Macrophage-derived matrix metalloproteinase-1 enhances aortic aneurysm formation in transgenic rabbits

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Abstract

Increased expression of matrix metalloproteinase-1 (MMP-1) has been observed in the lesions of atherosclerosis and aneurysms; however, it is not fully understood whether macrophage-derived MMP-1 affects these diseases. To investigate whether macrophage-derived MMP-1 participates in the development of vascular diseases, we generated transgenic (Tg) rabbits expressing human MMP-1 in the monocyte/macrophage lineage under the control of the human scavenger receptor enhancer/promoter. Tg rabbits exhibited no visible abnormalities throughout their bodies. Western blotting analysis revealed that the amount of MMP-1 proteins in the conditioned media secreted from peritoneal macrophages of Tg rabbits was up to 3-fold higher than that in non-Tg rabbits. For the first experiment, Tg and non-Tg rabbits were fed a cholesterol diet for 16 weeks, and aortic and coronary atherosclerosis were evaluated. The gross lesion area of aortic atherosclerosis in Tg rabbits was not significantly different from that in non-Tg rabbits, but Tg rabbits had marked destruction of the medial elastic lamina of the aortic lesions on microscopic examination. For the second experiment, we generated aortic aneurysms by incubating with elastase. Compared with non-Tg rabbits, Tg rabbits exhibited a significantly greater aortic dilation. Increased macrophage-derived MMP-1 led to increased medial destruction in both aortic atherosclerosis and aneurysms. These results demonstrate that MMP-1 plays a different role in the pathogenesis of atherosclerosis and aneurysms.

Keywords: matrix metalloproteinase-1, macrophage, atherosclerosis, aneurysm, transgenic rabbit
Introduction

The accumulation of macrophage-derived foam cells in the intima of large arteries is a hallmark of human and experimental animal atherosclerosis[1–3]. These macrophages play an important role in the development of atherosclerosis and its complications because they secrete a variety of bio-reactive substances such as cytokines, matrix metalloproteinases (MMPs), pro-coagulant protein tissue factor, and reactive oxygen species. These substances, either singly or collaboratively, function in the lesion progression[2,4]. Among them, MMPs are critically important in the process of vascular remodeling due to hydrolysis of the extracellular matrix (ECM)[5–6]. It was generally believed that increased hydrolysis of the ECM led to the disruption of the cell-basement membrane, which facilitates monocyte emigration from the circulation into the intima, thereby enhancing the lesion development[7–8]. In advanced atherosclerotic lesions, increased MMPs may result in the enhancement of plaque vulnerability, although this hypothesis has not been fully examined[8–11]. Recently, we performed a transcriptomic analysis of aortic lesions in hypercholesterolemic rabbits, and found that there were several MMPs that were specifically expressed in the lesions but not in normal aortas[12]. One was MMP-1, also known as interstitial collagenase, which is highly expressed by lesional macrophages[13].

MMP-1 can degrade several kinds of collagens, such as type I and type III, in the arterial wall, and has been implicated in many physiological and pathophysiological processes including vascular diseases[9,14]. Previous studies reported that MMP-1 expression was increased in human atherosclerotic plaques, but was undetectable in normal arteries[15–16]. Genetic studies revealed that MMP-1 gene mutations were associated with an increased risk of coronary heart disease[17–19]. In addition, MMP-1 was elevated in human aortic abdominal aneurysm specimens compared with normal aortic tissue[19–20]. Due to the substrate specificity of MMP-1 and its intimate co-localization with macrophages and degraded fibrillar collagens in the lesions, it has been hypothesized that this proteinase aids in the expansion and rupture of the plaque, although this notion has not been confirmed. Of note, unlike rabbits and humans, mice do not possess an MMP-1 gene[21]; therefore, it is not possible to make MMP-1 knock-out mice to investigate the functional roles of MMP-1. Using transgenic mice expressing the human MMP-1 gene, Lemaitre et al. demonstrated that macrophage-expressed MMP-1 is not involved in the lesion formation in apoE KO mice possibly due to the lack of appropriate ECM substrates in the mouse aorta[22].

