Temporal and quantitative analysis of expression of metalloproteinases (MMPs) and their endogenous inhibitors in atherosclerotic lesions

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Summary. Matrix metalloproteinases (MMPs) play an important role in the pathogenesis of vascular diseases, such as atherosclerosis, plaque rupture and aneurysms. Although several MMPs have been demonstrated in the lesions of atherosclerosis, their expression profiles during the initiation and progression of lesions have not been fully determined. We hypothesized that the expression of various MMPs, along with their endogenous inhibitors, may be differentially regulated dependent upon the lesion progression. Therefore, we made a temporal and quantitative analysis of the mRNA and protein expression of MMPs and tissue inhibitors of metalloproteinases expressed in the different stages of atherosclerotic lesions of rabbits and humans. We found that MMP-1, MMP-12 and MMP-13 expression was nearly absent in the normal arterial wall, but was remarkably increased with lesion progression. Furthermore, the expression of these MMPs in the lesions was closely associated with intimal macrophages and monocyte chemoattractant protein-1 expression, suggesting that the intimal macrophages are the major source of production of these MMPs. MMP-3 and MT1-MMP were also significantly upregulated in the early-stage lesions and fatty streaks compared to the normal aortas of rabbits. Our results indicate that MMP-1, -12, and -13 derived from intimal macrophages may play a

pivotal role in both lesion initiation and progression, and therefore are potential therapeutic targets for the treatment of plaque rupture and aneurysm formation.

Key words: MMPs, Atherosclerosis, Plaque stability, matrix degradation, macrophages

Introduction

The matrix metalloproteinases (MMPs) are zinc-endopeptidases that play important roles in many physiological and pathophysiological processes (Newby, 2005). Thus far, 25 members of the MMP family have been identified in humans, and they can degrade a broad spectrum of extracellular matrix components, cellular receptors, and other soluble proteins, such as growth factors and cytokines (Visse and Nagase, 2003; Lemaitre and D'Armiento, 2006).

In the arterial wall, abnormal upregulation of MMPs has been shown to be associated with, or to cause, cardiovascular diseases, such as atherosclerosis (Galis and Khatri, 2002) and abdominal aneurysm formation (Newman et al., 1994; Curci et al., 1998). The involvement of MMPs in the pathogenesis of atherosclerosis was initially suggested by the demonstration of a number of MMPs in the lesions of atherosclerosis in both humans and experimental animals (Henney et al., 1991; Galis et al., 1994, 1995a; Halpert et al., 1996; Rajavashisth et al., 1999; Schonbeck et al., 1999; Sukhova et al., 1999; Herman et al., 2001; Uzui et

al., 2002), and accumulating evidence indicates that increased MMP activity in the plaques is associated with the severity of unstable coronary syndrome (Galis and Khatri, 2002). Although increased MMP activity in the arterial wall is generally considered instrumental in the development of atherosclerosis, this notion has been challenged by recent observations using transgenic animals and unfavorable results from MMP inhibitor studies. For example, overexpression of MMP-1 in apoE KO mice decreased the extent of atherosclerosis (Lemaitre et al., 2001) whereas MMP-13 deficiency had no effect on atherosclerotic lesion formation (Deguchi et al., 2005). In addition, MMP-3 deficiency increased the lesion size (Silence et al., 2001), while MMP-12 deficiency either had no effect on aortic lesions (Luttun et al., 2004) or reduced the lesion size of brachiocephalic arteries in apoE KO mice (Johnson et al., 2005). Taken together, these results suggest that not all MMPs are pro-atherogenic, or some MMPs may be either pro-atherogenic or atheroprotective, possibly dependent upon the location of expression and/or lesion stages. This contention is also indicated by the negative results regarding the effects of broad-spectrum synthetic MMP inhibitors on the atherosclerotic lesions in both LDL-receptor and apoE KO mice (Manning et al., 2003; Johnson et al., 2006). Besides, the efficacy of these MMP inhibitors in the clinical setting is still in question (Brown et al., 2004). Therefore, elucidation of which particular MMP(s) and their inhibitors are altered in the different stages of the lesion formation is essential for the development of new therapeutic strategies.

Atherosclerosis develops gradually and many decades are required until clinical symptoms (such as coronary heart disease and stroke) become evident in humans. In this regard, many MMPs in the arterial wall may participate in the pathogenesis of atherosclerosis at different stages, such as initiation (monocyte adhesion and migration), progression (foam cell formation, smooth muscle cell migration and proliferation) and complications (stenosis, plaque rupture, and thrombosis). We hypothesized that the expression of various MMPs may be differentially mediated dependent upon the lesion stages. To investigate MMP expression patterns in the different stages of atherosclerotic lesions, we used two well-characterized atherosclerosis rabbit models because we can obtain sufficient amounts of aortic lesions, ranging from early-stage to advanced lesions for the simultaneous analysis of gene and protein expression, coupled with histological and immunohistochemical examination. Furthermore, unlike mice and rats, which lack homologues of human MMP-1 and MMP-13 (two important collagenases), rabbits express both MMP-1 and MMP-13 with greater homology to those of humans (Vincenti et al., 1998). We were particularly interested in investigating the expression changes of MMPs, along with their endogenous inhibitors, in these different lesions of both rabbits and humans. To the best of our knowledge, this is the first report to analyze the mRNA and protein

expression profiles of MMPs and TIMPs according to the lesion stage. These results may be helpful for designing selective MMP inhibition therapies to be administered at the appropriate time, to prevent progression and complications of atherosclerosis in the future.

Materials and methods

Rabbit samples

In this study, we collected aortas from cholesterol-fed rabbits which have hypercholesterolemia caused by increased levels of plasma remnant lipoproteins, ß-VLDLs, and WHHL rabbits, which have high levels of LDLs similar to human familial hypercholesterolemia (Shiomi et al., 2003). Male Japanese white (JW) rabbits (aged 16-20 wks) were fed a diet supplemented with 0.3~0.7% cholesterol and 3% soybean oil for 6 wks (n=5), 12 wks (n=5), or 16 wks (n=6). During the cholesterol feeding, we maintained the plasma total cholesterol levels in these rabbits at constant levels of 800~1000 mg/dl (equivalent to those of homozygous human FH patients and WHHL rabbits) by adjusting the cholesterol content in the diet. WHHL rabbits used in this study were from the colony as described previously (Shiomi et al., 2003). These WHHL rabbits were examined at 24 wks (n=4) when the aortic lesions became prominent. Normal JW rabbits (n=5) were fed a chow diet as normal control. All rabbits were sacrificed by venous injection of an overdose of sodium pentobarbital solution. The aortas were collected and the aortic arches were used for all analyses, including histological examination, immunostaining, and RNA and protein extraction. This study was approved by the Animal Care Committee of University of Yamanashi and also conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Human aortic samples

To compare the expression of MMPs in human aortic atherosclerotic lesions, we examined aortas from autopsy cases (Table 1), which were performed in the Department of Pathology and Cell Biology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu. The aortas with either normal-appearance (with histological feature of intimal thickening) and atheroma (fibrous plaques) were collected and histologically examined and total RNA was extracted as below.

Immunohistochemistry

Paraffin-embedded and fresh-frozen aortic specimens were sectioned and immunohistochemically stained using monoclonal antibodies (mAbs) against macrophages, smooth muscle cells, MMP-1, -2, -9, -12,

and TIMP-2 as described previously (Liang et al., 2006).

Real-time reverse transcriptase-polymerase chain reaction

Total RNA from the aortic arch was isolated using Trizol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) and then analyzed by real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) (DNA Engine Opticon; MJ Research, Tokyo, Japan) (Liang et al., 2006). The RNA expression levels of MMP-1, -2, -3, -9, -12, -13, MT1-MMP, TIMP-1, -2, -3, and MCP-1 were evaluated using SYBR$^{®}$ Premix Ex TaqTM kits (Takara Bio Inc., Tokyo) according to the manufacturer's instructions. The panel of primers used for these analyses is shown in Table. 2. Each RNA quantity was normalized by the endogenous GAPDH control (Sun et al., 2005).

Western blot analysis

Fresh aortic specimens were collected and homogenized in ice-cold suspension buffer (10 mM Tris-HCL, pH 7.6, 100 mM NaCl) supplemented with a proteinase inhibitor cocktail (Sigma, St. Louis, MO) as described previously (Fan et al., 2004). The supernatant was collected and the protein content was measured using a Bio-Rad protein assay kit. Equal amounts of protein (10 µg) were loaded in each lane and separated by 10% SDS-PAGE. The proteins were transferred to pure nitrocellulose membranes (Bio-Rad). The membranes were incubated at 4°C overnight in the presence of each Ab (Liang et al., 2006), and then washed three times with 0.1% Tween 20 in PBS and stained with horseradish peroxidase-conjugated secondary Abs. The ECL system (Amersham Life Science, Buckinghamshire, UK) was used for detection with an LAS-1000 plus gel documentation system (Fujifilm, Tokyo, Japan).

