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Matrix Metalloproteinase 12 Accelerates the Initiation of Atherosclerosis and Stimulates the Progression of Fatty Streaks to Fibrous Plaques in Transgenic Rabbits

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Whether fatty streaks are directly followed by fibrous plaque formation in atherosclerosis remains controversial. Disruption of the basement membrane and elastic layers is thought to be essential for this process. Matrix metalloproteinase 12 (MMP-12) can degrade a broad spectrum of substrates, but the role of MMP-12 in the early stage of atherosclerosis is unclear. To investigate MMP-12 function in the initiation and progression of atherosclerosis, we investigated macrophage migration and elastolysis in relation to fatty streaks in human MMP-12 transgenic (hMMP-12 Tg) rabbits. Fatty streaks in hMMP-12 Tg rabbits fed a 1% cholesterol diet for 6 weeks (cholesterol-induced model of atherosclerosis) were more pronounced and were associated with more significant degradation of the internal elastic layer compared with wild-type (WT) animals. Numbers of infiltrating macrophages and smooth muscle cells in the lesions were increased in hMMP-12 Tg compared with WT animals. In both cuff- and ligation-induced models of atherosclerosis, smooth muscle cell-predominant atherosclerotic lesions were elevated with significant elastolysis of the internal elastic lamina in Tg compared with WT animals; “microelastolytic sites” were recognized before

formation of the neointima in the cuff model only. These results indicate that MMP-12 may be critical to the initiation and progression of atherosclerosis via degradation of the elastic layers and/or basement membrane. Therefore, a specific MMP-12 inhibitor might prove useful for the treatment of progressive atherosclerosis. (Am J Pathol 2008, 172:1419–1429; DOI: 10.2353/ajpath.2008.070604)

In atherosclerosis, the extracellular matrix (ECM), mainly produced by smooth muscle cells (SMCs) of the synthetic phenotype in the arterial intima, includes collagen types I, III, IV, V, VIII, and laminin.^{1–2} Collagen types I and III are synthesized and located in the intima and fibrous caps, and the shoulder regions of the plaque are rich in type I procollagen-synthesizing cells.³ The basement membrane underlying the vascular endothelium is a complex structure that results from the interaction of laminin, entactin, and heparin sulfate proteoglycan along with type IV collagen.⁴ Elastin is a main and critical component of the arterial media, and it maintains the wall structure and function by its elastic nature. In addition to elastin in the media, relatively large amounts of elastin are also synthesized by SMCs that become located in increasing number in the intima with the progression of atherosclerosis.^{1,5}

We reported previously that matrix metalloproteinases (MMPs), which are a family of zinc-dependent proteinases, are synthesized and secreted by endothelial cells, SMCs, and macrophages and are involved in ECM turnover in relation to remodeling of the arterial wall.^{6–9} Recently, a vast amount of knowledge about the roles of

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MMPs in atherosclerosis has accumulated, and more than 20 members of this family are currently known.^{10–15} Several studies have revealed the following features of MMP-12 knockout mice: macrophages with a markedly diminished capacity to degrade ECM and an essential disability to penetrate reconstituted basement membranes,¹⁶ a significant reduction in the number of infiltrating macrophages in allergen-induced lung inflammation relative to that in wild-type (WT) mice,¹⁷ and reductions in atherosclerotic lesion size and macrophage number,¹⁸ suggesting strongly that macrophage-mediated basement membrane proteolysis by MMP-12 is critically necessary for cell invasion at inflammatory sites.

First identified as a potent elastolytic metalloproteinase specially synthesized by macrophages, MMP-12 can degrade a broad spectrum of ECM components.^{19,20} Therefore, human MMP-12 (hMMP-12) transgenic (Tg) rabbits, which specifically overexpress hMMP-12 in their tissue macrophages, were recently generated,²¹ and these rabbits were shown to be useful for investigating the role of MMP-12 in arthritis.²² The overexpression of macrophage-derived hMMP-12 was later shown to accelerate the degradation of the medial elastic laminae in advanced atherosclerosis and abdominal aortic aneurysms.²³ However, the role of MMP-12 in early atherosclerotic lesions was not conspicuous.

In humans, fatty streaks are thought to be a requisite event in the initiation of atherosclerosis, with additional factors needed for its progression. According to the compelling hypothesis known as the “response to injury theory,”²⁴ after immigration of monocytes/macrophages from the peripheral blood into the intima via degradation of the basement membrane, early atherosclerotic lesions (mainly fatty streaks) appear. Their appearance is followed by a microscopic event, ie, the migration of SMCs from the media to the intima, which probably results in the progression from fatty streaks to fibrous plaques. Considering this process, elastolysis of the internal elastic lamina would need to be accelerated for such a microscopic transition. In the current study, we investigated the details of elastolysis of the elastic layer over a relatively short period of time using hMMP-12 Tg rabbits in three different types of experimental models of atherosclerosis, ie, cholesterol induced, cuff induced, and ligation induced, and discussed the role of the disruption of the basement membrane and elastolysis of the internal elastic layer by MMP-12 in the early stage of atherosclerosis.

