Overexpression of Human Matrix Metalloproteinase-12 Enhances the Development of Inflammatory Arthritis in Transgenic Rabbits

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Increased proteolytic activity of matrix metalloproteinases (MMPs) may promote articular destruction such as occurs in rheumatoid arthritis and osteoarthritis. Recently, we reported that synovial tissue and fluid obtained from patients with rheumatoid arthritis contained higher activity of macrophage elastase (MMP-12). To examine the hypothesis that MMP-12 may potentially enhance the progression of arthritis, we investigated the effects of overexpression of MMP-12 on inflammatory arthritis in transgenic rabbits that express the human MMP-12 transgene in the macrophage lineage. Inflammatory arthritis was produced by articular injection of carrageenan solution and the degree of inflammatory arthritis in transgenic rabbits was compared with that in control rabbits. We found that overexpression of MMP-12 in transgenic rabbits significantly enhanced the arthritic lesions, resulting in severe synovial thickening, pannus formation, and prominent macrophage infiltration at an early stage and a marked destruction of articular cartilage associated with loss of proteoglycan at a later stage. These results demonstrate that excessive MMP-12 expression exacerbates articular connective tissue and cartilage degradation and thus plays a critical role in the development of inflammatory joint disease. (Am J Pathol 2004, 165:1375–1383)

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that possess the ability to degrade all of the extracellular matrix components associated with tissue destruction, and are believed to play important roles in many physiological and pathological processes. Among MMPs, MMP-12, also known as macrophage elastase (HME), is the predominant MMP produced by macrophages. Importantly, MMP-12 expression of macrophages is highly regulated by inflammatory mediators such as GM-CSF, MCP-1, and CD40 ligands, suggesting that in many inflammatory processes, once MMP-12 is up-regulated, there is a cascade of activation of other MMPs that leads to extracellular matrix degradation.

Rheumatoid arthritis (RA) is a chronic disease characterized by articular tissue destruction and irreversible joint damage. It is characterized by chronic inflammation of articular tissue, resulting in pain, swelling, and stiffness of joints. RA affects approximately 1% of the population worldwide and is two to three times more common in women than in men. The etiology of RA is not fully understood, but it is thought to be an autoimmune disease involving an interplay between genetic susceptibility, environmental factors, and immune responses.

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joint damage. The molecular mechanisms associated with rheumatoid tissue destruction have not been fully elucidated, but it seems that macrophage infiltration is directly involved in the severity of the articular destruction.\(^{13,14}\) Ample evidence has shown that in the RA synovial membrane, the number of macrophages rather than lymphocytes determines the outcome of joint destruction.\(^{15–17}\) A possible mechanism for this macrophage-mediated joint destruction has been proposed, and many proteolytic enzymes appear to be involved. It is generally believed that the joint destruction in RA is mediated by the concerted action of various proteinases, among which the MMPs appear to play a major role.\(^{18}\) During the past few decades, MMPs such as collagenase (MMP-1) and stromelysin (MMP-3) have been found to be elevated in the cartilage as well as in the synovial fluid of RA patients and have been considered the rate-limiting enzymes in the collagen degradation.\(^{15,16}\) More recently, MMP-9 and membrane-type MMPs have also been implicated in RA.\(^{20,21}\) suggesting that many kinds of MMPs may be associated with the destruction of cartilage and connective tissues.\(^{13}\) Because inflammatory macrophages can produce a large amount of MMPs, we postulated that it is likely that excess MMP-12 production by macrophages plays a crucial role in both the initiation and the progression of RA via a mechanism possibly similar to those responsible for atherosclerosis and aortic aneurysm formation.

Recently, our laboratory demonstrated that along with increased MMP-3 and MMP-9, there is increased MMP-12 in both synovial tissue and fluid from RA patients compared to those from patients with osteoarthritis.\(^{22}\) To examine the hypothesis that MMP-12 may act as an important mediator in the pathogenesis of RA, we used MMP-12 transgenic (Tg) rabbits that specifically overexpress human MMP-12 only in macrophages and investigated the effect of overexpressed MMP-12 on the development of experimentally induced inflammatory arthritis. To the best of our knowledge, the present study provides the first evidence showing that MMP-12 up-regulation affects the process of inflammatory arthritis. Our results not only shed fresh light on the mechanism of articular destruction but also have implications for the notion that inhibition of MMP-12 activity may be a potential therapeutic target for the treatment of joint destruction in RA.

