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The Role of Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Antibody-Induced Arthritis

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Matrix metalloproteinases (MMPs) are a large group of enzymes responsible for matrix degradation. Among them, the family of gelatinases (MMP-2/gelatinase A and MMP-9/gelatinase B) is overproduced in the joints of patients with rheumatoid arthritis. Because of their degradative effects on the extracellular matrix, gelatinases have been believed to play an important role in progression and cartilage degradation in this disease, although their precise roles are yet to be defined. To clarify these roles, we investigated the development of Ab-induced arthritis, one of the murine models of rheumatoid arthritis, in MMP-2 or MMP-9 knockout (KO) mice. Surprisingly, the MMP-2 KO mice exhibited severe clinical and histologic arthritis than wild-type mice. The MMP-9 KO mice displayed milder arthritis. Recovery from exacerbated arthritis in the MMP-2 KO mice was possible by injection of wild-type fibroblasts. These results indicated a suppressive role of MMP-2 and a pivotal role of MMP-9 in the development of inflammatory joint disease. ***The Journal of Immunology*****, 2002, 169: 2643–2647.**

Matrix metalloproteinases (MMPs)[2] are a group of zinc-dependent endopeptidases that can degrade every component of the extracellular matrix (ECM). Overexpression of MMPs has been implicated in the pathogenesis of various diseases, such as arthritis, atherosclerosis, and tumor invasion and metastasis (1–3). The mechanisms of action and the roles of individual MMPs in the pathophysiology of these and other conditions have yet to be clearly defined due to the lack of selective MMP inhibitors. However, recent progress with gene knockout (KO) mice offers a way to overcome this problem.

Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) may be especially important in collagen degradation, through digestion of denatured collagen (gelatin) generated by thermal denaturation at body temperature after specific cleavage of the triple helix region of the fibrillar collagen molecules by collagenases. These two enzymes also digest other substrates, such as type I collagen and aggrecan, which mainly exist in cartilage (4, 5). In rheumatoid arthritis (RA), synovial fluids from patients include enhanced levels of MMP-2 and MMP-9 (6–8). MMP-14 (MT1-MMP), an activator of MMP-2, is found in the synovium of RA patients (9). Such observations suggest that these enzymes have crucial roles for MMP-2 and MMP-9 in RA. Furthermore, MMP-9 from macrophages and neutrophils is thought to play a key role in the migration of these cells during inflammatory diseases such as RA (7, 8).

On the other hand, recent studies have suggested that MMPs can suppress inflammation by degrading biologically active molecules such as cytokines, chemokine, and growth factor receptor (10–13). Thus, the role of these enzymes in RA is controversial. To more directly examine the major roles of MMP-2 and MMP-9 in RA, we have used MMP-2 KO mice (14, 15) and MMP-9 KO mice (16). We investigated the development of Ab-induced arthritis (AbIA), one of the animal models of RA (17, 18), in these KO mice.

Materials and Methods

Mice

MMP-2$^{+/-}$ mice (14) and *MMP-9*$^{+/-}$ mice (16) were crossed five times with BALB/c mice and were crossed with each other to obtain *MMP-2*$^{+/-}$*MMP-9*$^{+/-}$ mice. We then crossed these mice with each other to obtain *MMP-2*$^{-/-}$*MMP-9*$^{+/+}$ (MMP-2 KO), *MMP-2*$^{+/+}$*MMP-9*$^{-/-}$ (MMP-9 KO), *MMP-2*$^{-/-}$*MMP-9*$^{-/-}$ (double knockout), and control wild-type mice.

Induction of AbIA and clinical assessment of arthritis

Arthritis was induced using methods described by Terato et al. (18). Female mice, 7–8 wk old, weighing 18–22 g, were injected i.v. with 2 mg of an arthritogenic mAb cocktail (Chondrex. LLC, Seattle, WA) on day 0. On day 3, LPS (2.5 mg/kg) was injected i.p. (18). The clinical severity of arthritis was graded as follows: 0, normal; 1, erythema; 2, slight swelling; and 3, severe swelling or deformity. Each limb was graded, allowing a maximum clinical score of 12 for each animal.