Materials and Methods

Experimental Designs

Three- or 4-month-old male hMMP-12 Tg and WT rabbits,²¹ weighing approximately 1.5 to 2.0 kg, were divided into three groups. The first experimental group was fed a 1% cholesterol diet (120 g/day and water) to induce hypercholesterolemia, which was assessed by weekly measurement of plasma lipids (total cholesterol, triglycerides, high-density lipoproteins, and low-density lipoproteins). Every morning, we checked their meal

boxes to ensure that the diet had been consumed and weekly measured their body weight. Then the animals were terminated by injection of an overdose of veterinary Ketalar[®] and Domitor[®] at 6 or 9 weeks and then autopsied. Rabbits with an extremely high or low cholesterol level were excluded from this experimental group.

The second and third experimental groups of rabbits were used to prepare femoral cuff-induced and carotid ligation-induced atherosclerosis models.²⁵ These rabbits were anesthetized with veterinary Ketalar[®] and Domitor[®], and the bilateral femoral or lateral carotid arteries were cuffed by use of a polyethylene tube (inside diameter, 2.0 mm; outside diameter, 3.0 mm; Natsume Co., Tokyo, Japan) or ligated by silk sutures, respectively, as described previously.²⁵ The animals were terminated at 1 or 3 weeks by injection of an overdose of veterinary Ketalar[®] and Domitor[®], and both cuffed femoral and ligated carotid arteries were excised. For excision, the bilateral femoral arteries were cut at both sides of the cuff point; and the lateral carotid arteries, close to the ligation point on one side and approximately 10 mm from it on the other side. All rabbits recovered and showed no symptoms of stroke. Arteries affected by significant artificial damage due to the surgical procedure were excluded from the study.

Animals

In this experiment, human MMP-12 (hMMP-12) transgenic (Tg) rabbits were used as the experimental animals²¹; and specific pathogen-free Japanese white rabbits (KBT Oriental Corporation, Saga, Japan) were used as the WT controls.

Immunohistochemistry

Immunohistochemical staining was performed using a Dako Envision kit (Dako Cytomation Co., Kyoto, Japan) according to the manufacturer's instructions. For this purpose, we used mouse monoclonal antibodies against smooth muscle actin (α -SMA; Dako Cytomation Co.), rabbit macrophages (RAM-11; Dako Cytomation Co.), and the hMMP-12 catalytic domain (MAB 919; R&D Systems, Minneapolis, MN), as reported previously.^{23,25} The number of smooth muscle cells positive for α -SMA or of macrophages positive for RAM-11 was determined by counting 10 high-power fields limited to the atherosclerotic lesion thoroughly and equally from each aorta (fatty streaks in hypercholesterolemia model) or artery (neointima or adventitia in cuff- or ligation-induced injury model).

Quantitative Assessment of Cholesterol-, Cuff-, and Ligation-Induced Atherosclerosis

The aortas and arteries were removed and immediately immersed in cold PBS, fixed in 10% neutral buffered formalin, and embedded in paraffin for histological examination. Thick-paraffin sections (4 μ m) were stained with hematoxylin and eosin (H&E) or elastica van Gieson (EVG) stains or by immunohistochemical techniques to

assess the degree of atherosclerosis and degradation of the elastic layers.^{23,25} Twenty-six aortas from rabbits ($n = 16$ and 10 for hMMP-12 Tg and WT, respectively) fed the 1% cholesterol diet were opened longitudinally, pinned out flat on Styrofoam sheets, and stained with oil red O after having been fixed in 10% neutral buffered formalin for 24 hours. To measure atherosclerotic lesions, we photographed the whole aortas with an Olympus Camedia E-10 digital camera (Olympus Co., Tokyo, Japan). The red area relative to the whole surface area was measured by use of computerized NIH imaging software for rough estimation of the percentage of the total aortic area that was atherosclerotic. After the calculation, the preparations were cut into cross sections, in particular, at the marked atherosclerotic sites, for H&E, EVG, and immunohistochemical staining. Ten aortas ($n = 5$ for hMMP-12 Tg and $n = 5$ for WT) from animals fed the 1% cholesterol diet for 6 weeks, in which just early-stage atherosclerotic lesions (mainly fatty streaks) had formed in the thoracic aorta, were additionally examined by preparing up to 2500 sequential 4- μ m cross sections to observe the details of elastolysis. Of each 10 serial sections, at least three sections were stained, either immunohistochemically or with H&E or EVG, to evaluate elastolysis in detail. In cuff- and ligation-induced models 1 and 3 weeks after the surgical treatment, the resected arteries ($n = 30$ and 32 for Tg and WT, respectively) were also cut into 4- μ m cross sections to observe and evaluate atherosclerotic lesions microscopically.