We hypothesized that increased MMP-1 expression by macrophages is involved in the initiation and progression of atherosclerosis and aneurysms. To test this hypothesis, we generated transgenic (Tg) rabbits overexpressing the rabbit MMP-1 gene specifically in the macrophage lineage and foam cells of atherosclerotic lesions. The rationale for using rabbits was three-fold. First, rabbits are sensitive to a cholesterol diet and develop atherosclerosis rapidly[23]. Second, atherosclerotic lesions in cholesterol-fed rabbits are rich in macrophage-derived foam cells, which facilitates the analysis of macrophage functions in the arterial wall[13]. Thirdly, rabbit atherosclerotic lesions contain high levels of MMP-1[12]. Our studies revealed that increased MMP-1 expression led to marked destruction of the medial elastic lamina in atherosclerotic lesions. In addition, MMP-1 overexpression exacerbated elastase-induced aneurysms in Tg rabbits.

Materials and methods

Generation of human MMP-1 transgenic rabbits

Tg rabbits were generated by the methods established in our laboratory, as reported previously[24–25]. The DNA construct used for micro-injection was composed of human MMP-1 cDNA under the control of a human scavenger receptor enhancer/promoter region along with four copies of the chicken β globin insulator (Fig. 1A), which prevents the positon effect of transgenes[26]. In total, 693 embryos were injected, and 567 embryos were implanted into 20 recipient female rabbits. Six recipients gave birth to 11 pups, and among them, 2 pups were found to carry the transgenes by PCR analysis with specific primers (forward, 5'-TGAGGTCAAGGGGATCAAGAC-3'; and reverse, 5'-AACTTGTGCGCAATTCCAG-3').

Tg founders were bred to provide F1 progeny. Northern blotting, Western blotting and zymography were performed, as described previously[25,27]. To evaluate MMP-1 protein expression and enzymatic activity, we collected alveolar macrophages and elicited peritoneal macrophages from the peritoneal cavity 4 days after injection of 4% Brewer's thioglycollate broth, as described previously[28]. In short, rabbits were anaesthetized by intramuscular injection of ketamine (25 mg/kg) + medetomidine hydrochloride (0.5 mg/kg) and restrained with the ventral side up. Thioglycollate broth loaded in 50 mL
syringes was injected into the peritoneal cavity. Four days later, rabbits were euthanized by injection of sodium pentobarbital solution (100 mg/kg) through an ear vein. The abdominal cavity was cut open along the middle line and washed three times using 100 mL of phosphate-buffered saline (pH 7.4) with heparin (10 U/L). After centrifugation, peritoneal macrophages \((1 \times 10^7)\) from either Tg or non-Tg rabbits were incubated in serum-free 1640 medium with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA) for 48 hours, and the conditioned media were then collected for Western blotting and zymographic analysis. The same aliquots of the conditioned media from each group were fractionated by electrophoresis on 10% SDS-polyacrylamide gels, transferred onto a nitrocellulose membrane, and then incubated with a monoclonal antibody (mAb) against human MMP-1. To evaluate MMP-1 activity, we performed β-casein gel zymographic analysis using the method reported previously [13].

Rabbits were fed with a chow diet (CR-3, CLEA Japan) containing 17.5% crude protein, 4.0% crude fat, and 11.7% crude fiber. In this study, rabbits at the age of 4–12 months were used. The rabbits were allowed access to diet and water \(ad libitum\). All animal experiments were performed with the approval of the Animal Care Committees of the University of Yamanashi and Saga University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

**Measurement of plasma lipids, biochemistry, and blood cells**

Plasma total cholesterol (TC), triglycerides (TG), and high density lipoprotein-cholesterol (HDL-C) were measured using commercial assay kits (Wako Pure Chemical Industries, Japan). Serum biochemistry was analyzed with a clinical chemistry analyzer JCA-BM2250 (JEOL, Japan). The blood cell count was measured with an automated hematology analyzer XE-2100 (Sysmex, Japan).

**Cholesterol diet-induced atherosclerosis**

Tg and non-Tg littermate rabbits were fed a diet containing 1.0% cholesterol and 3% soybean oil for 16 weeks. Blood was collected from an auricular artery of fasted rabbits for analysis of plasma lipids on a weekly basis.