Histologic analyses

For histologic analyses, about half of the KO and wild-type mice were randomly selected, and the hind paws were removed on day 11. The average arthritis scores of histologically analyzed mice were almost the same as those of all mice. After fixation with 10% formaldehyde in PBS, the paws were decalcified with EDTA and embedded in paraffin. The paraffin sections were stained with Safranin O-Fast Green. The grade of proteoglycan depletion determined by Safranin O staining, the erosion of ankle joint cartilage, and the extent of cellular infiltration were separately determined and graded from 0 to 3 (3 is the severest).

Fibroblast injection

Embryonic fibroblasts were extracted from day 12.5 embryos obtained by cesarean section. Following removal of extra-embryonic tissue and blood-containing embryonic organs, the embryos were rinsed with PBS and minced. Tissue clumps were removed by centrifugation, and fibroblast cultures were established in DMEM containing 10% FCS. The passage 2 cells were washed three times with PBS and suspended in PBS. Next, 2.5×10^5 cells (in 50 μl PBS) were injected into the ankle joint of each hind paw.

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[2] Abbreviations used in this paper: MMP, matrix metalloproteinase; AbIA, Ab-induced arthritis; ECM, extracellular matrix; KO, knockout; RA, rheumatoid arthritis; TIMP, tissue inhibitor of MMP.

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Gelatin zymography

Gelatin zymography was conducted as described previously (14). Briefly, whole mouse paws were removed proximal to the carpus or tarsus and homogenized in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Nonidet P-40 and centrifuged at 15,000 rpm for 10 min. The protein concentration of the supernatant was determined by the method of Bradford, and 20 μg supernatant proteins were applied to nonreduced SDS-PAGE using 7.5% gel containing 0.1% gelatin. After electrophoresis, the gel was soaked in 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 at room temperature with gentle shaking for 2 h and incubated overnight in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ at 37°C. The gels were then stained with Coomassie brilliant blue.

Competitive RT-PCR

Whole mouse paws were removed proximal to the carpus, and total cellular RNA was isolated with TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The RNA sample was subjected to first-strand cDNA synthesis by Superscript II (Life Technologies) using a random hexamer mix. The DNA competitors of MMP-3, MMP-8, MMP-13, MMP-14, tissue inhibitor of MMP (TIMP)-1, and TIMP-3 were generated with a Competitive DNA Construction kit (Takara Shuzo, Shiga, Japan) according to the manufacturer's instructions. The primers used for MMP-3 detection were 5′-TGTCCCGTTTCCATCTCTCT-3′ for the sense primer and 5′-ATCAAACCTCCAGTATTTGT-3′ for the antisense primer. Those for MMP-7 detection were 5′-CAGATGTTGCAGA ATACTCAC-3′ for the sense primer and 5′-ATGCCTGCAATGTCGTC CTTT-3′ for the antisense primer. Those for MMP-8 detection were 5′-CAGTACCTGAACACCTGGAA-3′ for the sense primer and 5′-TCTGC TTCTCCCTGTAAGAT-3′ for the antisense primer. Those for MMP-10 detection were 5′-GGGACCAACTTATTCCTGGT-3′ for the sense primer and 5′-GGAAGCCTTTATCCATAACA-3′ for the antisense primer. Those for MMP-11 detection were 5′-TCTGCCTAATACCTTGACAT-3′ for the sense primer and 5′-ATTCATGAGCCGCCACTTGC-3′ for the antisense primer. Those for MMP-12 detection were 5′-CAATAATC CAAAGTCAATAA-3′ for the sense primer and 5′-GGAAGAAATA GAAGTAATGT-3′ for the antisense primer. Those for MMP-13 detection were 5′-CGAACTTAACTTACAGGATTG-3′ for the sense primer and 5′-GCTGGGTCACACTTCTCTGGT-3′ for the antisense primer. Those for MMP-14 detection were 5′-GCCCAAGGCAGCAACTTCAG-3′ for the sense primer and 5′-AGCGCTTCCTCCGAACATTG-3′ for the antisense primer. Those for TIMP-1 detection were 5′-ACCACCTTATACCA GCGTTA-3′ for the sense primer and 5′-AAACAGGGAAACACTGT GCA-3′ for the antisense primer. Those for TIMP-2 detection were 5′-CACCCGCAACAGGCGTTTTG-3′ for the sense primer and 5′-ATCTTGCCATCTCCTTCTGC-3′ for the antisense primer. Those for TIMP-3 detection were 5′-CAAAGTGGTGGGAAAGAAGC-3′ for the sense primer and 5′-CCGGATGCAGGCGTAGTGTT-3′ for the antisense primer.