For the quantitative analysis, after staining, these sequential slides were scanned at a resolution of 300 dpi in 24-bit full color by use of an Epson ES-8500 color image scanner (Epson Co., Tokyo) or were captured by a 600ES digital camera (Pixera, Los Gatos, CA) attached to an Olympus BX51 light microscope and measured with Studio 3.0 (Pixera) and NIH image for the evaluation of the intima-to-media ratio (I/M ratio) and elastolysis ratio.^{23,24} For calculations of the I/M ratio, we measured the thickened intimal and normal medial areas of arteries; and for the elastolytic ratio, we measured the length of the elastolytic portions and of the whole internal elastic lamina in these serial sections.

Casein Zymography and mRNA Quantification

For zymography, 25 μ g of protein extracts of the atherosclerotic aortas was mixed with SDS sample buffer without reducing agent and loaded onto a 10% SDS-polyacrylamide gel containing 0.2% casein, as described in detail previously.²¹ Digestion bands were quantified using an image analyzer system (NIH image). Changes in the mRNA levels of MMPs in high-cholesterol diet-induced aortas, cuffed femoral arteries, or ligated carotid arteries were quantified by conducting the real-time RT-PCR on total RNA prepared with TRIzol reagent. The synthesized cDNA was quantified using TaqMan quantitative PCR analysis of each gene with the ABI PRISM 7700 Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Specifically, primer and probe sequences used were as follows:

for human MMP-12, 5'-AGCTCTCTGTGACCCCAATTG (forward), 5'-AGCCAGAAGAACCTGTCTTTGAAG (reverse), and 5'-TTTGATGCT-GTCACTACCGTGGGAAATAAGAT-CT (probe); for rabbit MMP-12 (rMMP-12), 5'-AACGACCAGTGTCCGTTTAATTTC (forward), 5'-ACTTGATGCTGTCTCCAATTTTCAT-AAG (reverse), and 5'-TGGCCAACCTTGCCCTTCAGG (probe); for rabbit MMP-1, 5'-AAAGATTCAGAAATTACAACCTGTATCG (forward), 5'-TC-AAAGCCCCAA-TATCAGTAGAATG (reverse), and 5'-CTCATGAACTGGGCCATTCCCTTGG (probe); for rabbit MMP-2, 5'-TCACCTTCCTGGGCAACAAG (forward), 5'-GAGG-TCGCGCACACATC (reverse), and 5'-CTGTAC-CAGCGCCGGCCGC (probe); for rabbit MMP-9, 5'-CCCTGATAAAGGATACAGCCTGTT (forward), 5'-CCCCTCTAG-GTAGCGGTACATG (reverse), and 5'-CATGCACTGGGCTTGGATCACTCCT (probe); and for rabbit glyceraldehyde-3-phosphate dehydrogenase, 5'-AGGGCGGAGCCAAAAGG (forward), 5'-TTGCTGACAATC-TTGAGAGATT-GTC (reverse), and 5'-ATGCCCCCATGT-TTGTGATGGGC (probe). Each RNA quantity was normalized to its respective glyceraldehyde-3-phosphate dehydrogenase mRNA quantity.

Statistical Analyses

All values were expressed as the mean $\pm$ SE, and statistical significance was analyzed using Student's *t*-test or the Mann-Whitney's *U*-test for nonparametric analysis. Statistical significance was set at $P < 0.05$.

Results

En Face and Cross-Section Analyses of Cholesterol-Induced Fatty Streaks in the Aortic Arch

As shown in Figure 1A, fatty streaks in the hMMP-12 Tg rabbit were grossly more evident in the aortic arch and in the upper portion of the thoracic aorta compared with those in the WT animals. In the thoracic aorta, WT animals showed a tendency for fatty streak distribution in the branches of the bronchial and intercostal artery branches. Fatty streaks in the hMMP-12 Tg animal, however, were relatively diffusely spread throughout the thoracic aorta compared with those in the WT rabbit.

Quantitative analysis of the whole aorta en face stained with oil red O showed that hMMP-12 Tg rabbits fed the 1% cholesterol diet developed more extensive atherosclerotic lesions than WT rabbits at 6 or 9 weeks (Figure 1A), and the difference was statistically noted at both times ($P < 0.05$; Figure 1B). Similar to those of the WT rabbits, the cholesterol levels of hMMP-12 Tg rabbits fed the high-cholesterol diet were increased up to 2000 mg/dl during the first 6 weeks (Figure 1C).

