

Suppression of Hyaluronan Synthesis Alleviates Inflammatory Responses in Murine Arthritis and in Human Rheumatoid Synovial Fibroblasts

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Objective. To clarify the roles of hyaluronan (HA) in joint inflammation and the process of joint destruction, using 4-methylumbelliferone (4-MU), an inhibitor of HA synthesis, in a mouse model of collagen-induced arthritis (CIA) and in a monolayer culture of fibroblast-like synoviocytes (FLS) derived from patients with rheumatoid arthritis.

Methods. DAB/1J mice were immunized with type II collagen. The effects of 4-MU were evaluated by the physiologic arthritis score, paw swelling, the histologic arthritis score, and expression of matrix metalloproteinase 3 (MMP-3) and MMP-13 in chondrocytes and synovial tissue. In vitro, the effect of 4-MU on messenger RNA and protein expression of MMP-1 and MMP-3 was determined. The effects of 4-MU on HA deposition and on serum/medium concentrations of HA were analyzed using biotinylated HA binding protein staining and an HA binding assay, respectively.

Results. Treatment with 4-MU in mice with CIA dramatically decreased the severity of arthritis (based on the arthritis score), paw thickness, and histopathologic changes. MMP-3 and MMP-13 expression in chondrocytes and synovial cells was significantly inhibited by 4-MU in vivo. Treatment with 4-MU also inhibited MMP-1 and MMP-3 expression in tumor necrosis factor

α -stimulated FLS, in a dose-dependent manner. The 4-MU-induced decreases in the serum HA concentration in mice with CIA and in “medium” and “pericellular” HA concentrations in cultured FLS support the contention that the inhibitory mechanism of 4-MU is mediated by HA suppression.

Conclusion. Reduced disease activity induced by 4-MU in mice with CIA revealed HA to be a crucial regulator in the course of arthritis. Therefore, 4-MU is a potential therapeutic agent in arthritis, and its inhibitory mechanism is possibly mediated by suppression of HA synthesis.

Rheumatoid arthritis (RA) is a progressive, destructive, systemic autoimmune disease characterized by chronic synovial joint inflammation, including synovial hyperplasia, infiltration of inflammatory cells, fibrin deposition, and joint destruction (1). The large amounts of proinflammatory cytokines and proteases that are secreted from synovial tissue cause changes in chondrocyte metabolism and matrix degradation, which lead to cartilage destruction. Recently, increasing evidence suggests that biologic disease-modifying antirheumatic drugs exert a significant beneficial effect in patients with RA. However, these inhibitory effects are limited to a certain patient population (2).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade many components of extracellular matrices and play an important role in tissue degradation in various physiologic and pathologic conditions (3). MMP-1 and MMP-3 are produced by synovial lining cells in RA and play a major role in the cartilage destruction in RA joints (4,5). Expression of these MMPs is a good parameter with which to evaluate disease activity in humans and to assess the effects of various agents on inflammatory conditions, in both in vitro and in vivo experiments.

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Previous reports suggested that the serum hyaluronan (HA) concentration in patients with RA is significantly higher than that in healthy control subjects (6), and that the serum levels of HA correlate with disease severity (7). HA produced by synovial cells is variably synthesized by hyaluronan synthase 1 (HAS-1), HAS-2, and HAS-3 according to the circumstances, such as the presence of inflammatory cytokines (8). In contrast, HA is a major component of the extracellular matrix (ECM) of articular cartilage and is synthesized mainly by HAS-2 (9). HA, together with aggrecan, maintains the high quality of the ECM in articular cartilage, giving the tissue its unique ability to resist compression. We hypothesized that among its diverse roles, HA (particularly HA that is synthesized by synovial cells) would stimulate the inflammatory reaction in the setting of arthritis.

A previous study demonstrated the significance of the CD44-HA interaction in the inflammatory response in a model of antigen-induced arthritis. Mikecz et al (10) demonstrated that systemic treatment of arthritic mice with anti-CD44 monoclonal antibodies eradicated joint edema and suppressed the migration of inflammatory leukocytes to the synovial tissue and joint cavities, suggesting that the interaction of CD44 with HA might be responsible for several events associated with joint inflammation, such as swelling and leukocyte recruitment. However, no study has yet investigated whether inhibition of HA synthesis exerts any inhibitory effects on the inflammatory response in antigen-induced arthritis.