Statistical analyses

All results were analyzed using the Mann-Whitney *U* test or Student's *t* test (StatView program 1992, Abacus Concepts, Berkeley, CA). Values of $p < 0.05$ were considered significant.

Results

MMP-2 KO mice showed exacerbated levels of AbIA, and MMP-9 KO mice showed mild levels of AbIA

To investigate the roles of MMP-2 and MMP-9 in AbIA, KO mice of each enzyme or both were used in this model. Arthritis appeared on day 5 and continued for at least 6 days. Fig. 1 shows the average arthritis scores of each KO mouse and control wild-type mouse. The MMP-2 KO mice showed exacerbated levels of AbIA compared with wild-type mice. On the other hand, reduced arthritis was observed in the MMP-9 KO mice (Fig. 1). On day 11, the average score was increased by 59% for the MMP-2 KO mice and was reduced by 45% for the MMP-9 KO mice ($p < 0.001$ and $p < 0.01$, respectively). The double KO mice showed no significant difference from the wild-type mice. The results for the double KO mice might reflect the effects of both MMP-2 deficiency and MMP-9 deficiency.

To examine the degradation of articular cartilage and cleavage of proteoglycan, histologic analyses were performed with the hind

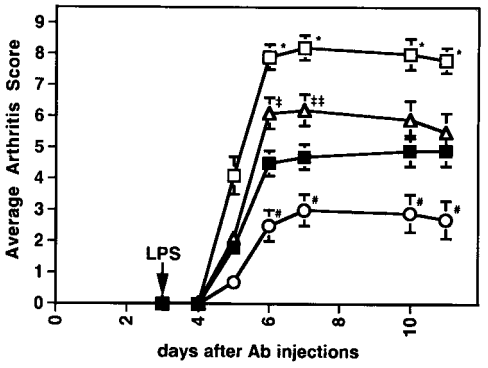

FIGURE 1. Average scores of AbIA in MMP-2 KO mice (□; $n = 26$), MMP-9 KO mice (○; $n = 43$), double KO mice (△; $n = 31$), and control wild-type mice (■; $n = 52$). Data represent the mean ± SEM per group. The mean arthritis scores on day 11 were 4.9 (wild-type), 7.8 (MMP-2 KO), 2.7 (MMP-9 KO), and 5.5 (double KO). ∗, $p < 0.001$; #, $p < 0.01$; ‡, $p = 0.10$; ‡‡, $p = 0.12$ (vs wild-type mice, by Mann-Whitney *U* test).

paws on day 11. As expected, the arthritic mice revealed extensive cellular infiltration and proteoglycan depletion (Fig. 2). Cartilage erosion was mild even in the severely arthritic mice (Fig. 2). In the MMP-2 KO mice, the average scores of cellular infiltration and proteoglycan depletion were significantly increased (Fig. 3). In contrast, the MMP-9 KO mice showed only mild disease, which was significantly less severe than that of the wild-type mice (Figs.