**Materials and Methods**

**Rabbits**

Human MMP-12 Tg rabbits were produced in our laboratory as described recently.\(^{23}\) The human MMP-12 transgene was under the control of the human scavenger receptor-A enhancer/promoter, a macrophage-specific promoter,\(^{24}\) and therefore, the hMMP-12 transgene was expressed in the macrophage lineage of Tg rabbits.\(^{23}\) In this study, a founder rabbit (designated as F2) was mated with non-Tg rabbits to obtain hemizygous rabbits for the following study. A total of 18 female Tg and 18 littermates (4 to 5 months old) fed a chow diet were used for the evaluation of MMP-12 effects on the experimentally induced inflammatory arthritis. All animal experiments were performed with the approval of the Animal Research Committee of the University of Tsukuba, Tsukuba, Japan.

**Induction of Experimental Arthritis by Carrageenan Injection**

We adopted the experimentally induced arthritis model in rabbits. For this purpose, rabbits were anesthetized with sodium pentobarbital solution and the right hind knee joint was intra-articularly injected with 0.3 ml of sterile \(\lambda\)-carrageenan solution (1%) (Wako Chemicals, Osaka, Japan) as described previously.\(^{25,26}\) The left knee joint was injected with the same amount of sterile saline as a sham control. Rabbits were sacrificed at 7, 14, and 35 days after carrageenan injection and both knee joints were excised. The whole knee joints were cut sagittally into four segments. Synovial samples were collected from the joint capsule for Western blot and Northern blot analysis. The synovial samples and joints were fixed in 10% neutral buffered formalin and embedded in paraffin for histological observation. The joints were decalcified in 14% ethylenediaminetetraacetic acid before the preparation of the sections. Paraffin sections (3 \(\mu\)m thick) were stained with hematoxylin and eosin (H&E) to assess the degree of inflammation and synovial proliferation. For the evaluation of articular cartilage degradation, sections of the joints were also stained with safranin O and counterstained with fast green (see below) and Masson’s trichrome. In addition, three rabbits from each group were used for collecting the synovial fluid for Western blot and zymographic analyses.

**Quantitative Assessment of Articular Synovitis and Cartilage Degradation**

Synovitis was histologically evaluated by scoring the changes of the synovial membrane: 0, no change; 1, synovial lining cell hyperplasia; 2, villous formation (<500-\(\mu\)m projections); and 3, papillary proliferation (>500-\(\mu\)m projections) based on the grading method reported by Rooney and colleagues.\(^{27}\) Three sagittal sections from each joint (anterior and posterior synovium) were observed and the average total score and distribution of each score were calculated. Macrophage infiltration and MMP-12 expression in the synovial tissues were assessed using immunohistochemical staining (see below). The size of pannus formation within the articular space was determined using the MacSCOPE image analysis system. To evaluate the articular cartilage damage, the PG content of the articular cartilage of the noncalcified layer of both the lateral and the medial sides of the tibia and femoral condyles was measured according to the method of Mankin and colleagues\(^{28}\) and quantitated using the MacSCOPE image analysis system. Three sagittal sections per knee were measured, and the results were expressed as the mean percentage of safranin O stain-
ing area relative to the whole cartilage PG compared to the PG content of the sham-operated group, which was defined as 100%.

Immunohistochemistry

Immunohistochemical staining was performed using a labeled streptavidin biotin kit (Nichirei Co, Tokyo, Japan) according to manufacturer’s instructions. After blocking of endogenous peroxidase activity, the sections were blocked with 10% goat serum for 60 minutes at room temperature. In this study, we were specifically interested in investigating macrophage infiltration and MMP-12 expression in the lesions of arthritis. For this purpose, we used monoclonal antibodies (mAbs) against rabbit macrophages (RAM11, 1:200) from Dako Cytomation (Carpinteria, CA) and against the human MMP-12 catalytic domain (MAB917, 1:20) from R&D Systems (Minneapolis, MN). Immunostained slides for macrophage and MMP-12 intensity were quantitated using the MacSCOPE image analysis system. The percentage of the total area with positive color in each section was recorded and expressed as percent for macrophage distribution or MMP-12 expression of the whole joint cavity.