It was previously reported that 4-methylumbelliferone (4-MU) inhibits HA synthesis in several cell types, in a dose-dependent manner (11,12). Although the mechanism of 4-MU underlying the inhibition of HA synthesis has not been completely elucidated, a recent study showed that 4-MU inhibited HA synthesis by depletion of cellular UDP-glucuronic acid and down-regulation of *HAS2* and/or *HAS3* in tumor cells (12). This agent facilitates analysis of the effects of HA inhibition on inflammatory responses, both in vitro and in vivo.

In this study, we used a mouse model of collagen-induced arthritis (CIA) to examine whether 4-MU inhibits the inflammatory response via suppression of HA synthesis, as evaluated in vivo by the arthritis score, the histologic score, MMP expression, and determination of the serum level of HA, and, if so, whether HA inhibition by 4-MU suppresses MMP expression in RA synovial fibroblasts in vitro. Further, we used genetic manipulation of HAS to investigate whether the inhibitory effects of 4-MU are mediated by suppression of HA.

MATERIALS AND METHODS

Reagents. The 4-MU was purchased from Wako Pure Chemical Industries; recombinant human tumor necrosis factor α (TNF α) was from PeproTech; Freund's complete adjuvant (CFA) was obtained from Chondrex; and hyaluronidase, bovine type II collagen, lipopolysaccharide (LPS), biotinylated HA binding protein (HABP), and HA (Artz; molecular weight $\sim 8 \times 10^5$ Da) were from Seikagaku.

Initiation of CIA. Seven-week-old DBA/1J mice were purchased from Japan SLC. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of our Institutional Animal Ethics Committee.

The mice were immunized subcutaneously at the base of the tail with 100 μ l of bovine type II collagen (2 mg/ml) dissolved in 0.01M acetic acid emulsified 1:1 with CFA (ultimately, each mouse received 100 μ g of bovine type II collagen). The mice received a subcutaneously administered booster injection of 50 μ g of bovine type II collagen emulsified in Freund's incomplete adjuvant in the tail 21 days later.

Treatment with 4-MU. In vivo, the treatment group ($n = 25$) was subdivided into 3 groups. In each group, 4-MU was administered orally with 300 μ l of 5% arabic gum as an aqueous suspension (0.5 mg/gm body weight [$n = 5$], 1.5 mg/gm body weight [$n = 5$], or 3 mg/gm body weight [$n = 15$]) daily from day 23 (primary immunization on day 0) to day 42 (end of the experiments). This schedule was based on preliminary results showing that arthritis almost always developed by day 23 using this protocol. As a control, 15 different mice with CIA were administered 5% arabic gum orally every day. These in vivo experiments were performed 3 times independently, and the results were reproducible. In vitro, fibroblast-like synoviocytes (FLS) derived from the synovial tissue of patients with RA were cultured in monolayers, treated concurrently with 10 ng/ml of TNF α and 4-MU (0–1.5 mM), and subjected to analyses of MMP expression, HA distribution, and knock-down of HAS messenger RNA (mRNA).

Clinical evaluation of arthritis. CIA was considered to have successfully developed when swelling was observed in at least 1 digit or paw. The severity of arthritis was graded in each paw on a 0–3-point scale as follows: grade 0 = normal, grade 1 = swelling of 1 digit, grade 2 = swelling of ≥ 2 digits, grade 3 = swelling of entire paw. The cumulative score for 4 paws of each mouse was used as the arthritis score (maximum score of 12 per mouse) to represent overall disease severity and progression in the analyzed mice (13). Swelling was measured with an electric caliper placed across the footpad at the widest point. The sum of increases in the diameter of the 4 footpads was determined every other day.

