5 cells per well; data not shown). In response to the presence of the chemoattractant MCP-1, the number of gel-invading macrophages from Tg rabbits was 3.2-fold greater than that from non-Tg rabbits (Figure 7). This result agrees with our finding that in lesions of Tg rabbits...
rabbits, overexpression of MMP-12 led to increased macrophage accumulation.

Detection of MMP-12 in Human Coronary Atherosclerosis

The finding that overexpression of MMP-12 in macrophages of Tg rabbits resulted in enhanced atherosclerosis prompted us to examine whether increased MMP-12 is associated with the lesions of human atherosclerosis. Although MMP-12 immunoreactive proteins were not present in the intimal thickening in which the major cells were those of SMCs, MMP-12 was consistently detected in areas with accumulated macrophages such as fatty streaks and the shoulder of the fibrous plaques (Figure 8A through 8D), in unstable plaque (Figure VI of the Data Supplement), and in ruptured plaque (data not shown).

Discussion

Despite the fact that increased expression of MMP-12 has been shown to be associated with a number of inflammatory processes, the pathophysiological roles of MMP-12 derived from macrophages in the formation of atherosclerotic lesions have not been elucidated. In the present study, using Tg rabbits overexpressing human MMP-12 specifically in the macrophage lineage,27 we examined the hypothesis that MMP-12 may be involved in the pathogenesis of atherosclerosis. Two types of atherosclerotic lesions (referred to as early and advanced stage) were created in Tg rabbits through 2 different dietary manipulations and compared with those in non-Tg littermate rabbits. In the first experiment, we did not observe any significant difference in lesions between Tg and non-Tg rabbits. Although this result may be surprising, it may be explained by the fact that monocytes other than differentiated tissue macrophages virtually do not express MMP-12.25 Therefore, it may suggest either that MMP-12 is not significantly involved in the initial process of atherosclerosis (such as monocyte adhesion to endothelial cells/emigration) or that the small quantity of lesions at the early stage present in these rabbits may not contain sufficient tissue macrophages to express substantial levels of MMP-12. This notion was substantiated by the second experiment in which rabbits developed more complicated lesions with many foam cells. It seems unlikely that MMP-12 was directly mediated by atherogenic lipoproteins in the intimal milieu because we did not demonstrate the significant stimulatory effects of either human oxidized low-density lipoprotein (LDL) (Figure VII in the Data Supplement) or rabbit β-very-low-density-lipoprotein (data not shown) on MMP-12 mRNA expression using U937-derived macrophages.

In the second experiment, we found not only that Tg rabbits had more extensive aortic and coronary atherosclerosis but also that the lesions of aorta were characterized by the marked accumulation of macrophages and SMCs accompanied by remarkable destruction of the medial elastic laminae. This finding supports the prevailing view that increased elastolytic activity in the arterial wall accelerates the progres-
tion of atherosclerosis. It has been reported that enhanced elastin fragmentation is required for SMC migration and proliferation; therefore, increased elastic destruction of the tunica media may provide a mechanistic explanation for the finding shown here that the lesions contain more SMCs in Tg rabbits. It is notable that the lesions of Tg rabbits also were enriched in macrophages compared with those of non-Tg rabbits, raising the possibility that increased MMP-12 in the lesions may lead to the enhanced recruitment of monocyte/macrophages into the lesions. Two mechanisms may aid in the enhancement of macrophage accumulation in the lesions. First, increased MMP-12 activity may augment the degradation of elastin and generate elastin fragments. It is well known that elastin peptides generated through hydrolysis of elastin are a potent chemoattractant for monocytes and macrophages. Second, migration of macrophages requires elastin fragmentation is required for SMC migration and activation of other MMPs in the pathogenesis of atherosclerosis. Our data not only shed fresh light on the functional roles of MMP-12 but also have implications for the notion that specific inhibition of MMP-12 activity may be a candidate therapeutic target for the treatment of atherosclerosis and its complications in the future.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Although many matrix metalloproteinases (MMPs) have been detected in the lesions of human atherosclerosis, their pathophysiological roles in the lesion formation have not been fully defined. In this study, we focused on MMP-12 (also called macrophage elastase) in terms of its roles in atherosclerosis using transgenic rabbits expressing human MMP-12 in macrophage lineage. We found that increased macrophage-derived MMP-12 significantly enhanced the progression of atherosclerosis in cholesterol diet–fed transgenic rabbits. Compared with the lesions of control rabbits, the lesions of transgenic rabbits were characterized by (1) greater lesion area of both aortic and coronary atherosclerosis, (2) increased number of macrophages, and (3) remarkable destruction of the medial elastic laminae with occasional formation of aneurysm-like lesions. In addition, increased MMP-12 in the lesions also was associated with upregulation of other MMPs such as MMP-3, suggesting that MMP-12 plays a pivotal role in the cascade activation of other MMPs, thereby exacerbating extracellular matrix degradation. Our results not only provide a fresh insight into understanding the roles of MMP-12 in atherosclerosis but also imply that inhibition of MMP-12 may become a therapeutic target for the treatment of atherosclerotic complications such as plaque ruptures and aortic aneurysms.
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