In situ zymography

To localize the cellular site of MMP enzymatic activity in the lesions, liquid nitrogen-frozen specimens of aortas were embedded in Tissue-Tek OCT compound (Sakura Finetek Japan Co., Tokyo). Cryostat sections (8 µm thick) were made and mounted onto slides coated with a resorufin-labeled casein, a substrate for stromelysin and metalloelastase (EMD Biosciences, Inc., La Jolla, CA). The slides with sections were immediately placed in a moisture chamber at 37°C for 48 hours using the method as described by others (Galis et al., 1995b). The proteolytic areas of the sections were examined under a fluorescent microscope. To validate the specific nature of the casein breakdown by MMPs, control slides were pre-treated with Tris-buffer containing 10mM 10-phenanthroline, a specific inhibitor of MMP enzymatic activity (Molecular Probes, Eugene, OR).

MMP expression of U937-derived macrophages

To investigate the expression of different MMPs in macrophages stimulated by various cytokines, human U937 cells were differentiated into macrophages by incubation with 50 ng/ml PMA for 3 days. U937-derived macrophages were incubated in a serum-free medium in the presence of the following cytokines for 48h: MCP-1 (200 ng/ml), IL-6 (50 ng/ml), IL-10 (100 ng/ml), TNF-α (100 ng/ml), and GM-CSF (150 ng/ml). The optimal doses were selected according to the previous study (Wu et al., 2000). The conditioned media were collected for Western blotting and gelatin zymography and cellular mRNA was extracted for real-time RT-PCR and Western blotting.

Statistical analysis

All values were expressed as mean ± SEM and statistical significance was determined using Mann-Whitney's U-test for nonparametric analysis. Student's t-test was used to compare the result of other assays. In all cases, statistical significance was set at $P < 0.05$.

Results

Histological features of atherosclerotic lesions of rabbits

Through manipulation of the cholesterol content in the diet and the length of feeding, we were able to obtain different degrees of aortic atherosclerotic lesions from cholesterol-fed rabbits. We selected three time points to collect rabbit aortas. Rabbits fed a cholesterol diet for 6 wks developed very tiny fatty dots on the surface of the aorta (Fig. 1A). These lesions were basically composed of a single layer or a few layers of macrophages on the intima, representing the earliest events in the arterial intima during the pathogenesis of atherosclerosis in experimental animals (Watanabe et al., 1985). For the convenience of description, we defined these lesions as

Table 1. Clinical data of autopsy cases used for the current study

No.	Age	Sex	Clinical diagnosis
1	78	F	Interstitial pneumonia
2	80	F	Pneumonia + Pulmonary edema
3	77	M	Acute myocardial infarction
4	84	M	Acute myocardial infarction
5	61	M	Pancreatic head carcinoma
6	72	M	Malignant lymphoma
7	55	M	Acute myocardial infarction

All autopsies were performed at the Department of Pathology, School of Med, University of Occupational and Environmental Health. Aortas were collected during the autopsy and they were used for the analysis of mRNA expression by real-time RT-PCR.

early stage lesions. Accumulation of foam cells in the subendothelial space was consistently observed and became prominent after rabbits were fed a cholesterol diet for 12 wks or 16 wks. At these stages, in addition to the accumulation of foam cells, many smooth muscle cells along with extracellular matrix were intermingled
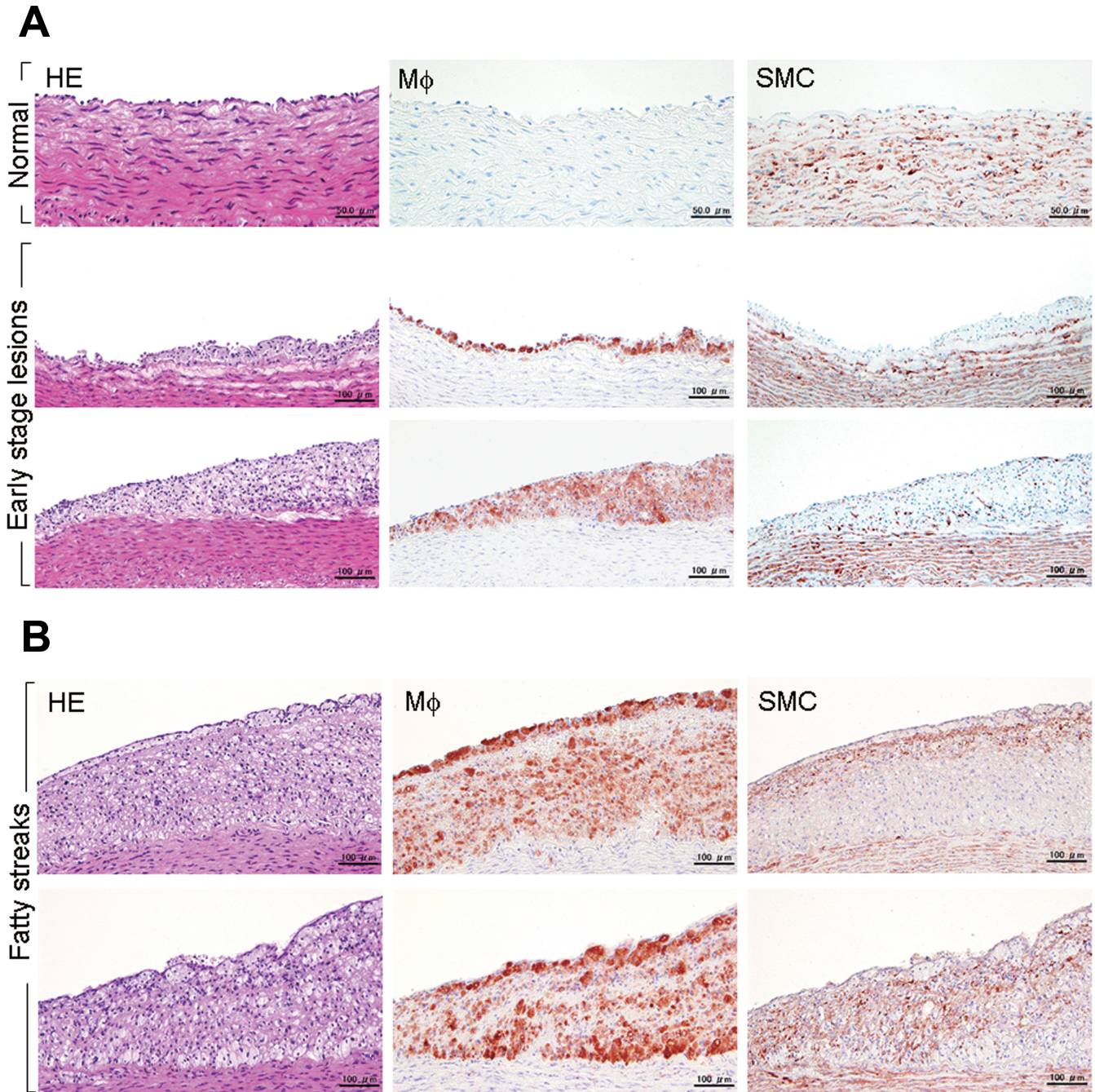

Fig. 1. Representative micrographs of early-stage lesions and fatty streaks in cholesterol-fed rabbits. **A.** Serial paraffin sections of the aorta of normal rabbits (top panel) and rabbits fed cholesterol for 6 wks (middle and bottom panels) were stained with either H&E or mAbs against macrophages (Møs) and smooth muscle cells (SMCs). The lesions are composed of a single or several layers of Møs and a few SMCs in cholesterol-fed rabbits. These lesions are designated "early-stage lesions". **B.** The fatty streaks of aortas from rabbits fed a cholesterol diet for 12 wks. Compared to the early-stage lesions above, the lesions become larger and contain many Møs intermingled with SMCs, either on the lesion surface (top), or throughout the lesions (bottom). These lesions are similar or equivalent to those of fatty streaks and preatheromas (types II and III lesions) in humans.

as the lesions progressed (Fig. 1B). These lesions are similar or equivalent to those of fatty streaks or preatheromas (types II and III lesions) in humans (Stary et al., 1995).

In WHHL rabbits at the ages of 24-wks, the lesions were composed of lipid cores and thick fibrous caps, while accumulation of macrophages was focally present

(Fig. 2). These lesions mimic human advanced lesions of fibroatheroma and complicated lesions (types V and VI lesions) (Stary et al., 1995).