Similar to these gross findings, microscopic analysis of cross sections of the aortic arch and the upper portion of the thoracic aorta revealed that hMMP-12 Tg rabbits developed much more pronounced aortic atherosclero-

Figure 1. Quantitative analysis of gross observations of the high-cholesterol diet-induced fatty streaks. **A:** Representative oil red O-stained aortas prepared from hMMP-12 Tg and WT rabbits fed the diet containing 1% cholesterol for 6 or 9 weeks. The fatty streaks in the hMMP-12 Tg rabbits in the aortic arch and the upper portion of the thoracic aorta were grossly more extensive than those in the WT animals. Fatty streaks of WT animals were mainly located in the bronchial artery branches and in the distal portion of the intercostal artery branches, whereas fatty streaks in the hMMP-12 Tg rabbits were diffusely spread throughout the aorta. **B:** En face quantitative analysis of the oil red O-stained portion of whole aortas. Data are expressed as the mean $\pm$ SE (n , number of aortas). * $P < 0.05$. **C:** Total cholesterol levels in hMMP-12 Tg and WT rabbits fed a diet containing 1% cholesterol up to 9 weeks are shown in the graph. Data are expressed as the mean $\pm$ SE (n , number of rabbits).

sis than WT rabbits, wherein a large number of RAM-11-reactive macrophages and a much smaller number of α -SMA-positive SMCs had accumulated and synthesized hMMP-12 in the fatty streaks (Figure 2A). Statistical microscopic analysis by atherosclerosis grading of the aortic arch and the upper portion of the thoracic aorta revealed that the intimal lesion area or I/M ratio was significantly increased about 2.3- to 3.0-fold in hMMP-12 Tg rabbits fed the high-cholesterol diet for 6 or 9 weeks compared with that for the WT rabbits ($P < 0.05$; Figure 2B). α -SMA-positive SMCs were also diffusely distributed in the fatty streaks, and some of these cells were located in the deep portion of the lesions close to the media. The numbers of macrophages and SMCs in fatty streaks of hMMP-12 Tg rabbits were significantly larger than those in WT animals ($P < 0.05$; Figure 2C).

Figure 2. Quantitative analysis of microscopic observations of the high-cholesterol diet-induced fatty streaks in the aortic arch and the upper portion of the thoracic aorta. **A:** Serial paraffin cross sections of aortas stained immunohistochemically with antibodies against macrophages (RAM-11), SMCs (α -SMA), and hMMP-12. **B:** Quantitative microscopic measurements indicated that the intimal lesion area was increased about 3.0-fold (6 weeks) or 2.3-fold (9 weeks) in hMMP-12 Tg aortas compared with that for the WT aortas. Data are expressed as the mean $\pm$ SE (n , number of aortas; number of sections = 9 for Tg and WT). * $P < 0.05$. **C:** Immunohistochemical quantification of the lesional cellular components revealed a significantly larger number of RAM-11-reactive macrophages and α -SMA-positive SMCs in hMMP-12 Tg rabbits than in the WT rabbits. Data are expressed as the mean $\pm$ SE (n , number of aortas). * $P < 0.05$.

Quantitative Analysis of Fatty Streaks and Elastolysis in the Distal Portion of the Thoracic Aorta

To study the relationship between fatty streaks and elastolysis of the most inner elastic layer below the endothelium, we examined fatty streaks in the thoracic aorta from hMMP-12 Tg and WT rabbits fed the 1% cholesterol diet for 6 weeks, by which time relatively slight fatty streaks had appeared, and we limited the observation area to about 10 mm in length, as shown in Figure 3A. For investigation of disruption of the elastic layers (Figure 3B), of up to 2500 serial 4- μ m-thick sections, up to 250 were stained with H&E or EVG. The

Figure 3. Quantitative microscopic analysis of the high-cholesterol diet-induced fatty streaks and elastolysis in the distal portion of the thoracic aorta. **A:** A schema for preparation of sequential sections of the intercostal artery area. **B:** Micrographs represent elastolytic portion of the internal elastic layer (arrows) observed in a fatty streak, particularly in EVG-stained sections of an hMMP-12 Tg rabbit. **C and D:** Quantitative microscopic measurements of intimal lesion area (**C**) and elastolysis ratio (**D**). The significantly increased intimal lesion area of hMMP-12 Tg rabbits was associated with approximately 3.2-fold more progressive elastolysis of the internal elastic layer compared with that for the WT rabbits. Data are expressed as the mean $\pm$ SE (n , number of aortas; total number of sections = 74 and 51 for intimal lesion area and elastolysis, respectively). * $P < 0.05$.

quantitative analysis of the intimal lesion area for these serial sections confirmed the area to be significantly larger in hMMP-12 Tg animals ($P < 0.05$; Figure 3C) and showed approximately 2.5- to 3.2-fold more pro-

gressive elastolysis of the elastic layer in these animals than in the WT rabbits ($P < 0.05$; Figure 3D). The general relationship between fatty streaks and elastolysis is shown in Figure 4.