At the end of the experiment, all rabbits were sacrificed for evaluation of atherosclerosis. Aortic gross and microscopic lesions were analyzed as described previously[29]. Briefly, aortas were stained with Sudan IV for evaluation of the gross lesion size using an image analysis system. For microscopic evaluation of the lesion area, the aortic arch was dissected into eight sections and embedded in paraffin. Serial sections (3 μm thick) were stained with hematoxylin-eosin (HE) and Elastica van Gieson (EVG),

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**Fig. 1 Generation of human MMP-1 transgenic rabbits.** A: Transgenic DNA construct for microinjection. The 12-kb transgenic construct consisted of a human scavenger receptor enhancer/promoter region, human MMP-1 cDNA sequence, and human growth hormone genomic DNA poly-A tail with four copies of the chicken β-globin insulator. B: PCR analysis for examining the human MMP-1 transgene using specific primers. C: Northern blotting analysis of human MMP-1 mRNA expression. The membrane was hybridized with a \(^{32}\)P-labeled human MMP-1 cDNA probe. Expression of human MMP-1 mRNA in the alveolar and peritoneal macrophages was detected in Tg rabbits. D: Western blotting (upper) and β-casein zymography (bottom) analysis of macrophage-conditioned media. Tg: transgenic; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MΦ: macrophage.
and immunohistochemically stained with monoclonal antibodies against macrophages (RAM11, Dako, Japan) and smooth muscle α-actin (HHF35, Dako, Japan). The microscopic lesion area, and macrophage and SMC content in the lesions were quantified using an image analysis system.

Elastic lamina destruction was analyzed as described previously,[29] with modifications. Elastic lamina destruction was defined by more than 3-layer fragmentation or the disappearance of normal structure in the tunica media. The length of destruction was quantified with an image analysis system, and elastic lamina destruction was expressed as a percentage of the length of the atherosclerotic lesion.

Coronary atherosclerosis was analyzed as described previously.[28] The coronary lesion was expressed as stenosis percentage.

**Elastase-induced abdominal aortic aneurysms**

Tg and non-Tg littermate rabbits were fed a diet containing 1.0% cholesterol and 3% soybean oil before the 4-week aneurysm induction. Abdominal aortic aneurysms were induced as reported previously,[30-31], with modifications. Rabbits were anesthetized by intramuscular injection of ketamine (25 mg/kg) + medetomidine hydrochloride (0.5 mg/kg), and a 15-mm-long stretch of the abdominal aorta proximal to the left renal artery was exposed. The isolated aorta was subjected to perivascular incubation with 1 U/μL of elastase (70 μL) and 0.5 mol/L CaCl₂ (70 μL) for 20 minutes.

To assess dilatation of abdominal aorta, the maximum diameter of the abdominal aorta was measured by ultrasonography SONOS 5500 (Philips Medical Systems, Japan) before and after (one or two weeks) aneurysm induction. At the end of the experiment, rabbits were sacrificed for histological evaluation of aortic aneurysms. The abdominal aorta was embedded in paraffin. Serial sections (3 μm thick) were stained as described above.

**Statistical analysis**

All data are expressed as the mean±SEM. The Student’s t-test (for parametric data) or Mann-Whitney U-test (for non-parametric data) was used for statistical analyses. P<0.05 was considered significant.

**Results**

**Characterization of human MMP-1 transgenic rabbits**

We created two Tg founder rabbits, as confirmed by PCR analysis (Fig. 1B). One founder rabbit was bred to provide F1 progeny for the current study. The Northern blotting analysis revealed that Tg rabbits expressed high levels of human MMP-1 mRNA in peritoneal and alveolar macrophages, but non-Tg rabbits did not (Fig. 1C). With the Western blotting analysis of the conditioned media, the peritoneal macrophages from Tg rabbits secreted approximately 3-fold more MMP-1 protein than control macrophages measured by densitometry (Fig. 1D). β-casein zymography demonstrated that the MMP-1 proteins secreted by macrophages were enzymatically active (Fig. 1D). Tg rabbits did not exhibit any visible abnormalities in whole-body appearance or organs compared with non-Tg rabbits. There were no significant differences in body weight, plasma TC, TG, HDL-C, free fatty acids, glucose, total protein, enzyme markers of liver functions, and blood cells (Supplementary Table 1, available online).