MMP and TIMP expression in the lesions of rabbits

We first characterized the mRNA expression profiles

Table 2. Primers for real-time RT-PCR.

| Gene	Sequence	Product (bp)	TA (°C)	Accession #
Human MMP-1	(F) CTGGGAGCAAACACATCTGA; (R) CTGCTTGACCCTCAGAGACC	149	58.5	NM_002421
Human MMP-2	(F) ACGACCGCGACAAGAAGTAT; (R) ATTTGTTGCCCAGGAAAGTG	111	56.3	NM_004530
Human MMP-3	(F) GAGGACACCAGCATGAACCT; (R) TCACCTCCAATCCAAGGAAC	148	57.0	NM_002422
Human MMP-9	(F) TTGACAGCGACAAGAAGTGG; (R) CCCTCAGTGAAGCGGTACAT	148	57.0	NM_004994
Human MMP-12	(F) CCCGATCTCCATCATTTCAG; (R) TCACGGTTCATGTCAGGTGT	101	56.3	NM_002426
Human MMP-13	(F) TTGAGCTGGACTCATTGTCG; (R) CGCGAGATTTGTAGGATGGT	126	56.3	NM_002427
Human MT1-MMP (MMP-14)	(F) TGCCCAATGGAAAGACCTAC; (R) TGAATGACCCTCTGGGAGAC	138	57.0	U41078
Human TIMP-1	(F) ACATCCGGTTCGTCTACACC; (R) TGATGTGCAAGAGTCCATCC	120	57.0	NM_003254
Human TIMP-2	(F) AAGCGGTCAGTGAGAAGGAA; (R) TCTCAGGCCCTTTGAACATC	108	56.3	NM_003255
Human TIMP-3	(F) CCTGCTACTACCTGCCCTTGC; (R) GGCGTAGTGTTTGGACTGGT	104	58.5	NM_000362
Human MCP-1	(F) AGCAAGTGTCCCAAAGAAGC; (R) GAGTTTGGGTTTGCTTGTCC	121	56.3	S71513
Human GAPDH	(F) CAATGACCCCTTCATTGACCTC; (R) AGCATCGCCCCACTTGATT	172	60.9	BC029340
Rabbit MMP-1	(F) TTCCAAAGCAGAGAGGCAAT; (R) GGAGTGAGGACGAACTGAGC	160	59.0	M17820
Rabbit MMP-2	(F) GGCATTCAAGAGCTCTACGG; (R) CCTGTGTGCAGATCTCAGGA	103	59.0	D63579
Rabbit MMP-3	(F) CGTTCCTGATGTTGGTCACTTC; (R) TTGGCAGATCCGGTGTGTAA	101	57.5	M25664
Rabbit MMP-9	(F) TGAGCTTTGACATCCTGCAC; (R) AAGAAAGGCGAGGAGAGAGG	186	53.0	D26514
Rabbit MMP-12	(F) TGAAGCGTGAGGATGTTGAG; (R) AAAGCATGGGCTATGACACC	181	59.0	AB006779
Rabbit MMP-13	(F) GGAGAGGCAGCAGTCTCCAG; (R) CCTCGGACAAATCATCATCC	135	59.0	AF059201
Rabbit MT1-MMP (MMP-14)	(F) TGCCCAATGGAAAGACCTAC; (R) GCCTTCCCACACTTTGATGT	110	56.3	U83918
Rabbit TIMP-1	(F) GGTTCCCTGGAACAGTCTGA; (R) GGTCTGTCCACAAGCAATGA	143	57.4	AY829730
Rabbit TIMP-2	(F) GCACATCACGCTCTGTGACT; (R) CCGGAGAGGAGATGTAGCAC	143	59.4	AF069713
Rabbit TIMP-3	(F) CTACTACCTGCCCTGCTTCG; (R) GGCGTAGTGTTTGGACTGGT	100	59.4	AF069714
Rabbit MCP-1	(F) AGCACCAAGTGTCCCAAAGA; (R) TGTGTTCTTGGGTTGTGGAA	163	60.0	M57440
Rabbit GAPDH	(F) ATCACTGCCACCCAGAAGAC; (R) GTGAGTTTCCCGTTCAGCTC	146	58.3	L23961

TA, annealing temperature
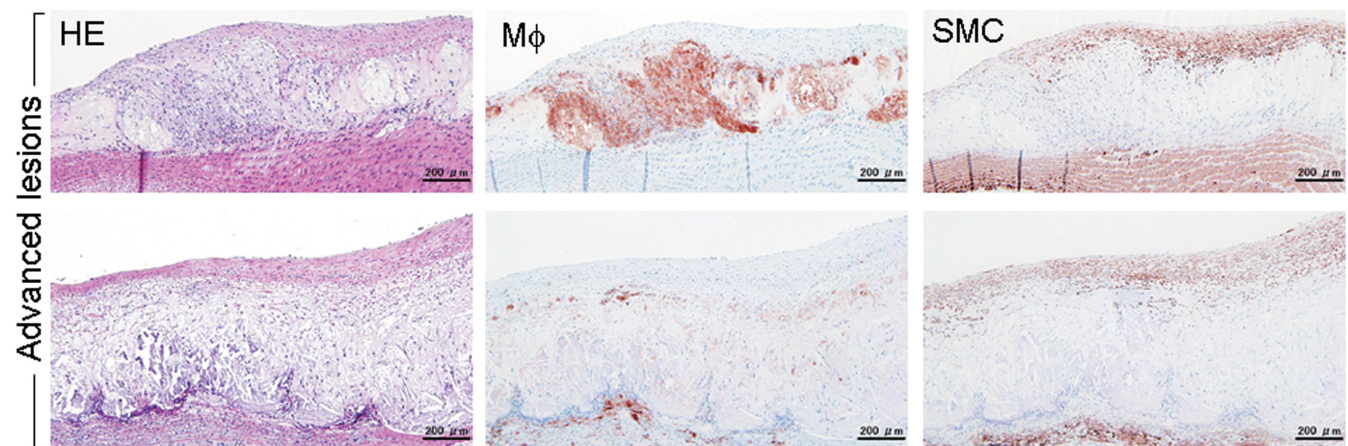
Fig. 2. Representative micrographs of advanced lesions of WHHL rabbits at 24 wks old. The lesions are characterized by the presence of a fibrous cap on the surface and lipid or necrotic cores, in which either Møs (top) or necrotic materials and calcium deposition (bottom), are present. SMCs are contained in the fibrous cap. These lesions mimic human advanced lesions ("fibroatheroma") and complicated lesions (types V and VI lesions).

of MMPs and TIMPs that have been detected in the lesions (Newby, 2005). By real-time RT-PCR analysis, we found that there were three distinct expression patterns with regard to changes in these MMPs. As shown in Fig.3A, the mRNA expression of MMP-1, -12, and -13 was barely detectable in the normal aorta. Once the lesions formed, the expression of these MMPs was evident, and it increased as the lesion progressed: starting from the early-stage lesions (6 wks) to fatty streaks (12 and 16 wks) of cholesterol diet-fed rabbits and WHHL rabbits at age 24-wks. Interestingly, increased expression of these MMPs was simultaneously associated with high expression of MCP-1 in the lesions (Fig. 3B, bottom panel). Immunohistochemical staining showed that macrophages were the major cellular sources of MMP-1 and -12 proteins in both early lesions and fatty streaks (Fig. 4A,B).

The second pattern of MMP expression was seen for MMP-3 and MT1-MMP (Fig. 3B, top and middle panels). Although their expression was normally present in the normal aorta (a pattern which clearly differed from those of MMP-1, -12, and -13), expression levels were markedly upregulated as the lesions progressed to the fatty streaks (in cholesterol-fed rabbits at 12 and 16 wks)

and advanced lesions (in WHHL rabbits at 24 wks) compared to the normal aorta.

The third expression pattern in the lesions was that of gelatinases A and B (MMP-2 and MMP-9). The expression of both MMPs was detected in the normal aortas; however, their expression was not significantly changed, regardless of the presence or absence of the lesions, although MMP-2 expression was slightly elevated in the lesions of fatty streaks of 16-wk-cholesterol-fed rabbits (Fig. 3C). Immunohistochemical staining showed that MMP-2 proteins were faintly stained in intimal smooth muscle cells (Fig. 4B), and MMP-9 showed the same staining pattern (data not shown).

Finally, we examined the expression of TIMP family members in the lesions (Fig. 3D). Although all TIMPs were present in the normal arterial wall, TIMP-1 and -2 appeared to be increased in the fatty streaks, but TIMP-3 was not changed throughout. Immunohistochemical staining revealed that TIMP-2 was mainly expressed by intimal macrophages (data not shown).