Figure 4. Scheme of relationship between fatty streaks, elastolysis, and intercostal artery branches in the thoracic aorta. Photographs show elastolytic areas (blue lines) in fatty streaks (red lines) in high-cholesterol diet-fed hMMP-12 Tg (nos. 781 to 785) and WT rabbits (nos. 788 to 790 and 794 to 795). Fatty streaks were relatively developed in relation to intercostal artery branches in WT animals, whereas in hMMP-12 Tg, the lesion was spread diffusely in the distal thoracic aorta. Elastolytic lesions were almost entirely located in fatty streaks.

Figure 5. Real-time RT-PCR analysis and activity of hMMP-12 expression and rMMPs expression in cholesterol-induced atherosclerosis. **A:** In hMMP-12 Tg rabbits, analysis of real-time RT-PCR demonstrated the expression of hMMP-12 mRNA in atherosclerotic aortas fed a diet containing 1% cholesterol for 6 weeks but not in WT animals. Data are expressed as the mean $\pm$ SE (n , number of aortas). * P < 0.05. **B:** Zymography of hypercholesterolemia-induced atherosclerotic aortas showed that clear bands of hMMP-12 (with estimated sizes of 54-kDa) in samples prepared from Tg rabbits could digest casein, indicating that these proteins were enzymatically active. No definitive bands were found in samples from non-Tg animals. **C:** Rabbit MMPs, including rMMP-1, -9, and -12, from the Tg atherosclerotic aortas were more significantly expressed than those from WT aortas. Expression levels of all MMP mRNA were normalized by those of rabbit glyceraldehyde-3-phosphate dehydrogenase mRNA. Data are expressed as the mean $\pm$ SE (n , number of aortas). * P < 0.05.

Expression of hMMP-12 and rMMPs in hMMP-12 Tg Rabbits

The aortas from hMMP-12 Tg rabbits fed the 1% cholesterol diet also showed a quite increased expression of hMMP-12 mRNA, but those from WT animals did not, as evaluated by the real-time RT-PCR method (Figure 5A). In the vascular cuff and ligation models, the arteries from hMMP-12 Tg rabbits also revealed the increased expression of hMMP-12 (Figure 6A); but these levels were lower than those in the aortas of the cholesterol-fed rabbits, and those from the WT animals showed no expression of it. Zymographic assays of these aortas showed that caseinolytic activities were present in the 54-kDa band obtained with the hMMP-12 Tg hypercholesterolemia-induced atherosclerotic aortas and cuff-injured arteries were markedly increased compared with those in WT aortas (Figures 5B and 6B). These results confirmed that the atherosclerotic lesion from Tg rabbits consistently contained high levels of hMMP-12 proteins that were enzymatically active.

Additionally, except for rMMP-2, other rMMPs including rMMP-1, -9, and -12 from the Tg atherosclerotic aortas were significantly expressed more than those from WT aortas (Figure 5C) but not in the case of cuff- and ligation-induced arteries (Figure 6, C and D).

Atherosclerosis Caused by Cuff- or Ligation-Induced Injury in Muscular Arteries

For statistical analysis of cuff- or ligation-induced atherosclerosis, step or sequential sections (up to 900) were

Figure 6. Real-time RT-PCR analysis and activity of hMMP-12 and rMMPs expression in cuff- and ligation-induced atherosclerosis. **A:** Analysis by real-time RT-PCR demonstrated the expression of hMMP-12 mRNA in cuff-injured femoral arteries and in ligation-injured carotid arteries after 3 weeks in hMMP-12 Tg rabbits but not in WT animals. Data are expressed as the mean $\pm$ SE (n , number of arteries). * P < 0.05. **B:** Zymography shows clear proteolytic activity of hMMP-12 (with estimated sizes of 54-kDa) only from cuffed arteries of Tg rabbits, indicating the digestion of casein as seen in Figure 5. No definitive clear bands were detected in WT rabbits. **C and D:** Rabbit MMPs, including rMMP-1, -9, and -12, from the Tg rabbits with cuff-induced (**C**) and ligation-induced (**D**) atherosclerosis were tested by real-time RT-PCR. Unlike cholesterol-induced atherosclerosis, no significant difference in the expression of rMMPs between Tg and WT animals was detected for any of the rMMPs tested. Expression levels of all MMP mRNAs were normalized by those of rabbit glyceraldehyde-3-phosphate dehydrogenase mRNA. Data are expressed as the mean $\pm$ SE (n , number of arteries).