Histologic analysis of knee joints. On day 42, the mice were killed under general anesthesia, by systemic perfusion of 4% paraformaldehyde. The knee joints (30 from the control group, 10 from mice receiving 0.5 mg/gm of 4-MU, 10 from mice receiving 1.5 mg/gm of 4-MU, and 30 from mice receiving 3 mg/gm of 4-MU) were dissected and subjected to histologic analysis. Histopathologic changes in the joints were scored using the parameters described in a previous report (14). All sections from each mouse were graded separately in a blinded manner on a 0–3-point scale as follows: grade 0 = normal, grade 1 = infiltration of inflammatory cells, grade 2 = synovial

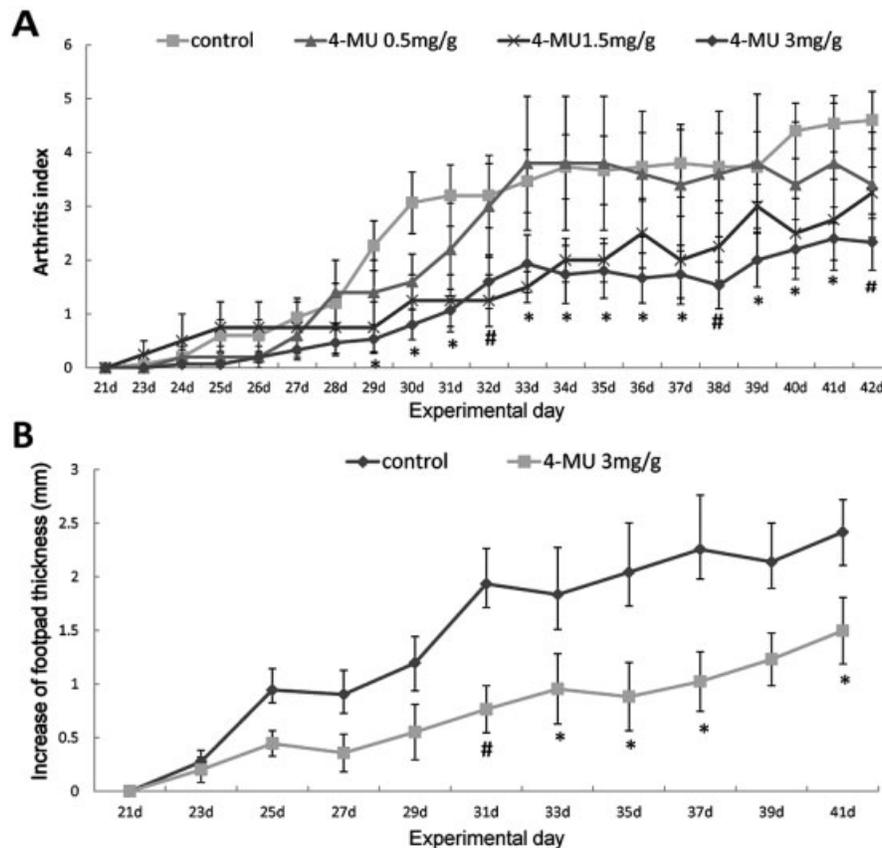


Figure 1. Effects of 4-methylumbelliferone (4-MU) on arthritis symptoms in mice with collagen-induced arthritis (CIA). **A**, Severity of arthritis symptoms as evaluated using the arthritis score in untreated (control) mice and mice treated with different concentrations of 4-MU. **B**, Total increases in footpad thickness in control mice and mice treated with 4 mg/gm 4-MU. Values are the mean \pm SEM. * = $P < 0.05$, 4-MU 3 mg/gm versus control; # = $P < 0.01$, 4-MU 3 mg/gm versus control, by Mann-Whitney U test.

hyperplasia and pannus formation, grade 3 = bone erosion and destruction. Thus, for each mouse, the maximum possible score was 6 (sum of the score for both knee joints).

Immunohistochemical analysis. The knee joint sections were also subjected to immunohistologic analysis using specific antibodies or 2.0 μ g/ml of a biotinylated HARP. The primary antibodies used were mouse anti-MMP-3 monoclonal antibody and mouse anti-MMP-13 monoclonal antibody (both 1:100 dilution; Protein Technologies). Stained sections incubated without primary antibodies were used as negative controls.