To evaluate the expression of MMP proteins in the lesions, we also performed Western blotting analysis. Consistent with the findings of real-time RT-PCR,
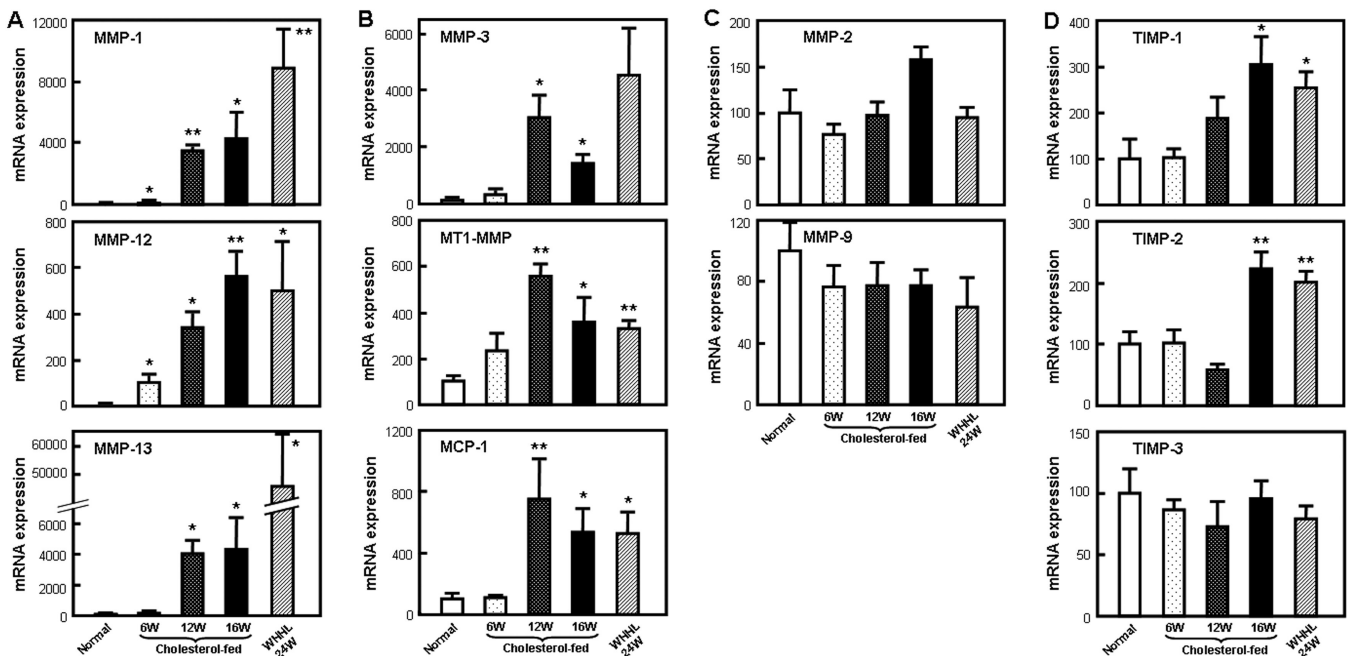