also prepared and used for the observation of atherosclerosis coupled with degradation of the internal elastic lamina. In histological examination coupled with immunohistochemical staining, the results demonstrated that in the cuff and ligation models, the hMMP-12 Tg rabbits 3 weeks after the surgical treatment showed significantly more atherosclerosis than the WT rabbits (Figure 7A), although there was no significant difference between hMMP-12 Tg and WT rabbits 1 week after the surgical treatment (data not shown). Determination of the I/M ratio revealed an approximate 2.3-fold (cuff model) or 2.2-fold (ligation model) greater value for the hMMP-12 Tg rabbits than for the WT rabbits (P < 0.05; Figure 7B).

Immunohistochemically, the neointima caused by the cuff- or ligation-induced injury was predominantly composed of SMCs positive for α -SMA and a few scattered macrophages reactive with the RAM-11 antibody (Figure 8A). In contrast, in the tunica adventitia of the cuff-injured femoral arteries, a larger number of infiltrating monocytes/macrophages was found; and their number was

Figure 7. Microscopic observation of cuff-induced and ligation-induced atherosclerotic lesions in muscular arteries. **A:** Representative micrographs show cuffed-femoral and ligated-carotid arteries of hMMP-12 Tg and WT rabbits after 3 weeks of cuffing or ligation (H&E staining). The cuff-induced and ligation-induced intimal thickening of hMMP-12 Tg animals was greater than that of WT rabbits. In hMMP-12 Tg rabbits, inflammation in the tunica adventitia (arrows) was more conspicuous than that in WT rabbit. **B:** Quantitative I/M ratio was increased about 2.3- and 2.2-fold in cuff- and ligation-induced model, respectively, at 3 weeks for hMMP-12 Tg rabbits compared with that for the WT animals. Data are expressed as the mean $\pm$ SE (*n*, number of arteries; number of sections = 11–17 for artery). **P* < 0.05.

greater in the hMMP-12 Tg than in the WT rabbits (Figure 8A, left middle row). hMMP-12-positive cells in the tunica adventitia appeared to overlap with these macrophages only in the cuff-injured hMMP-12 Tg animals (Figure 8A, left bottom row). The number of infiltrating macrophages in the tunica adventitia of the cuffed hMMP-12 Tg rabbits was about 2.2-fold larger than that for the WT rabbits (*P* <

0.05; Figure 8B). In the ligation model, except for the ligation site with foreign body-type inflammation caused by the suture, adventitial inflammation was extremely weak compared with that in the cuff-injury model (Figure 8A, right); and there was no significant difference between hMMP-12 Tg and WT rabbits 1 or 3 weeks after the surgical treatment (data not shown).

Figure 8. Immunohistological observation of cuff-induced and ligation-induced atherosclerotic lesions in muscular arteries. **A:** Immunohistochemical staining revealed the neointima to be composed predominantly of SMCs positive for α -SMA and a few macrophages reactive with RAM-11. In the tunica adventitia of the hMMP-12 Tg rabbits, a relatively larger number of macrophages (arrows) and SMCs (arrows) had infiltrated than in the case of that of the WT animals. Additionally, a number of hMMP-12-positive inflammatory cells (arrows) appeared to overlap with these RAM-11-reactive macrophages but only in the cuff-injured hMMP-12 Tg rabbits. In the ligation model, near the ligation site but not in other areas, foreign body-type inflammation was recognized. **B:** In the tunica adventitia, the number of macrophages in cuff-injured hMMP-12 Tg rabbits was about 2.2-fold larger than that for the WT animals. Data are expressed as the mean $\pm$ SE (*n*, number of arteries). **P* < 0.05.

Figure 9. Quantitative analysis of elastolysis of cuff-induced and ligation-induced atherosclerotic lesions in muscular arteries. **A:** Elastolytic lesions (**white arrows**) in cuffed femoral arteries after 3 weeks. The elastolytic ratio was significantly greater for cuff-injured hMMP-12 Tg than for WT rabbits. Data are expressed as the mean $\pm$ SE (n , number of arteries; total number of sections = 45 and 33 for Tg and WT, respectively). $*P < 0.05$ (EVG staining). **B:** Elastolytic activity (**white arrows**) in ligated carotid arteries after 3 weeks. Elastolytic activity in the internal elastic lamina was detected in the vessel wall just proximal to the ligation site, where foreign body-type inflammation caused by the suture (**arrowheads**) was prominent; and the elastolysis was more conspicuous in hMMP-12 Tg rabbits than in the WT rabbits. Data are expressed as the mean $\pm$ SE (n , number of arteries; total number of sections = 17 and 12 for Tg and WT, respectively). $*P < 0.05$ (EVG staining).