Northern Blot and Real-Time Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) Analysis

Total RNA from various tissues and isolated peritoneal and alveolar macrophages was rapidly isolated using Trizol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA). Northern blot analysis was performed using Trizol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) and used as an internal control. The bands of MMP-12 detected on X-ray film were scanned using a GS-700 imaging densitometer and the results were expressed relative to the control β-actin signals in the same samples. The expression of the hMMP-12 in lung, liver, spleen, and bone marrow in Tg rabbits was confirmed using the Quiagen OneStep RT-PCR system. MMP-12 mRNA expression levels in peritoneal and alveolar macrophages were evaluated by real-time RT-PCR (DNA Engine Opticon; MJ Research, Tokyo, Japan) using DyNAmo SYBR Green qPCR kit (Finnzymes Bioworks, Inc., Espoo, Finland) according to the manufacturer’s instructions. The primers for hMMP-12 (forward, 5′-ACA CAT TTC GCC TCT CTG CT-3′; reverse, 5′-CCT TCA GCC AGA AGA ACC TG-3′; 191 bp; nucleotides,749 to 940) were used. Rabbit endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward, 5′-GCT GAA CGG GAA ACT CAC TG-3′; reverse, 5′-CCA GCA TCG AAG GTA GAG GA-3′, 266 bp) was used as an internal control.

Western Blot Analysis

To identify MMP-12 and MMP-3 proteins in the synovium and synovial fluid, 40 mg of frozen synovium in liquid nitrogen was homogenized in ice-cold suspension buffer (10 mmol/L Tris-HCl, pH 7.6, 100 mmol/L NaCl) supplemented with a proteinase inhibitor cocktail (Sigma, St. Louis, MO). The supernatant was collected and the protein content was measured using a Bio-Rad protein assay kit. Ten-μg aliquots of the crude proteins from synovium and synovial fluid were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for Western blotting and probed with an Ab against hMMP-12 and mouse monoclonal Ab against hMMP-3 (Fuji Chemical, Toyama, Japan). The immunocomplexed proteins were identified by reaction with a horseradish peroxidase-conjugated goat antibody to rabbit IgG followed by enhanced chemiluminescent detection (Amersham, Piscataway, NJ). To quantitate the relative change of MMP-12 and MMP-3 levels, MMP-12 and MMP-3 bands were scanned using a GS-700 imaging densitometer and the results were expressed relative to the β-actin signals.

Zymography

Substrate gel zymography of the activity of MMPs of crude proteins from synovial fluid was performed using the method described previously.

Quantitative Macrophage Migration Assay

To evaluate the effect of MMP-12 on macrophage migration ability, we performed a chemotaxis assay using Biocoat cell culture inserts coated with murine laminin (Becton Dickinson Labware, Bedford, MA). Alveolar macrophages (0.5 ml) (2.5 × 10⁵ cells/ml) in 1640 medium (Invitrogen) were plated on the upper wells. The lower compartments were loaded with the same medium containing recombinant human monocyte chemotactic protein-1 (MCP-1) at 10 ng/ml (Pepro Tech, London, UK). After 48 hours of incubation (37°C, 5% CO₂), the number of macrophages having penetrated the gels was determined by counting 10 high-power fields at random from each well.

Statistical Analyses

All values were expressed as mean ± 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

Northern blot analysis of eight different tissues showed that Tg rabbits had higher MMP-12 expression in macrophages isolated from the peritoneal cavity, lung, and carrageenainduced subcutaneous granulomas than those from control rabbits (Figure 1, A and B). In the lung, liver, spleen, and bone marrow, which contain significant numbers of macrophage-lineage cells, weak expression of MMP-12 was detected after a longer exposure (data not shown) and the hMMP-12 expression was detected by RT-PCR (Figure 1C). Quantification of the MMP-12

expression levels in macrophages by real-time RT-PCR showed that there was significant increase of MMP-12 expression in both peritoneal and alveolar macrophages of Tg rabbits (Figure 1D).