Fig. 3. MMP and TIMP gene expression in various lesions of cholesterol-fed and WHHL rabbits. The mRNA expression of each MMP and TIMP was analyzed by real-time RT-PCR as described in Materials and Methods, and expressed as a percentage of the control of normal rabbits. Each sample was analyzed in triplicate and data are expressed as the mean ± SEM. N=3 to 6 for each group. *$P<0.05$, **$P<0.01$ vs normal aortas. Expression patterns of MMP were arbitrarily classified into three categories: Pattern A is characterized by no expression in normal aorta, but markedly increased expression, including MMP-1, -12, and -13 expression in the lesions **(A)**. Pattern B includes MMP-3 and MT1-MMP, which are normally present in the aorta but are significantly increased with the progression of the lesions **(B)**. Both patterns A and B were accompanied by the simultaneous increase of a chemokine, monocyte chemoattractant protein-1 (MCP-1) (B, at the bottom). Pattern C includes the expression of gelatinases A and B (MMP-2 and MMP-9) **(C)**. Both were expressed in the normal aorta; however, their expression was not significantly affected by the presence of the lesions. Expression changes of TIMPs are shown in **D**.
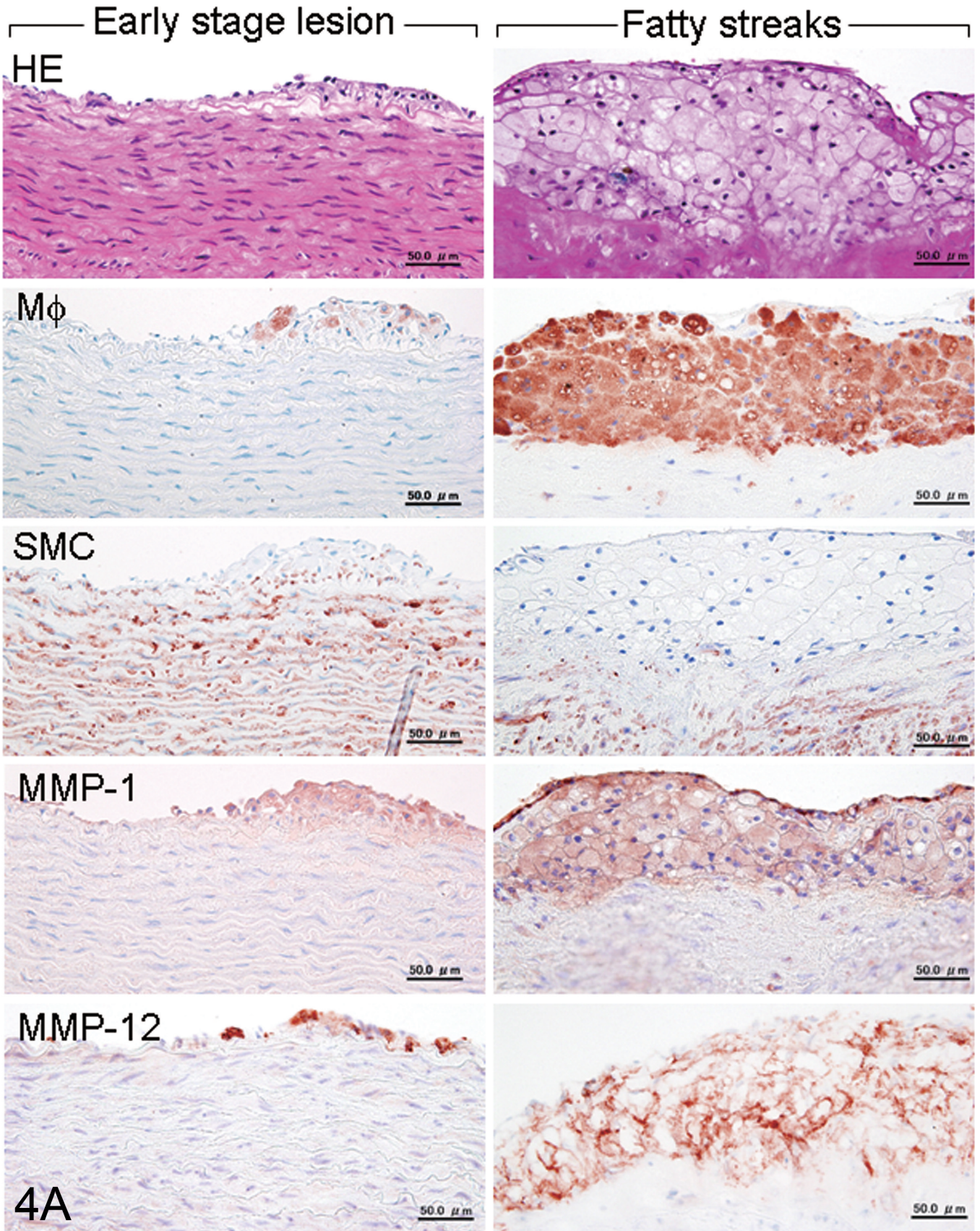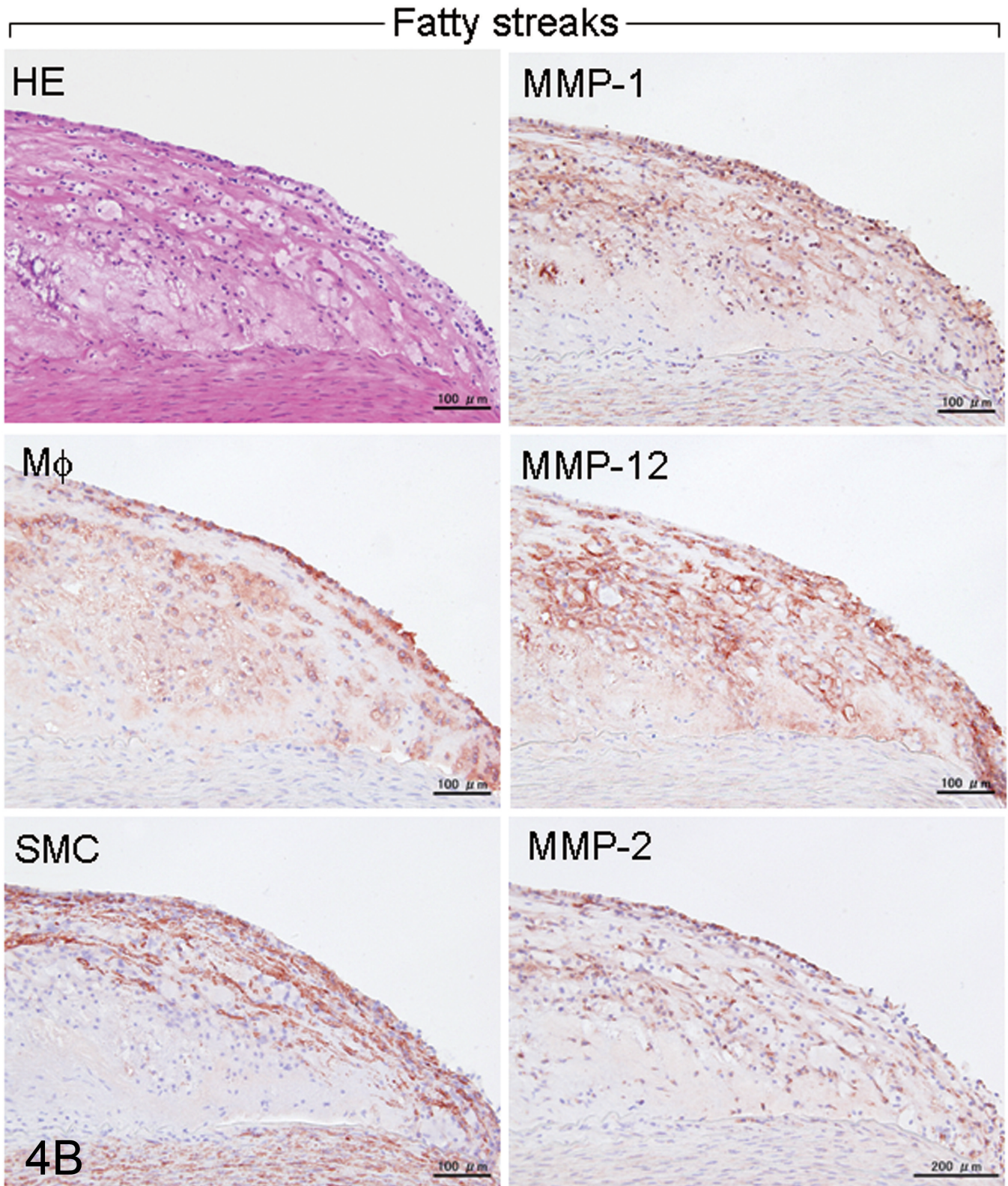
Fig. 4. Immunohistochemical demonstration of MMPs in the lesions of cholesterol-fed rabbits **(A, B)**. **A.** Serial paraffin sections of lesions were stained with H&E or immunostained with each mAb. Immunoreactive proteins of MMP-1 and -12 are present in the early stage lesions (left) and fatty streaks (right) and they are closely associated with macrophage-derived foam cells. **B.** In another lesional area of fatty streaks where smooth muscle cells and extracellular matrix were increased (compared to the lesions above), MMP-1 and MMP-12 were also colocalized with macrophages, but MMP-2 was colocalized with smooth muscle cells.
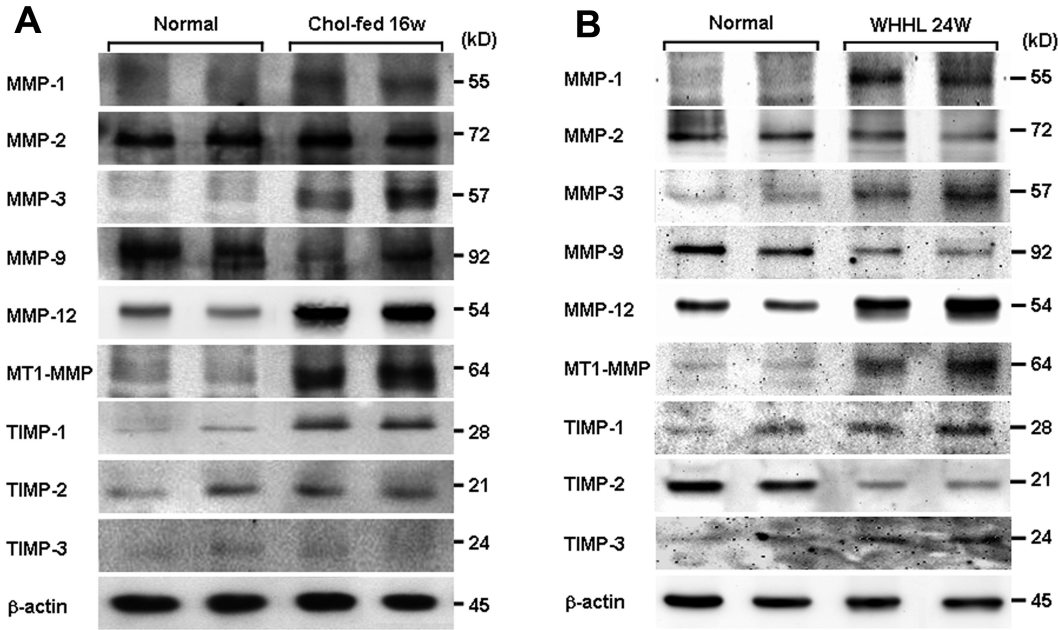
Fig. 5. Western blotting analysis of MMPs and TIMPs in the lesions. Total proteins isolated from aortas of normal rabbits and 16-wk-cholesterol-fed rabbits **(A)** and 24-wks WHHL rabbits **(B)** were fractionated by 10% SDS-PAGE, followed by immunoblotting using each Ab as described in Materials and Methods. Molecular size is shown on the right. ß-actin proteins are shown at the bottom to indicate that equal amounts of proteins were loaded in each lane. Compared to normal aortas, MMP-1, -3, -12, MT1-MMP, and TIMP-1 were increased, whereas MMP-2 and -9 were either unchanged or slightly reduced. Representative data from three separate analyses are shown.
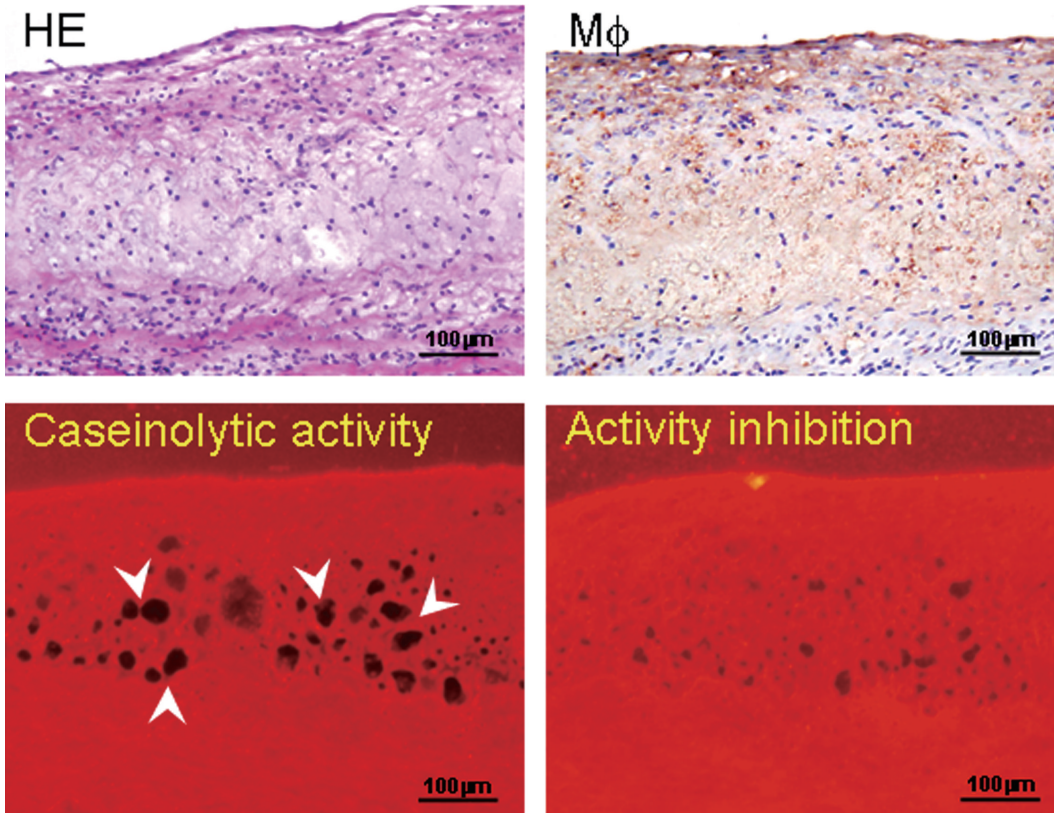

Fig. 6. *In situ* zymographic demonstration of MMP enzymatic activity in the lesions. *In situ* zymography was performed using cryostat sections of aortic arch of cholesterol-fed rabbits. The lesions are composed of many foam cells, as shown in H&E and immunohisto-chemical staining of macrophages (Møs) (upper panel). Caseinolytic activity was characterized by fluorescent substrate degradation (black areas indicated by white arrow heads), which can be dramatically inhibited by incubation with MMP inhibitor (bottom panel).