Quantitative analysis of elastolysis at the internal elastic lamina showed that the elastolysis ratio was more significantly pronounced, about 2.2-fold, in hMMP-12 Tg than in WT rabbits in both models (Figure 9, A and B). The elastolysis in the carotid arteries, however, was relatively less than that in the femoral arteries and was limited to just the portion close to the ligation site with foreign body-type inflammation (Figure 9B).

Another type of elastolysis was found at 1 and 3 weeks but only in the cuff model. In particular, 1 week after the surgical treatment, "microelastolytic points" were recognized in the nonatherosclerotic portions before formation of the neointima (Figure 10). Immunohistochemically identified SMCs seemed to be associated with these microelastolytic points (not shown). There was no significant difference in the number of these points between hMMP-12 Tg and WT rabbits at 1 or 3 weeks after the surgical treatment (data not shown).

Discussion

In our previous study on atherosclerosis induced in hMMP-12 Tg rabbits consuming a diet including 0.2% cholesterol for 16 weeks, there was no significant difference in the early stage of atherosclerosis between hMMP-12 Tg and WT "lower hypercholesterolemia" animals.²³ Considering the actions of hMMP-12 in advanced atherosclerosis, these data were unexpected. The hMMP-12 transgene was under the control of the human scavenger receptor-A enhancer/promoter, which is not the promoter for the MMP-12 gene, as a macrophage-specific promoter^{21,26} in hMMP-12 Tg animals. The hMMP-12 Tg rabbits were generated to investigate pathological events in relation to atherosclerosis under overexpression of MMP-12. Therefore, in current study, we studied pronounced fatty streaks composed of larger number of foam cells under the condition of

a higher serum cholesterol than was present in the previous study. The current data reveal a significant role of hMMP-12 even in the formation of early atherosclerotic lesions.

The quantitative analysis of fatty streaks in en face (Figure 1) showed that the early atherosclerotic lesions in whole aortas of the Tg rabbits were significantly more progressive than those of the WT rabbits, indicating that a larger number of monocytes/macrophages had migrated from the peripheral blood to the intima to phagocytose lipid in the former animals. The analysis of cut sections in aortic arches, the upper portion of the thoracic aorta, and the distal portion of the intercostal artery branches arising from the thoracic aorta also revealed significantly more pronounced lesions in terms of the intimal area and showed immunohistochemically that a larger number of monocytes/macrophages had infiltrated in hMMP-12 Tg than in WT rabbits (Figure 2). These results strongly suggest that hMMP-12 expression accelerated the migration of monocytes/macrophages into the intima by basement membrane proteolysis.

Currently, it is thought that lipid deposition is a necessary but not sufficient condition for the development of human atherosclerosis. Because elastin is a critical matrix component of the artery not only to maintain its elastic nature but also to divide structurally the intima from the media, disruption of the elastic fibers by MMP-12 within fatty streaks could cause a morphologically minor but critical microenvironmental change affecting SMCs in the media. Therefore, the quantitative analysis of elastolysis associated with fatty streaks was performed by examining sequential sections of the lower portion of the thoracic aorta from rabbits fed the high-cholesterol diet for 6 weeks, at the end of which time, the extremely early fatty streaks have appeared. The results showed that disruption of the internal elastic layer in hMMP-12 Tg rabbits was also significantly increased compared with that in

FA (Cuffing)

Figure 10. Initiation of elastolysis in cuff-induced atherosclerosis. H&E and EVG staining revealed microelastolytic sites (**connected arrows**) in the internal elastic lamina in nonatherosclerotic portions of Tg arteries.

WT rabbits (Figures 3 and 4), indicating that the macrophage-expressed hMMP-12 participated in elastolysis of the internal elastic layer within fatty streaks. Moreover, other MMPs, including not only rMMP-12 but also rMMP-1 and -9, in Tg atherosclerotic aortas were expressed more increasingly than those in WT aortas (Figure 5). According to another study, MMP-12 undergoes self-activation through autolytic processing and then activates other MMPs.¹⁵ It was also reported that elastin peptides generated through hydrolysis of elastin are a potent chemoattractant for monocytes and macrophages.^{27,28} These observations suggest that there is a cascade of MMP activation that leads to degradation of elastic fibers, once MMP-12 is overexpressed or up-regulated. From these indications, it is very likely that excess MMP-12 production by macrophages plays important and essential roles in both the initiation and the transition from fatty streaks to fibrous plaques during the progression of atherosclerosis.