Pathological Observations of Carrageenan-Induced Arthritis

Inflammatory arthritis was investigated at 7, 14, and 35 days in an attempt to evaluate the effect of overexpressed MMP-12 on the lesions from various stages. Grossly, the treated joints in both Tg and control rabbits were slightly swollen for up to 3 days (data not shown). At 7 days, the synovium was significantly thickened because of a variable amount of synovial lining cell hyperplasia and extensive inflammatory infiltration consisting mainly of macrophages, with few lymphocytes and neutrophils (Figure 2A). A cluster of focal fibrin deposition on the synovial surface was observed occasionally (data not shown). The synovium of Tg rabbits contained more macrophages associated with increased MMP-12 immunoreactive proteins (Figure 2A). Synovial thickening reached a peak at 14 days but was constantly severe in Tg rabbits. Quantification of the synovitis score revealed that the synovitis was pronounced in Tg rabbits at all stages: 1.5-fold at 7 days, 1.46-fold at 14 days, and 1.24-fold high at 35 days over control rabbits (Figure 2B). The increased synovial thickness found in carrageenan-induced arthritis was not found in saline-injected sham joints of either Tg or control rabbits (Figure 2A).
Effect of Increased MMP-12 Expression on Pannus Formation

The second major pathological features examined were those of the pannus formation in the articular cavity, which became most evident at 14 days (Figure 3A). Compared to the lesions in control rabbits, the pannus lesions in Tg rabbits were significantly larger in size and contained more macrophages accompanied by increased levels of MMP-12 proteins. Quantification of the pannus lesions revealed that there was an increase of 1.27-fold at 7 days, 1.63-fold at 14 days, and 1.17-fold at 35 days in the lesion size in Tg rabbits compared to that in control rabbits (Figure 3B). Of note, the increased synovial thickening and pannus formation found in Tg rabbits were clearly correlated with both macrophage infiltration and MMP-12 staining intensity (Figure 4).

Effect of Increased MMP-12 Expression on Cartilage Destruction

At 35 days, while the inflammatory components of both synovial thickening and pannus formation tended to regress, the lesions were characterized by cartilage destruction as shown by reduced intensity of safranin O staining (Figure 5). At this stage, we assessed the articular cartilage damage by comparing the cartilage PG depletion between the two groups. We found that PG contents as stained by safranin O were significantly reduced at 14 days and 35 days in Tg rabbits compared to control rabbits (Figure 5). Histological observation revealed that the cartilage destruction in Tg rabbits was so evident that the articular surface was rough, necrotic, or eroded accompanied by the reduction of PG and collagen (Figure 6).

Increased MMP-12 Expression in Arthritis of Tg Rabbits

To further examine whether the increased arthritic lesions in Tg rabbit joints were correlated with MMP-12 overexpression, we investigated the MMP-12 in the synovial tissue and fluid by Northern blotting, Western blotting, and zymography. Northern blot analysis of the synovial tissue detected weak expression in both groups, but the expression of MMP-12 transcripts in Tg synovial tissue was higher than that in control rabbits (data not shown). In accordance with the findings of immunohistochemical staining, Western blot analysis confirmed that at all stages of the analyses, synovial tissue as well as synovial fluid from Tg rabbits consistently contained high levels of MMP-12.
MMP-12 associated with higher levels of MMP-3, suggesting that MMP-12 may be involved in the activation of other MMPs (Figure 7A). Moreover, increased amounts of the active types of MMP-12 and MMP-3 were found in the synovial fluid of Tg rabbits (Figure 7B). Zymographic assays showed that increased MMP-12 proteins (especially at 14 days) were associated with β-casein digestion, indicating that these proteins were enzymatically functional (Figure 7C). Gelatinolytic activity of synovial fluid in Tg rabbits was also higher than that in control rabbits during the whole period of experiment.

**Macrophage Migration Assay**

To determine whether the MMP-12 transgene affects the migration and chemotaxis properties of the macrophages, we compared their capacity to invade an immobilized extracellular matrix, laminin, in vitro. Without a chemoattractant, neither Tg nor control rabbit macrophages showed migratory activity (less than five cells in the whole well, data not shown). In response to the presence of a chemoattractant, MCP-1, the number of gel-invading macrophages from Tg rabbits was 3.26-fold greater than that from control rabbits (Figure 8).