MMP-1 and MMP-3 proteins were almost totally undetectable, but pro-type MMP-12 proteins were faintly detected in the normal aorta (Fig. 5A). In spite of this, their protein expression, along with that of MT1-MMP and TIMP-1, was markedly increased in fatty streaks of cholesterol-fed rabbits (Fig. 5A) and advanced lesions of WHHL rabbits (Fig. 5B). MMP-2 and TIMP-3 protein expression patterns were the same as their mRNA expression patterns, while MMP-9 was reduced as the lesions progressed.

To localize the site of MMPs enzymatic activity in the lesions we performed in situ zymography. As shown in Fig.6, the casienolytic activity in the lesions can be visualized (arrow heads) and overlapped with macrophages, suggesting that macrophages are the major cells that produce MMP activity in the lesions.

MMP expression in human atherosclerotic lesions

Since the lesions of rabbits may not completely mirror those seen in humans, we compared the MMP expression patterns of rabbits with those of human specimens. Increased expression tendency of MMP-1, -12, -13, -2, -3, along with MCP-1, were almost identical to those of rabbits (although not statistically significant), while MT1-MMP tended to be decreased (Fig. 7). In
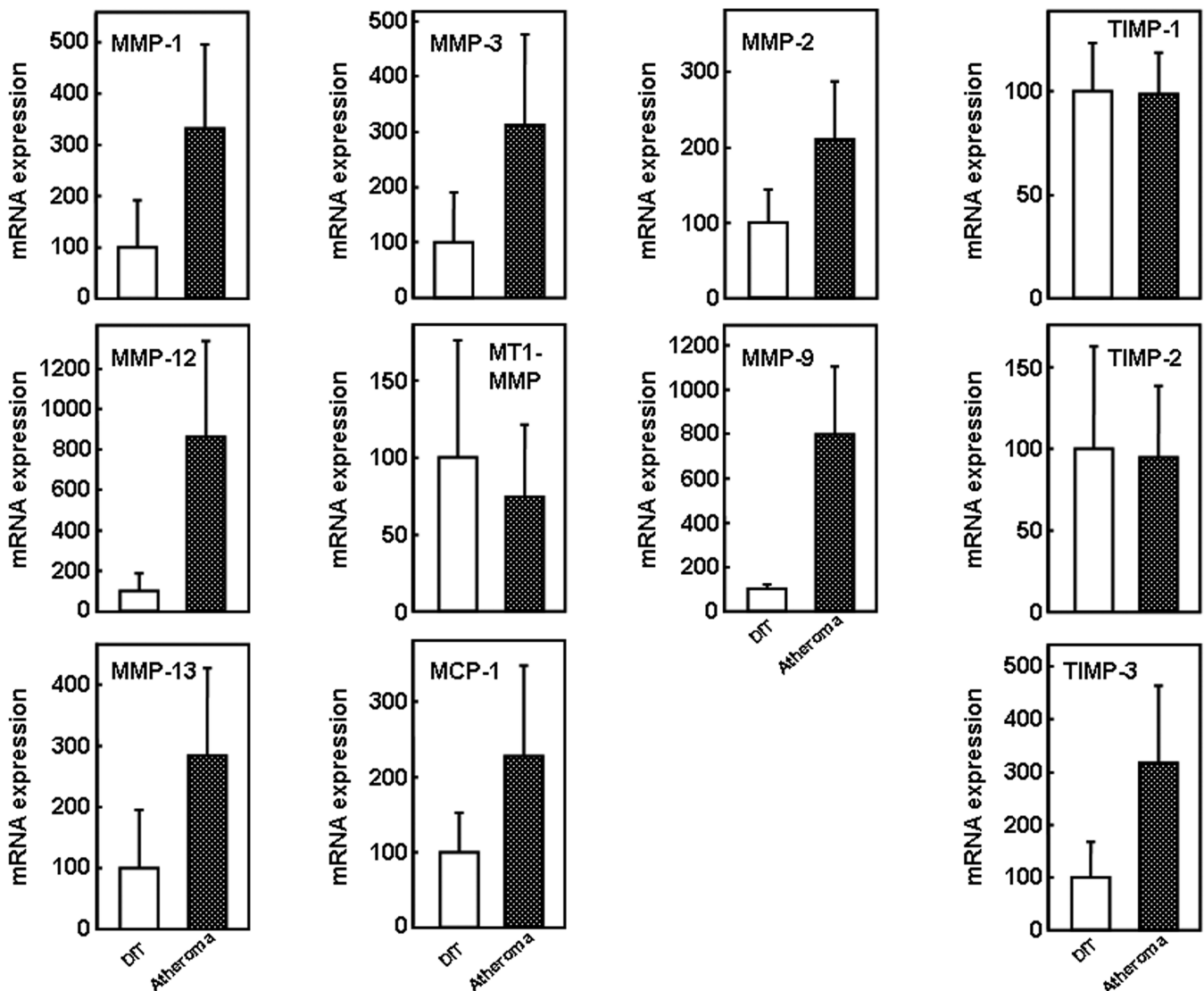

Fig. 7. MMP and TIMP expression profiling in the lesions of humans. mRNA expression of each MMP and TIMP was analyzed by real-time RT-PCR, as described in Materials and Methods. The samples were collected from 7 autopsy cases shown in Table 1, and grossly classified into normal-appearance (diffuse intimal thickening, DIT) and atheroma (fibrous plaques). Each sample was analyzed in triplicate and data are expressed as the mean ± SEM. N=7 for each group.
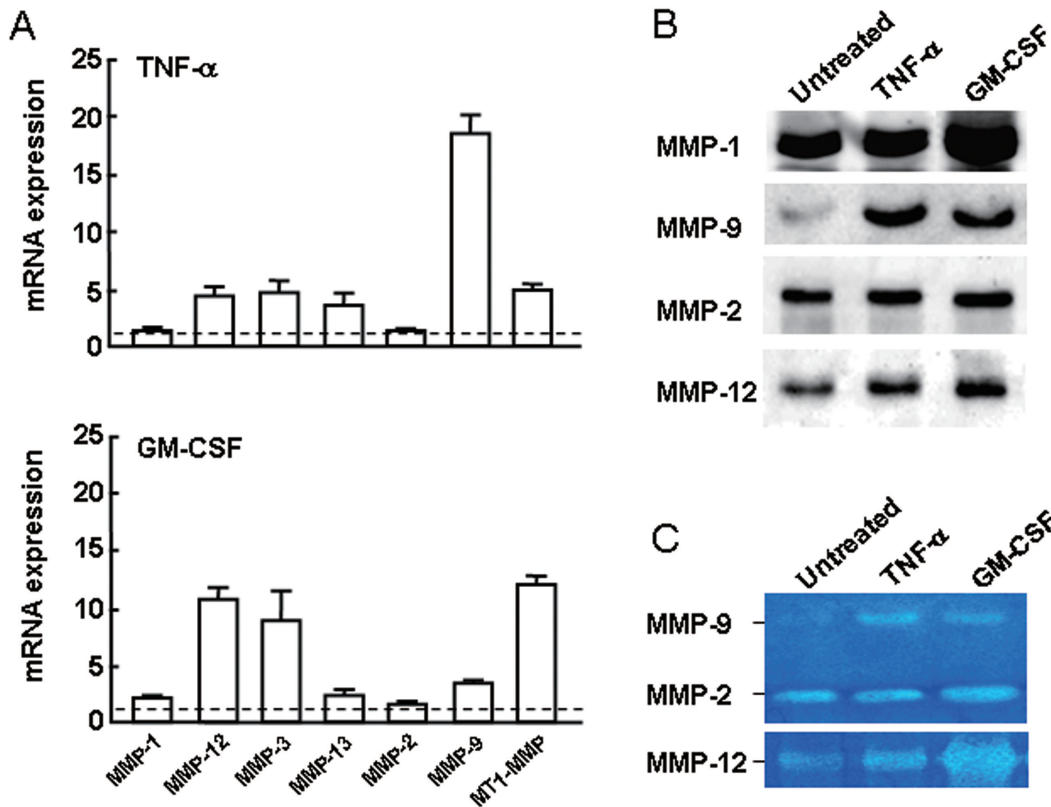
Fig. 8. MMP expression in U937-derived macrophages U937-derived macrophages were incubated with either TNF-α or GM-CSF for 48h, then the cells were homogenized and RNA was analyzed for the expression of different MMPs **(A).** The conditioned media were used for the analyses of proteins by Western blots **(B)** and gelatin and casein zymography **(C)** (Wu et al., 2000). Each experiment was performed at least twice and the values are the mean ± SEM of triplicate analyses. The dotted lines indicate the levels of each gene expression in the condition without cytokines (control level).