Serum HA levels in mice with CIA. To examine the serum levels of HA in each group of mice ($n = 10$), we collected blood from the hearts of the mice before they were killed. As a nonarthritis control, blood was collected from normal nonimmunized mice ($n = 10$). The concentration of HA in serum was determined using an HA binding assay, as described previously (15).

FLS culture. FLS were isolated from the synovial tissue of 5 patients with RA who were undergoing joint replacement surgery. Informed consent was obtained from all patients, and our institutional ethics committee approved this

human subject study. Tissue specimens from the patients were subjected to monolayer culture.

The FLS were placed in 6-well plates (1×10^5 cells/well), stimulated with 10 ng/ml of $\text{TNF}\alpha$, and concurrently treated with 4-MU (0–1.5 mM) or hyaluronidase (10 units/ml) for 12 hours. In addition, after the cells were treated with $\text{TNF}\alpha$ (10 ng/ml) and 1.0 mM 4-MU for 12 hours, 4-MU was washed out, and the cells were restimulated with $\text{TNF}\alpha$ for 12 hours. To investigate the direct effects of 4-MU on $\text{TNF}\alpha$ expression in FLS, the cells were stimulated with 200 ng/ml of LPS and concurrently treated with 1.0 mM 4-MU. Total RNA was extracted and subjected to real-time polymerase chain reaction (PCR) analysis to determine the mRNA expression of *MMP1*, *MMP3*, and the $\text{TNF}\alpha$ gene. The primer sequences (forward and reverse) were as follows: for MMP-1, 5'-TGG-ACCTGGAGGAAATCTTG-3' and 5'-AGTTCATGAGCT-GCAACACG-3'; for MMP-3, 5'-TTCCTTGGATTGGAGG-TGAC-3' and 5'-TGCCAGGAAAGGTTCTGAAG-3'; for $\text{TNF}\alpha$, 5'-TGTAGCCCATGTTGTAGCAAACC-3' and 5'-AATGGCGTGGAGCTGAGAGAT-3'; for GAPDH, 5'-TG-AACGGGAAGCTCACTGG-3' and 5'-TCCACCACCTG-TTGCTGTA-3'.

Western blot analysis. The effect of 4-MU on the expression levels of MMP-1 and MMP-3 protein in FLS in vitro was evaluated by Western blot analysis. FLS were treated with or without TNF α (10 ng/ml) in the presence or absence of 4-MU (0.1–1.5 mM) for 24 hours. The extracted protein (40 μ g per lane) was subjected to Western blot analysis using rabbit anti-MMP-1, rabbit anti-MMP-3, and anti- β -actin antibodies.

Small interfering RNA (siRNA) inhibition of HAS mRNA. To determine whether the effects of 4-MU are mediated by inhibition of HA synthesis, FLS were stimulated with TNF α (10 ng/ml) and treated with 4-MU after knockdown of *HAS1*, *HAS2*, or *HAS3* separately or *HAS1*, *HAS2*, and *HAS3* in combination. *MMP1* and *MMP3* mRNA expression was evaluated by real-time PCR. Small interfering RNAs specific for human *HAS1*, *HAS2*, and *HAS3* were purchased from Sigma-Aldrich. As a control siRNA, MISSION siRNA Universal Negative Control (Sigma-Aldrich) was used. FLS were transfected with siRNA using Lipofectamine 2000 (Invitrogen). During 12 hours of incubation, the efficiency of knockdown with siRNAs was confirmed using real-time PCR. The effects of 4-MU on *MMP1* and *MMP3* mRNA expression were analyzed by real-time PCR under the condition of *HAS1*, *HAS2*, or *HAS3* knockdown separately or knockdown of *HAS1*, *HAS2*, and *HAS3* in combination.

HA deposition in FLS. The FLS cells (1×10^4) were incubated in chamber slides for 6 hours and then stimulated with 10 ng/ml of TNF α and concurrently incubated with or without 1.0 mM 4-MU for 12 hours. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 2 hours and subjected to HA visualization using 2.0 μ g/ml of a biotinylated HABP probe for 2 hours at room temperature (15).