**Effects of MMP-1 on cholesterol diet-induced atherosclerosis**

Tg and non-Tg rabbits had similar plasma TC levels throughout the experimental period (Fig. 2A). Based on the analysis of aortic gross lesion areas, the atherosclerotic lesions in Tg rabbits were not significantly different (P=0.566) from those in non-Tg rabbits (Fig. 2B).

On histological examination, the aortic lesions in both Tg and non-Tg rabbits were composed of infiltrating macrophages and smooth muscle cells intermingled with the extracellular matrix. The size of aortic microscopic lesions, and the macrophage- and smooth muscle cell-positive areas in Tg rabbits were not significantly different from those in non-Tg rabbits (Fig. 2C).

Analysis of coronary atherosclerosis revealed that Tg and non-Tg rabbits had similar lesions (Supplementary Fig. 1, available online).

Although the atherosclerotic lesion size and cellular composition were not significantly different between Tg and non-Tg rabbits, histological examinations revealed that elastic lamina disruption of aortic lesions in Tg rabbits had significantly increased by 2.3-fold compared with those in non-Tg rabbits according to EVG staining (Fig. 3). In these areas, the collagen content was lower in Tg rabbits than in non-Tg rabbits. We did not observe plaque rupture in the aortas of Tg rabbits.

**Effects of MMP-1 on elastase-induced abdominal aortic aneurysms**

Next, we investigated whether MMP-1 affects...
Fig. 2 Cholesterol-rich diet-induced atherosclerosis. A: Plasma total cholesterol levels in non-Tg rabbits (n=10–11) and Tg rabbits (n=15). B: Representative aortas from non-Tg and Tg rabbits stained with Sudan IV (left). Atherosclerotic lesions defined by the sudanophilic area were quantified with an image analysis system (right). Each dot represents the lesion area of an individual animal. C: Representative micrographs of the aortic arch lesions from non-Tg and Tg rabbits (left). Serial paraffin sections were stained with hematoxylin-eosin (HE) and Elastica van Gieson (EVG), and immunohistochemically stained with antibodies against both macrophages (MΦ) and smooth muscle cells (SMC). Intimal lesions on EVG-stained sections and positively immunostained areas of MΦ were quantified with an image analysis system (right).
elastase-induced abdominal aortic aneurysms. Abdominal aortic aneurysms were induced by perivascular incubation with elastase and CaCl$_2$, and the abdominal aortic diameter was compared by ultrasonography. As shown in Fig. 4A, dilatation of abdominal aortic aneurysms in Tg rabbits was significantly greater than that in non-Tg rabbits at two weeks. Histological examinations revealed that dilatation of abdominal aortas in Tg rabbits was associated with increased medial elastic lamina disruption and macrophage infiltration (Fig. 4B).