addition, MMP-9 was increased in the lesions compared to diffuse intimal thickening in human aortas (Fig. 7), which is different from those found in rabbit lesions. We next examined whether the expression of MMPs in macrophages could be induced by inflammatory cytokines, presumably present in the arterial milieu. Among these cytokines, GM-CSF and TNF-α were a potent stimulator for upregulation of many MMPs, including MMP-1, -12, -3, -13, -9 and MT1-MMP, whereas MMP-2 was unchanged (Fig. 8A). These changes were also shown in the Western blots and zymography (Fig. 8B,C). However, incubation with MCP-1, IL-6 and IL-10 did not lead to the upregulation of MMPs in macrophages (data not shown).

Discussion

In this study, we demonstrated for the first time that the expression patterns of MMPs and TIMPs varied dependent upon the lesion progression. First, we found that MMP-1, -12, and -13 expression (barely present in the normal aortas) was markedly increased as the lesion appeared and progressed in both rabbit and human atherosclerotic lesions. This result suggests that these three MMPs are either associated with, or directly participate in, the lesion formation or stability by facilitating SMC entry. Because macrophages are the major cellular components in the early-stage lesions, it is very likely that the increased expression of these MMPs was caused by increased intimal macrophages in the lesions (Galis et al., 1995a). As many cytokines (e.g. GM-CSF and TNF-α) are virtually present in the lesions, upregulation of these MMPs derived from macrophages may be mediated through both autocrine and paracrine mechanism. In addition, enhanced accumulation of macrophages in the lesions driven by MCP-1 may lead to the vicious circle of the MMP upreguation and macrophage recruitment as the lesions progressed.

The functional roles of MMP-1, -12, and -13 in lesion formation are not completely clear, but enhanced enzymatic activity of MMP-12 in the intima may result in the excessive degradation of the basement membrane and elastic lamina, which subsequently facilitates lesion expansion through increased macrophage and smooth muscle migration and proliferation. This notion has been supported by recent studies using both MMP-12 transgenic rabbits and KO mice. Increased expression of MMP-12 in macrophages accelerates the progression of aortic and coronary atherosclerosis in transgenic rabbits (Liang et al., 2006), whereas deficiency of MMP-12 in apoE KO mice results in a reduction of the lesion size and buried fibrous layers of the branchiocephalic arteries (Johnson et al., 2005). The functional role of MMP-1 in atherosclerosis is not fully understood yet. In the current

study, we found that MMP-1 immunoreactive proteins are indeed present in human fatty streaks and unstable coronary plaques (data not shown). Because this enzyme is not normally present in mice, it is impossible to investigate its functional phenotype in KO mice (Vincenti et al., 1998). Instead, transgenic mice expressing human MMP-1 were created by Lemaitre and coworkers (Lemaitre et al., 2001). Their studies showed that increased MMP-1 expression in macrophages of apoE KO mice led to a reduction of aortic atherosclerosis, suggesting that MMP-1 may play an atheroprotective role (Lemaitre et al., 2001). It seems that MMP-13 deficiency in apoE KO mice did not affect the lesion growth either, although collagen accumulation was enhanced (Deguchi et al., 2005). Despite these observations, further studies will be required to clarify the physiological functions of MMP-1 and -13 in the pathogenesis of atherosclerosis (Vincenti et al., 1998).

In addition to MMP-1, 12, and -13, we found that MMP-3 and MT1-MMP (also called MMP-14) were upregulated in rabbits, but MT1-MMP was not changed in human atherosclerotic lesions. However, both of these MMPs were present in normal arteries, suggesting that they are essential for maintaining normal arterial remodeling and functions. Silence and coworkers reported that MMP-3 deficiency in apoE KO mice fed a cholesterol-rich diet for 30 wks increased the thoracic aortic lesion size (Silence et al., 2001) but it is not known whether MMP-3 deficiency affects early-stage lesion formation. MT1-MMP has been implicated in the lesions of human atherosclerotic lesions, but its precise functions have not been examined. MT1-MMP KO mice were developed (Atkinson et al., 2005) and it has been shown that MT1-MMP has a significant role in smooth muscle cell migration (Filippov et al., 2005).

In contrast to the above MMPs (which were upregulated in the lesions), MMP-2 and -9 were normally expressed in the arterial wall but remained unchanged (both mRNA and protein levels) regardless of the presence of the lesion progression in rabbits. This result observed in rabbits was surprising and unexpected because such an expression pattern in rabbits is apparently different from that of human atherosclerotic lesions, in which MMP-9 tended to be upregulated. This discrepancy between rabbit and human MMP-9 expression suggests that there is a species difference between the nature of atherosclerotic lesions and/or macrophage protease repertoire. This difference was also seen in the expression of TIMPs of the lesions: upregulation of TIMP-1 and -2 in rabbits, yet upregulation of TIMP-3 in humans. MMP-2 and MMP-9 are physiologically essential for arterial remodeling in the tunica media, by mediating smooth muscle cell migration (Galis et al., 2002; Kuzuya et al., 2003). The low expression of MMP-9 in rabbits may help explain why the advanced lesions of coronary arteries in WHHL rabbits were seldom ruptured (Shiomi et al., 2003a). It was reported that MMP-9 was not significantly changed in apoE KO mice fed a high fat diet for 16 wks (Jeng et al., 1999). On the other hand, it has been reported that MMP-2 and -9 are both required, and work in concert to produce aortic aneurysms (Longo et al., 2002) and protect against the lesion formation in apoE KO mice (Luttun et al., 2004; Kuzuya et al., 2006). Recently, Gough et al. reported that macrophage-specific expression of the active form of MMP-9 led to the rupture of aortic plaques in apoE KO mice (Gough et al., 2006).

In the current study, we also characterized the expression patterns of TIMPs and showed that TIMP-1 and -2 were upregulated as the lesions advanced in rabbits. This finding is also consistent with the study by Zaltsman et al., who demonstrated increased secretion of TIMP-1 and -2 from the aorta explants of cholesterol-fed rabbits by zymography (Zaltsman et al., 1999). Therefore, TIMP-1 and -2 may be the major endogenous inhibitors that counterbalance increased MMP activity in the lesions, although this hypothesis remains unverified.

In conclusion, we have demonstrated for the first time that MMPs, along with their inhibitors, are differentially upregulated in the different types of atherosclerotic lesions of both rabbits and humans. Although further studies are still needed to clarify the functional roles played by each of the MMPs and TIMPs during lesion formation, our results may provide important clues for the development of MMP inhibitors in the future. Because the currently available synthetic MMP inhibitors suffer from lack of specificity, poor oral bioavailability, and strong unrelated side-effects, the development of effective and specific MMP inhibitors is essential. Our data also suggests that atherosclerotic lesions are different in terms of MMPs expression between rabbits and humans, therefore care should be taken for testing specific MMPs inhibitors when using animal models.

References

Atkinson J.J., Holmbeck K., Yamada S., Birkedal-Hansen H., Parks W.C. and Senior R.M. (2005). Membrane-type 1 matrix metalloproteinase is required for normal alveolar development. Dev. Dyn. 232, 1079-1090.

Brown D.L., Desai K.K., Vakili B.A., Nouneh C., Lee H.M. and Golub L.M. (2004). Clinical and biochemical results of the metalloproteinase inhibition with subantimicrobial doses of doxycycline to prevent acute coronary syndromes (MIDAS) pilot trial. Arterioscler. Thromb. Vasc. Biol. 24, 733-738.

Curci J.A., Liao S., Huffman M.D., Shapiro S.D. and Thompson R.W. (1998). Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms. J. Clin. Invest. 102, 1900-1910.

Deguchi J.O., Aikawa E., Libby P., Vachon J.R., Inada M., Krane S.M., Whittaker P. and Aikawa M. (2005). Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques. Circulation 112, 2708-2715.

Fan J., Wang X., Wu L., Matsumoto S.I., Liang J., Koike T., Ichikawa T., Sun H., Shikama H., Sasaguri Y. and Watanabe T. (2004).