Quantification of HA. The subconfluent FLS were stimulated with 10 ng/ml of TNF α concurrently with 4-MU (0–1.5 mM) or hyaluronidase for 24 hours. HA was collected according to a previously described protocol (16). Briefly, the conditioned medium was collected and designated as the “medium” pool. To remove cell surface-associated HA, the cells were incubated for 10 minutes at 37°C with trypsin-EDTA and washed with PBS. The trypsin solution and combined washes were designated as the “pericellular” pool. After the cells were counted, they were placed in proteinase K solution (0.15M Tris HCl, pH 7.5, 0.15M NaCl, 10 mM CaCl₂, and 5 mM deferoxamine mesylate containing 20 units of proteinase K) and incubated for 2 hours at 55°C, with the solution designated as the “intracellular” pool. The HA concentrations were measured using an HA binding assay (15).

Statistical analysis. Data are expressed as the mean \pm SEM. Statistical significance was analyzed using Dunn’s test with Bonferroni adjustment and the nonparametric Mann-Whitney U test. *P* values less than 0.05 were considered significant.

RESULTS

Amelioration of the clinical symptoms of CIA by treatment with 4-MU. Previous studies using the same immunization protocol as that in this study showed that the onset of arthritis symptoms usually occurred

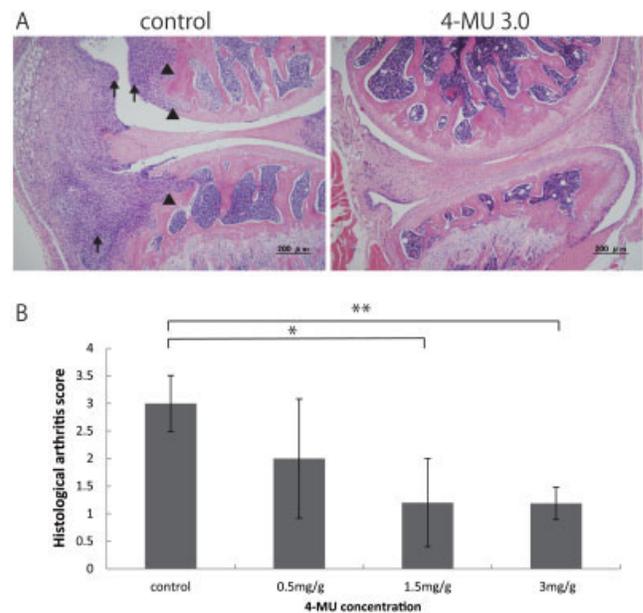


Figure 2. Histologic evaluation of arthritis in mice with CIA. The severity of arthritis was evaluated histologically in knee joint specimens obtained from control mice and mice treated with different concentrations of 4-MU. Each section was stained with hematoxylin and eosin. Serial sagittal sections (4.5 μ m) were examined, and the maximally affected section was evaluated and scored. Typically, the midline of the medial condyle was examined. **A**, Infiltration of inflammatory cells, synovial hyperplasia (arrows), cartilage destruction, and bone erosion (arrowheads) in control mice and mice treated with 3 mg/gm 4-MU. **B**, Mean \pm SEM histologic arthritis scores in control mice and mice treated with different concentrations of 4-MU. * = *P* < 0.05; ** = *P* < 0.01, by Dunn’s test with Bonferroni adjustment. See Figure 1 for definitions.

~21–28 days after the primary immunization, followed by a period of rapid disease progression (16). Because we sought to determine the effect of 4-MU in the early phase of arthritis onset, treatment of mice with 4-MU was initiated 23 days after the primary immunization. In practice, CIA developed in almost 100% of the control mice (without 4-MU treatment) by days 23–30 after primary immunization, validating the accuracy of the experiments.