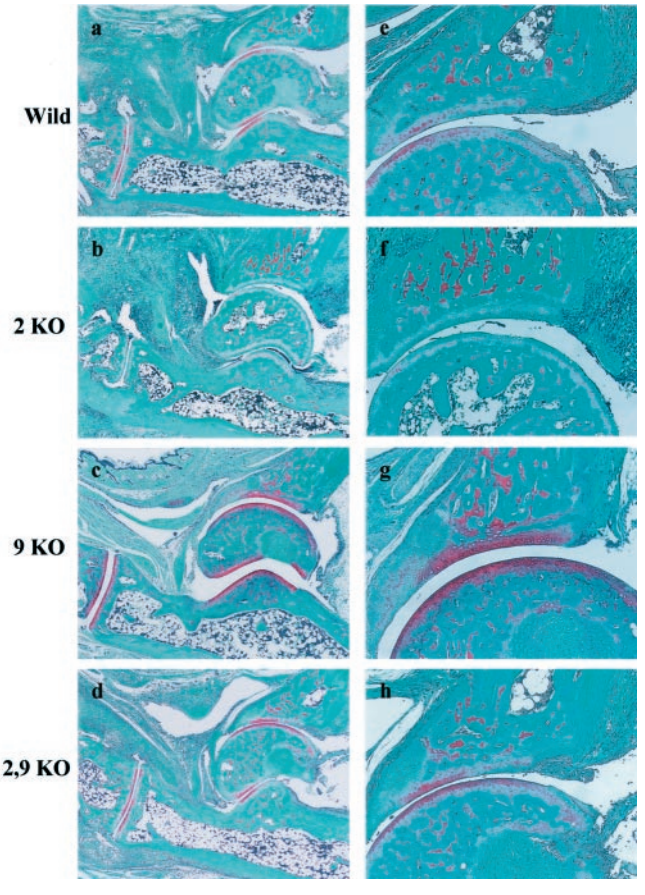

FIGURE 2. Effects of MMP deficiency in the progression of AbIA. A hind paw was removed (day 11) from a wild-type mouse (*a* and *e*) and from MMP-2 (*b* and *f*), MMP-9 (*c* and *g*), and double KO (*d* and *h*) mouse. The paraffin sections were stained with Safranin O-Fast Green. Original magnification, ×10 (*left panels*) and ×25 (*right panels*).

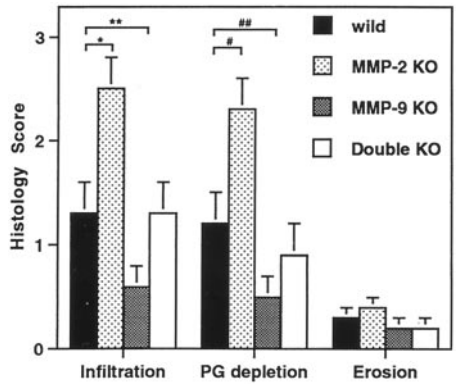

FIGURE 3. Articular inflammation and destruction of arthritic mice. Hind paws were removed (day 11) from wild-type mice ($n = 23$) and from MMP-2 ($n = 13$), MMP-9 ($n = 24$), and double KO ($n = 17$) mice. The grade of proteoglycan depletion estimated by Safranin O staining, the erosion of ankle joint cartilage, and the extent of cellular infiltration were separately determined and graded from 0 to 3. Data represent the mean ± SEM. ∗, $p = 0.0004$; ∗∗, $p = 0.017$; #, $p = 0.0007$; ##, $p = 0.018$ (vs wild-type mice, by Student's t test).

2 and 3). The average score of proteoglycan depletion in the double KO mice was lower than that in the wild-type mice, but the difference was not significant ($p = 0.33$, by Student's t test).