**Discussion**

In a recent study, we demonstrated that synovial membrane and fluid from patients with RA contained higher MMP-12 activity than those from patients with osteoarthritis. Although these results indicate that MMP-12 may be associated with RA, it is not clear whether MMP-12 directly participates in the pathogenesis of RA. In this study, we examined the hypothesis that increased MMP-12 potentially enhances the progress of inflammatory arthritis. We used Tg rabbits that express a human MMP-12 transgene in a macrophage-specific pattern and used the carrageenan-induced inflammatory arthritis model. This arthritis model has several advantages: it is reproducible and is rapidly induced by a single injection of a small amount of carrageenan (carrageenan poly-
saccharide), which is relatively nontoxic to the animal as a whole and thus is considered an appropriate model for elucidating the relationship between the inflammatory response and the destruction of cartilage matrix. The lesions present in this experimentally induced arthritis model possess many features that mimic those of human RA, such as synovitis, pannus formation, and degradative changes in the articular cartilage matrix associated with loss of PG. The lesions in control rabbits, the lesions in Tg rabbits showed severe synovitis, pannus formation, and cartilage destruction, suggesting that increased MMP-12 is involved in the enhancement of the lesion formation.

Several mechanisms may be operative in the enhancement of inflammatory arthritis found in MMP-12 Tg rabbits. First, in the joint lesions of Tg rabbits, there was higher expression of MMP-12 protein derived from macrophages. Increased MMP-12 enzymatic activity may directly induce the degradation of connective tissue (mainly collagen and PG) and cartilage. Although the major substrates for MMP-12 are elastin, MMP-12 also has the ability to digest other extracellular matrix compo-
ments such as type IV collagen, fibronectin, laminin, vitronectin, PGs, and chondroitin sulfate. Besides, MMP-12 can activate other MMPs such as MMP-2 and MMP-3, and consequently, exacerbate the proteolytic process. In this study, we also demonstrated that MMP-3 expression is increased in Tg rabbits (Figure 7). Therefore, it is likely that MMP-12 directly and/or indirectly (via concerted action with other MMPs) affects the processes of inflammatory arthritis. Second, there were more macrophages in the lesions of Tg rabbits than in those of control rabbits (Figure 4), which raises the possibility that increased MMP-12 from macrophages may result in the enhanced degradation of extracellular matrix surrounding the cells, thereby facilitating the migration of macrophages themselves toward chemoattractants. This notion is supported by the results of a chemotaxis assay showing that Tg rabbit macrophages had higher capacity of invading the extracellular matrix in vitro (Figure 8). A noteworthy finding in this study was that increased MMP-12 expression in the lesions was apparently associated with the increased number of macrophages and the lesion severity. In a separate study, we examined this issue using carrageenan-induced subcutaneous granuloma models in Tg rabbits and found that overexpression of MMP-12 significantly increased the size of subcutaneous granulomas.

Finally, enhanced expression and production of MMP-12 may also be mediated by local cytokines and growth factors via paracrine and/or autocrine pathways in the synovial milieu. For example, GM-CSF and MCP-1 are known to be important activators of MMP-12, whereas PPAR-γ and transforming growth factor-β along with TIMPs are able to repress the expression of MMP-12. In this regard, one can state that up-regulation of these cytokines in arthritis may subsequently elevate the expression of MMP-12 because these cytokines are ubiquitously present in RA tissue. Nevertheless, direct contacts or interactions between T lymphocytes and macrophages, which are diffusely present in the RA synovial tissue, may also markedly induce MMP-12 expression. This assumption is supported by the demonstration that CD40/CD40 ligand signaling significantly augments the expression of MMPs. It is currently unknown, however, whether all these inflammatory processes are involved in the regulation of MMP-12 expression in vivo. In future studies, we need to investigate whether blocking MMP-12 activity will ameliorate the progression of RA and whether inhibition of MMP-12 can be used as a therapeutic strategy to treat RA. In this aspect, several MMP inhibitors have been developed and it will be interesting to test the efficacy of these inhibitors.

In conclusion, the increased expression of MMP-12 in Tg rabbits dramatically exacerbated synovial hyperplasia, pannus formation, and the degeneration of articular cartilage in an experimentally induced inflammatory arthritis. These results provide evidence for a potential role of MMP-12 in the pathogenesis of RA and imply that the inhibition of MMP-12 may be potentially therapeutic for the treatment of inflammatory joint diseases.

References