Macrophage-specific overexpression of human matrix metalloproteinase-12 in transgenic rabbits. Transgenic Res. 13, 261-269.

Filippov S., Koenig G.C., Chun T.H., Hotary K.B., Ota I., Bugge T.H., Roberts J.D., Fay W.P., Birkedal-Hansen H., Holmbeck K., Sabeh F., Allen E.D. and Weiss S.J. (2005). MT1-matrix metalloproteinase directs arterial wall invasion and neointima formation by vascular smooth muscle cells. J. Exp. Med. 202, 663-671.

Galis Z.S. and Khatri J.J. (2002). Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ. Res. 90, 251-262.

Galis Z.S., Sukhova G.K., Lark M.W. and Libby P. (1994). Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J. Clin. Invest. 94, 2493-2503.

Galis Z.S., Sukhova G.K., Kranzhofer R., Clark S. and Libby P. (1995a). Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. Proc. Natl. Acad. Sci. USA 92, 402-406.

Galis Z.S., Sukhova G.K. and Libby P. (1995b). Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. FASEB J. 9, 974-980.

Galis Z.S., Johnson C., Godin D., Magid R., Shipley J.M., Senior R.M. and Ivan E. (2002). Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. Circ. Res. 91, 852-859.

Gough P.J., Gomez I.G., Wille P.T. and Raines E.W. (2006). Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. J. Clin. Invest. 116, 59-69.

Halpert I., Sires U.I., Roby J.D., Potter-Perigo S., Wight T.N., Shapiro S.D., Welgus H.G., Wickline S.A. and Parks W.C. (1996). Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. Proc. Natl. Acad. Sci. USA 93, 9748-9753.

Henney A.M., Wakeley P.R., Davies M.J., Foster K., Hembry R., Murphy G. and Humphries S. (1991). Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. Proc. Natl. Acad. Sci. USA 88, 8154-8158.

Herman M.P., Sukhova G.K., Libby P., Gerdes N., Tang N., Horton D.B., Kilbride M., Breitbart R.E., Chun M. and Schonbeck U. (2001). Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling. Circulation 104, 1899-1904.

Jeng A.Y., Chou M., Sawyer W.K., Caplan S.L., Von Linden-Reed J., Jeune M. and Prescott M.F. (1999). Enhanced expression of matrix metalloproteinase-3, -12, and -13 mRNAs in the aortas of apolipoprotein E-deficient mice with advanced atherosclerosis. Ann. NY Acad. Sci. 878, 555-558.

Johnson J.L., Fritsche-Danielson R., Behrendt M., Westin-Eriksson A., Wennbo H., Herslof M., Elebring M., George S.J., McPheat W.L. and Jackson C.L. (2006). Effect of broad-spectrum matrix metalloproteinase inhibition on atherosclerotic plaque stability. Cardiovasc. Res. 71, 586-595.

Johnson J.L., George S.J., Newby A.C. and Jackson C.L. (2005). Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. Proc. Natl. Acad. Sci. USA 102, 15575-15580.

Kuzuya M., Kanda S., Sasaki T., Tamaya-Mori N., Cheng X.W., Itoh T.,

Itohara S. and Iguchi A. (2003). Deficiency of gelatinase a suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia. Circulation 108, 1375-1381.

Kuzuya M., Nakamura K., Sasaki T., Cheng X.W., Itohara S. and Iguchi A. (2006). Effect of MMP-2 deficiency on atherosclerotic lesion formation in apoE-deficient mice. Arterioscler. Thromb. Vasc. Biol. 26, 1120-1125.

Lemaitre V. and D'Armiento J. (2006). Matrix metalloproteinases in development and disease. Birth Defects. Res. C. Embryo. Today 78, 1-10.

Lemaitre V., O'Byrne T.K., Borczuk A.C., Okada Y., Tall A.R. and D'Armiento J. (2001). ApoE knockout mice expressing human matrix metalloproteinase-1 in macrophages have less advanced atherosclerosis. J. Clin. Invest. 107, 1227-1234.

Liang J., Liu E., Yu Y., Kitajima S., Koike T., Jin Y., Morimoto M., Hatakeyama K., Asada Y., Watanabe T., Sasaguri Y., Watanabe S. and Fan J. (2006). Macrophage metalloelastase accelerates the progression of atherosclerosis in transgenic rabbits. Circulation 113, 1993-2001.

Longo G.M., Xiong W., Greiner T.C., Zhao Y., Fiotti N. and Baxter B.T. (2002). Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. J. Clin. Invest. 110, 625-632.

Luttun A., Lutgens E., Manderveld A., Maris K., Collen D., Carmeliet P. and Moons L. (2004). Loss of matrix metalloproteinase-9 or matrix metalloproteinase-12 protects apolipoprotein E-deficient mice against atherosclerotic media destruction but differentially affects plaque growth. Circulation 109, 1408-1414.

Manning M.W., Cassis L.A. and Daugherty A. (2003). Differential effects of doxycycline, a broad-spectrum matrix metalloproteinase inhibitor, on angiotensin II-induced atherosclerosis and abdominal aortic aneurysms. Arterioscler. Thromb. Vasc. Biol. 23, 483-488.

Newby A.C. (2005). Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol. Rev. 85, 1-31.

Newman K.M., Jean-Claude J., Li H., Scholes J.V., Ogata Y., Nagase H. and Tilson M. D. (1994). Cellular localization of matrix metalloproteinases in the abdominal aortic aneurysm wall. J. Vasc. Surg. 20, 814-820.

Rajavashisth T.B., Xu X.P., Jovinge S., Meisel S., Xu X.O., Chai N.N., Fishbein M.C., Kaul S., Cercek B., Sharifi B. and Shah P.K. (1999). Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators. Circulation 99, 3103-3109.

Schonbeck U., Mach F., Sukhova G.K., Atkinson E., Levesque E., Herman M., Graber P., Basset P. and Libby P. (1999). Expression of stromelysin-3 in atherosclerotic lesions: regulation via CD40-CD40 ligand signaling in vitro and in vivo. J. Exp. Med. 189, 843-853.

Shiomi M., Ito T., Yamada S., Kawashima S. and Fan J. (2003). Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). Arterioscler. Thromb. Vasc. Biol. 23, 1239-1244.

Silence J., Lupu F., Collen D. and Lijnen H.R. (2001). Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. Arterioscler. Thromb. Vasc. Biol. 21, 1440-1445.

Stary H.C., Chandler A.B., Dinsmore R.E., Fuster V., Glagov S., Insull W. Jr, Rosenfeld M.E., Schwartz C.J., Wagner W.D. and Wissler R.W. (1995). A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report

from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 92, 1355-1374.

Sukhova G.K., Schonbeck U., Rabkin E., Schoen F.J., Poole A.R., Billinghurst R.C. and Libby P. (1999). Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. Circulation 99, 2503-2509.

Sun H., Koike T., Ichikawa T., Hatakeyama K., Shiomi M., Zhang B., Kitajima S., Morimoto M., Watanabe T., Asada Y., Chen Y.E. and Fan J. (2005). C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. Am. J. Pathol. 167, 1139-1148.

Uzui H., Harpf A., Liu M., Doherty T.M., Shukla A., Chai N.N., Tripathi P.V., Jovinge S., Wilkin D.J., Asotra K., Shah P.K. and Rajavashisth T.B. (2002). Increased expression of membrane type 3-matrix metalloproteinase in human atherosclerotic plaque: role of activated macrophages and inflammatory cytokines. Circulation 106, 3024-3030.

Vincenti M.P., Coon C.I., Mengshol J.A., Yocum S., Mitchell P. and Brinckerhoff C.E. (1998). Cloning of the gene for interstitial collagenase-3 (matrix metalloproteinase-13) from rabbit synovial fibroblasts: differential expression with collagenase-1 (matrix metalloproteinase-1). Biochem. J. 331 (Pt 1), 341-346.

Visse R. and Nagase H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ. Res. 92, 827-839.

Watanabe T., Hirata M., Yoshikawa Y., Nagafuchi Y., Toyoshima H. and Watanabe T. (1985). Role of macrophages in atherosclerosis. Sequential observations of cholesterol-induced rabbit aortic lesion by the immunoperoxidase technique using monoclonal antimacrophage antibody. Lab. Invest. 53, 80-90.

Wu L., Fan J., Matsumoto S. and Watanabe T. (2000). Induction and regulation of matrix metalloproteinase-12 by cytokines and CD40 signaling in monocyte/macrophages. Biochem. Biophys. Res. Commun. 269, 808-815.

Zaltsman A.B., George S.J. and Newby A.C. (1999). Increased secretion of tissue inhibitors of metalloproteinases 1 and 2 from the aortas of cholesterol fed rabbits partially counterbalances increased metalloproteinase activity. Arterioscler. Thromb. Vasc. Biol. 19, 1700-1707.