Exacerbated arthritis in MMP-2 KO mice was recovered by MMP-2 derived from fibroblasts

We next investigated whether recovery was possible from the exacerbated AbIA in the MMP-2 KO mice by the injection of wild-type cells. Embryonic fibroblasts from the wild-type mice were injected into the ankle joint on days 0, 3, and 6. These cells secrete MMP-2 in vitro (14). Recovery from the exacerbated arthritis in the MMP-2 KO mice occurred following the injection of wild-type fibroblasts (Fig. 4). As little effect was observed by the injection of

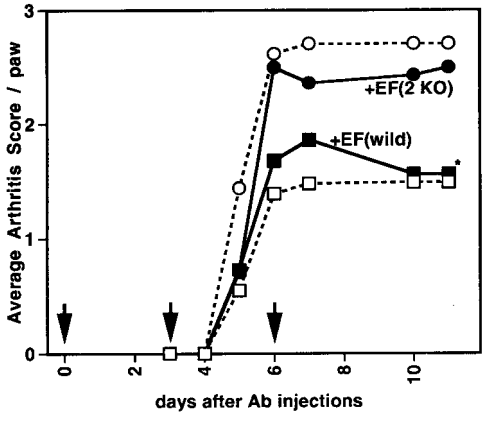

FIGURE 4. Recovery from exacerbated arthritis in MMP-2 KO mice by injection of wild-type fibroblasts. AbIA was induced in the MMP-2 KO mice as described in *Materials and Methods*. The mice were injected three times with wild-type fibroblasts (■; $n = 11$) or MMP-2 KO fibroblasts (●; $n = 7$) into the ankle joint of hind paws (arrows). The first injections were performed after administration of mAb cocktail on day 0. The second injections were given after LPS administration on day 3. The third injections were made after determination of the arthritis score on day 6. Data represent the mean arthritis score of each paw. Average arthritis scores of hind paws in wild-type mice (□; $n = 104$) and MMP-2 KO mice (○; $n = 52$) are shown. ∗, $p = 0.05$ (wild-type fibroblasts vs MMP-2 KO fibroblasts, by Mann-Whitney U test).

MMP-2 KO fibroblasts, recovery from the exacerbated AbIA occurred due to the fibroblast-derived MMP-2.

Secretion of MMP-2 and MMP-9 increased in parallel with AbIA development

To ensure that the expression levels of MMP-2 and MMP-9 were up-regulated with the development of arthritis in AbIA as in human RA, the lysates of the paws were examined by gelatin zymography. On days 3 and 4, little MMP-2 and MMP-9 activity was seen similar to that on day 0, indicating that the expression of the two enzymes in the paw was not affected by Ab or LPS administration in this model (Fig. 5). If arthritis developed, MMP-2 and MMP-9 were overproduced (Fig. 5). This MMP-2 or MMP-9 expression was not compensated for by deficiency of the other gelatinase (Fig. 5).

Expression levels of the other MMPs and TIMPs in each KO mouse showed no large changes

MMPs have overlapping substrate specificity, so the effect of loss of specific activities of one or two enzymes may be caused by compensation by other enzymes. Therefore, we examined MMP and TIMP expression in nonarthritic paws and arthritic paws (score 3) from the wild-type or each KO mouse by competitive RT-PCR. Fig. 6 shows the average copy number of MMP-3 (stromelysin-1), MMP-8 (neutrophil collagenase), MMP-13 (collagenase-3), MMP-14 (MT1-MMP), TIMP-1, and TIMP-3. The expressions of mRNA of MMP-3, -8, -13, and -14 and TIMP-1 were increased in arthritic mice, but TIMP-3 expression was not changed (Fig. 6). In these MMPs and TIMPs, no significant difference was seen between the wild-type and each KO mouse (Fig. 6). The expression levels of mRNA of MMP-7 (matrilysin), MMP-10 (stromelysin-2), MMP-11 (stromelysin-3), MMP-12