In the cuff- and ligation-induced models, the quantitative analysis of cross sections of hMMP-12 Tg rabbits showed not only a significantly more pronounced neoin-

tima but also significantly increased diffuse elastolysis compared with the neointima and elastolysis in WT animals (Figures 7 and 9). Because only a few monocytes/macrophages infiltrated into the neointima that had been formed almost entirely by the proliferation of SMCs in the cuff-model, such conspicuous elastolysis of the internal elastic layer was unexpected (Figure 8). In addition, before neointima formation, microelastolytic sites of the internal elastic lamina appeared in nonatherosclerotic femoral arteries in the cuff model (Figure 10) but not in the ligation model. Such a microelastolytic site in pre-atherosclerotic lesions strongly suggests it to be an initiation site for remodeling of the intima in this model. In contrast, in the tunica adventitia of the cuffed artery, a larger number of monocytes/macrophages was observed there than in the neointima, and their number was significantly greater in hMMP-12 Tg than in the WT animals (Figure 8). These results lead us to consider that such a significant difference in neointima formation and elastolysis between hMMP-12 Tg and WT rabbits could be attributable to the inflammation involving macrophages in the tunica adventitia. In fact, hMMP-12 was expressed in the cuffed artery

(Figure 6, A and B). Among MMPs expressed, however, unlike in the case of cholesterol-induced atherosclerosis, only hMMP-12 expression was up-regulated in both cuffed and ligated arteries. We considered that such a difference of rMMP expression between cholesterol-induced atherosclerosis and these two types of models may be dependent on the number of infiltrating foam cells (macrophages). Therefore, we concluded that hMMP-12 should also be essentially involved in atherosclerosis in these two models.

The ligated arteries showed results similar to those for the cuffed arteries, but the formation of the neointima was weaker in the ligated arteries than in the cuffed arteries; and elastolysis was limited to the portion close to the ligation site (Figure 9B), suggesting participation of marked foreign body-type inflammation caused by the suture in the elastolysis. No microelastolytic sites were detected in either hMMP-12 Tg or WT rabbits of the ligation model. Thus, it is likely that in the cuff- and ligation-induced models, the mechanisms responsible for atherosclerosis must be fundamentally different from the mechanism at play in the high-cholesterol diet-induced aortas and that the atherosclerotic mechanisms are also different between cuff- and ligation-induced atherosclerosis.

In the cuff-induced atherosclerotic model, the mechanism of atherosclerosis is controversial in relation to the origin of the SMCs, because SMCs are the dominant cells in the neointima. Potential origins of neointimal SMCs are as follows: arterial media,²⁹ circulating SMC precursor cells,³⁰ skeletal muscle- or heart-resident SMC progenitors,^{31,32} and adventitial SMC progenitors.³³ In balloon overstretch-induced atherosclerosis, a model similar to the cuff-induced type, proliferating α -SMA-positive cells or fibroblasts in the tunica adventitia migrate into the neointima and differentiate to myofibroblastic cells.^{34,35} Based on the current data, we propose here that hMMP-12 and SMC progenitor cells in the tunica adventitia of femoral arteries could be transferred to the neointima and activated in this site to degrade the internal elastic lamina. Thus, microelastolytic sites would be advantageous to adventitial SMC progenitors for their migration into the intima.

We should consider the functional single nucleotide polymorphism of the hMMP-12 gene. A cluster of eight MMP genes, those of MMP-1, -3, -8, -10, -12, -13, -20, and -27, is located on human chromosome 11q 22.3.³⁶ Currently, 96 single nucleotide polymorphisms of MMPs are listed in the single nucleotide polymorphism database of the National Center for Biotechnology Information, and 2 single nucleotide polymorphisms of the MMP-12 gene were reported to be associated with diseases. One was found by analysis of multiple cancer-associated genetic variants, which suggested an important role of a MMP-1, -3, and -12 gene cluster in lung cancer development and progression³⁷; and the other is a common functional polymorphism (-82A) that influences the binding of the transcriptional factor activation protein (AP-1) to the AP-1 site of the MMP-12 gene and is related to the coronary artery luminal dimension in diabetic patients with manifest coronary artery disease.³⁸ It

is likely that the up-regulation of the MMP-12 gene by that polymorphism is modulated by the binding of AP-1 protein to the AP-1 site. MMP-12 production is up-regulated by granulocyte macrophage-colony-stimulating factor and platelet-derived growth factor via the AP-1 site.^{39,40} Thus, these data indicate strongly also the importance of AP-1 site in atherosclerotic processes.

Our serial study of hMMP-12 Tg rabbits showed potential role of increased expression of MMP-12 not only in the progression but also in the initiation of atherosclerosis. However, considering the broad spectrum of substrates of MMP-12, all results obtained in the serial experiments may not be attributable only to the elastolytic activity of the enzyme.

Nonetheless, a specific inhibitor of MMP-12 activity and regulation might prove to be potentially therapeutic for atherosclerosis throughout the course of the disease, from initiation to advanced stages.

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