Discussion

In the current study, we generated Tg rabbits expressing human MMP-1 specifically in the macrophage lineage to investigate whether increased MMP-1 affects atherosclerosis and aortic aneurysm formation in cholesterol-fed rabbits. As MMP-1 was considered to be a key proteinase in lesion progression, we initially expected overexpression of macrophage-derived MMP-1 to cause plaque rupture. However, although Tg rabbit macrophages expressed higher levels of MMP-1, MMP-1 did not directly affect atherosclerosis formation because both gross and microscopic lesions did not show a considerable difference between Tg and non-Tg rabbits. These results suggest that MMP-1 is not involved in the macrophage accumulation in the lesions even though MMP-1 can indeed disrupt the elastic lamina. This observation does not support the notion that increased macrophage MMP-1 enhances atherosclerosis. However, compared with other MMPs, such as MMP-9, gelatinase, MMP-12, or elastase secreted from macrophages in the lesions, MMP-1 plays little role in the progression of atherosclerosis[29,32]. In transgenic mice expressing human MMP-1, it was reported that MMP-1 delays lesion development and is beneficial for atherosclerosis[29]. Using transgenic rabbits expressing either MMP-12 or MMP-9, we demonstrated that increased MMP-12 drastically enhances the progression of advanced atherosclerosis[29], whereas increased MMP-9 led to the enhancement of atherosclerosis and vascular calcification[32]. Enhanced atherosclerosis induced by MMP-9 and MMP-12 was mainly caused by elastin peptides generated by either MMP-9 or MMP-12 hydrolysis, which exerts potent chemoattractant effects on monocytes, thereby expanding the lesions. In addition, MMP-12 can activate other MMPs, thus enhancing lesion development[33]. In this aspect, MMP-1 may not have such a function due to the differences in substrate specificities. It is likely that after the 16-week cholesterol dieting the rabbits developed even more advanced lesions and MMP-1 functions were overwhelmed by the high cholesterol levels or MMP-1 only affects early-stage lesions, such as monocyte adhesion and migration. To examine this possibility, we performed another experiment in which rabbits were fed a cholesterol diet for six weeks (short-term), and we compared the lesions in Tg rabbits with those in non-Tg rabbits. However, even in the early stage, MMP-1 exerted mild effects on lesion formation (Supplementary Fig. 2, available online). Our study also demonstrates that expression of MMP-1 alone does not cause plaque rupture in Tg rabbits. This observation suggests that other MMPs, acting alone or
**Fig. 4** Analysis of abdominal aortic aneurysms. A: Representative ultrasound images at two weeks after aneurysm induction (left); red dashed lines indicate the lumen boundary. The maximum diameter of the abdominal aorta is shown on the right. Mean±SEM, non-Tg (n=6) and Tg (n=9). **P<0.01 vs. non Tg. B:** Representative micrographs of the abdominal aorta stained with Elastica van Gieson (EVG), and immunohistochemically stained with antibodies against macrophages (MΦ) from non-Tg and Tg rabbits. Both the abdominal aorta of the infrarenal intact region and aneurysm region from non-Tg and Tg rabbits are shown. Black dashed boxes are enlarged in the bottom panels.
together with MMP-1, are required to destroy the fibrous cap of lesions.

On the other hand, we found that MMP-1 enhances aneurysm formation in cholesterol-fed rabbits in the second study. The diameter of the abdominal aorta measured by ultrasonography was larger in Tg rabbits than in non-Tg rabbits, suggesting that increased degradation of the collagen matrix by MMP-1 is involved in vascular remodeling. In support of this notion, histological analysis revealed that marked medial destruction was associated with macrophage infiltration in Tg rabbits. Taken together with the results of the atherosclerosis study, we can conclude that MMP-1 plays different roles in different vascular diseases, which strengthens the argument that the function of MMP-1 depends upon specific pathophysiological conditions such as ECM specificity and macrophage numbers.

Although increased MMP-1 expression was observed in both human and experimental atherosclerotic lesions, the current study demonstrated that MMP-1 alone plays a minor role in the progression of atherosclerosis or plaque rupture but functions in aortic aneurysm formation. We cannot exclude the possibility that increased MMP-1 expression is involved in tissue repair or healing processes during atherosclerosis, and this needs to be clarified in the future.

In conclusion, different from other MMPs, macrophage expression of MMP-1 is not involved in the development of atherosclerosis and instead functions in aortic aneurysm formation. Thus, targeting MMP-1 alone may not help prevent the progress of atherosclerosis.

Acknowledgments

This work was supported in part by research grants from JSPS KAKENHI (JP26460486 to MN and JP15H04718 to JF) and NIH grants (R01HL117491 and R01HL129778 to YEC). MN, YEC and JF designed the research. ZJ prepared the transgenic and R01HL129778 to YEC). MN, YEC and JF generated the transgenic and R01HL117491 to JSPS KAKENHI (JP26460486 to MN and JP15H04718 to JF) and NIH grants (R01HL117491 to JSPS KAKENHI (JP26460486 to MN and JP15H04718 to JF) and NIH grants (R01HL117491 to JSPS KAKENHI (JP26460486 to MN and JP15H04718 to JF) and NIH grants (R01HL117491 to JSPS KAKENHI (JP26460486 to MN and JP15H04718 to JF) and NIH grants (R01HL117491 to JSPS KAKENHI (JP26460486 to MN and JP15H04718 to JF). MN, YEC and JF conducted the experiments. MN and JF analyzed the data. MN and JF wrote the manuscript. We thank Dedong Kang, Bo Ning, Tomonari Koike, Ying Yu, Yuko Nakagawa and Akiko Sumii for technical assistance.

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