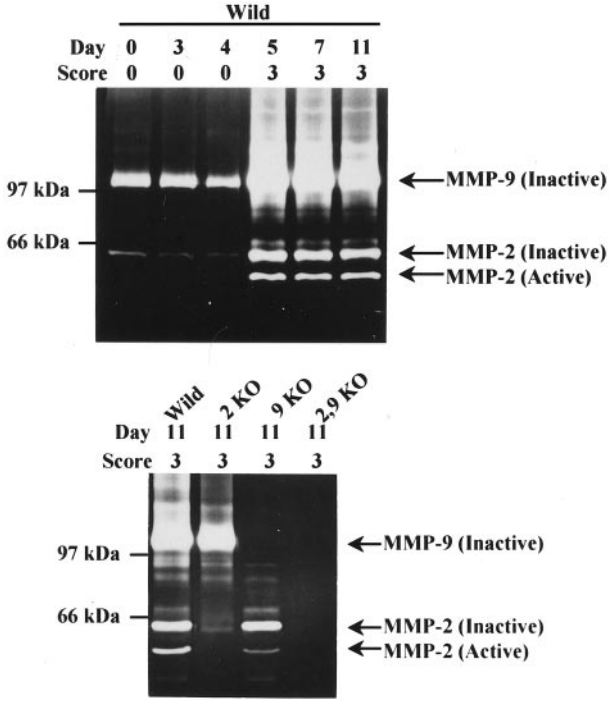

FIGURE 5. Gelatinase secretion in the paws with AbIA. Lysates of the paws of days 0 (before Ab administration), 3 (before LPS administration), 4, 5, 7, and 11 from wild-type mouse and lysates on day 11 from each KO mouse were analyzed by gelatin zymography. Gelatinolytic bands of MMP-2 and MMP-9 are indicated by arrows.

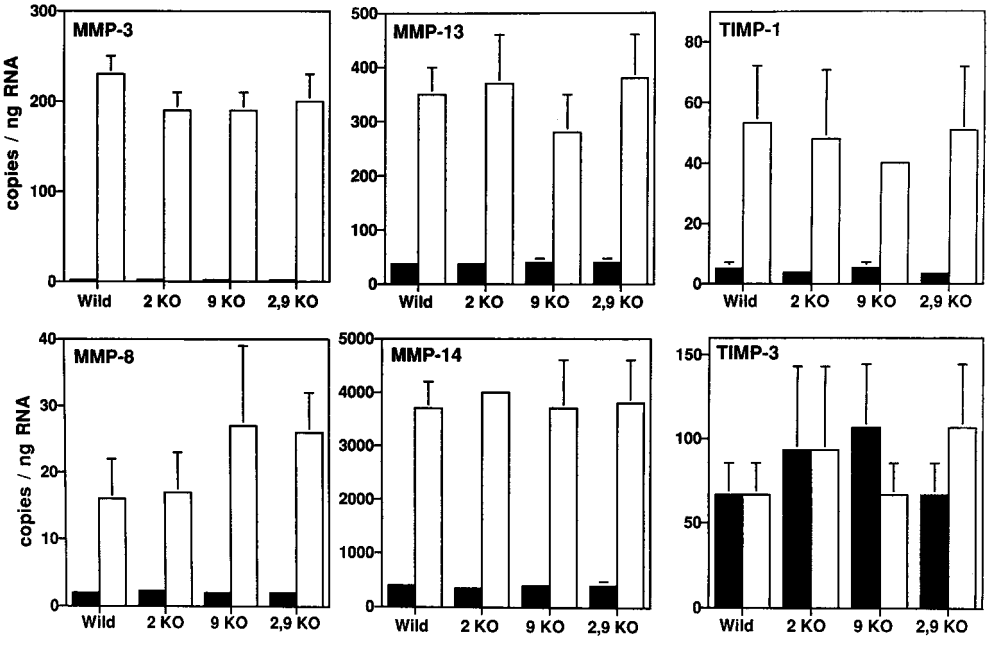

FIGURE 6. Analysis of mRNA levels of *MMP-3*, *MMP-8*, *MMP-13*, *MMP-14*, *TIMP-1*, and *TIMP-3* expressed in normal or arthritic paws of each KO mouse. mRNAs from paws of wild-type ($n = 3$), MMP-2 KO ($n = 3$), MMP-9 KO ($n = 3$), and double KO ($n = 4$) mice with (□) or without (■) arthritis (score 3) were prepared, and the copy numbers of each MMP or TIMP RNA were estimated by competitive RT-PCR. Data represent the mean ± SD.

(macrophage elastase), and TIMP-2 were small, and no large difference was seen between the wild-type mice and each KO mouse elucidated by RT-PCR (data not shown).

Discussion

The data presented here have important implications for the roles of MMP-2 and MMP-9 in the progression of this RA model. MMP-2 and MMP-9 were overproduced when arthritis appeared in AbIA. In MMP-2 KO mice, AbIA levels were significantly exacerbated. Generally, overproduction of MMPs in RA is believed to enhance arthritis by degrading the joint matrix because of their matrix-degrading activities (19, 20). Therefore, we confirmed that the exacerbated AbIA could be recovered by injection of wild-type fibroblasts and that the other MMPs would not be up-regulated in the KO mice. These results directly show that exacerbated AbIA in MMP-2 KO mice was caused by MMP-2 deficiency, and thus that MMP-2 plays a suppressive role in the progression of AbIA. Although MMP-2 was overproduced in the arthritic joint of AbIA, its ECM-degrading activities might have little effect on AbIA development. Because MMP-2 activity is controlled by its activation from proenzyme (1–3, 21) and endogenous inhibitors (1–3), this enzyme may function in a specific region. Recently, a number of non-matrix MMP substrates that potently influence cellular functions (13), such as cytokines (10), growth factor receptor (12), and chemokine (11), have been identified. The major role of MMP-2 in AbIA may be to degrade inflammatory factors or to activate anti-inflammatory factors, although the substrates have not been determined.

Unlike MMP-2 KO mice, MMP-9 KO mice showed reduced levels of AbIA, indicating that MMP-9 enhances arthritis in this model. Overproduction of MMP-9 has been observed in the synovial fluid of patients with various inflammatory diseases; a correlation between the increased level of MMP-9 activity in the synovial fluid of RA and the severity of the disease has been found (7, 8). MMP-9 may enhance arthritis by degrading the ECM surrounding the joint or anti-inflammatory factors or by activating inflammatory factors (22). MMP-9 may also contribute to migrations of inflammatory cells by degrading the ECM surrounding the cells because MMP-9 is mainly secreted by inflammatory cells, such as macrophages or neutrophils (7, 8).

Why do the two MMPs have such opposite roles in the progression of AbIA? As the two enzymes have very similar substrate specificities for matrix protein (1, 2), the opposite roles may be caused by the difference of non-matrix substrates. For example, MMP-9 is not able to cleave the MMP-2 cleavage sites of monocyte chemoattractant protein-3 (11) and fibroblast growth factor receptor 1 (12). Elucidation of the pathways downstream from MMP-2 and MMP-9 will be important for defining new molecular targets.

The double KO mice showed no significant difference from the wild-type mice. The results may reflect the effects of both exacerbated arthritis by MMP-2 deficiency and reduced arthritis by MMP-9 deficiency. As proteoglycan depletion was not significantly suppressed by inhibition of both enzymes, these two enzymes were not essential for the degradation of proteoglycan. Because proteoglycan degradation is mediated by many MMPs and aggrecanase (23, 24), the compensatory or redundant activities of the other MMPs or aggrecanase probably have a greater influence on proteoglycan depletion in the double KO mice.

In AbIA cartilage erosion was mild even in severely arthritic mice. Therefore, we could not elucidate the roles of MMP-2 and MMP-9 in cartilage erosion. We have been crossing these MMP KO mice with a strain that supports a more erosive arthritis model, such as collagen-induced arthritis (25).

We have shown the opposite roles of MMP-2 and MMP-9 for the progression of AbIA. If MMPs function in human RA as they do in mouse AbIA, our results point out the difficulty of using MMP inhibitors for the treatment of RA. Although inhibition of MMPs (including MMP-2) is believed to be the primary therapy for RA (20, 26), highly specific MMP-9 inhibitors would be essential for the inhibition of RA. Perhaps MMPs may act as both enhancers and suppressors in the progression of RA as discussed

above. To determine the dominant function of each MMP is important for anti-RA therapy.

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