Clinical arthritis was evaluated from day 21 to day 42. Oral administration of 4-MU decreased the arthritis score in a dose-dependent manner (Figure 1A). The beneficial effect was maximal in mice treated with 3 mg/gm of 4-MU. As a parameter other than the arthritis score, footpad thickness was measured to quantify the severity of joint inflammation. The increases in footpad swelling were significantly lower in mice treated with 3 mg/gm of 4-MU compared with control mice (Figure 1B). Mice treated with other concentrations of 4-MU

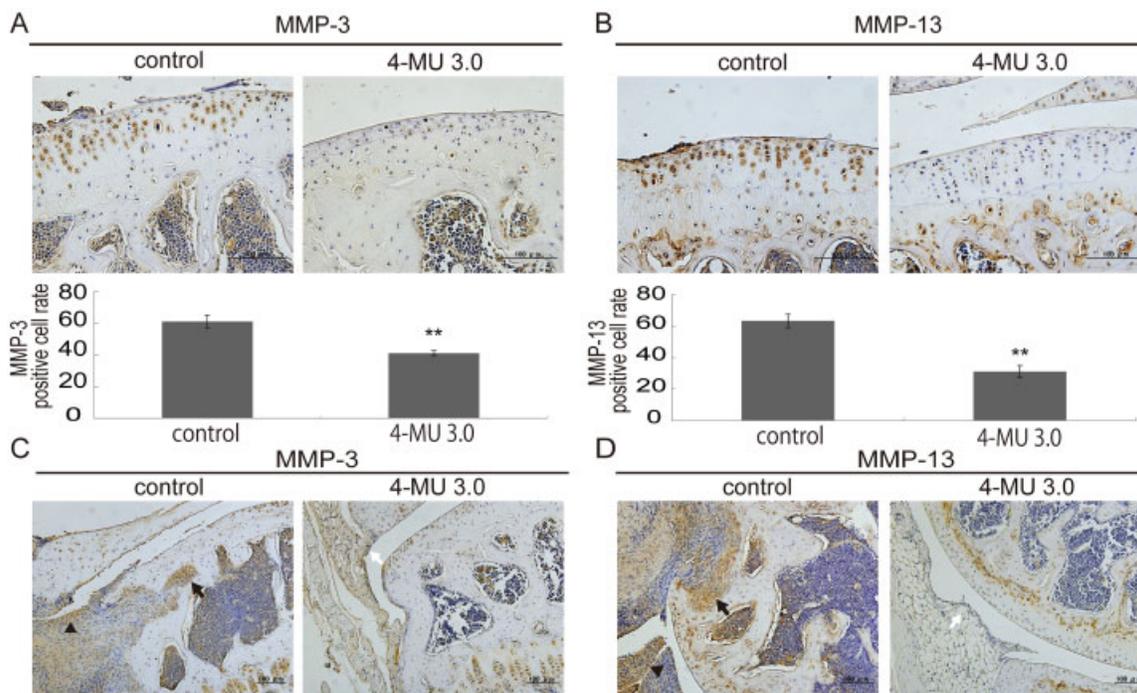


Figure 3. Immunohistochemical staining of matrix metalloproteinase 3 (MMP-3) and MMP-13 in knee joint specimens from control mice with CIA and mice with CIA treated with different concentrations of 4-MU. Immunopositive cells were counted individually in 3 different areas under a light microscope at 400 \times magnification; the number of positively stained cells was then averaged. The percentage of immunopositive chondrocytes was determined by dividing the number of positive chondrocytes by the total number of chondrocytes in all cartilage layers within the field. **A** and **B**, Increased expression of MMP-3 (**A**) and MMP-13 (**B**) in the control group compared with that in the group treated with 3 mg/gm of 4-MU. The numbers of MMP-3–positive and MMP-13–positive cells were significantly lower in the 4-MU–treated group compared with the control group. Bars show the mean \pm SEM. ** = $P < 0.01$, by Mann-Whitney U test. **C** and **D**, MMP-3 (**C**) and MMP-13 (**D**) expression at sites of synovial invasion (arrows) and in synovial tissue (arrowheads) in control mice and mice treated with 4-MU. MMP expression at sites of synovial invasion and in synovial tissue was markedly increased in control mice, while expression of MMPs in synovial tissue was suppressed in 4-MU–treated mice. See Figure 1 for other definitions.

(0.5 mg/gm and 1.5 mg/gm) did not show any significant differences in footpad thickness compared with control mice (results not shown).

All mice survived throughout the experiment. On day 42, there were no significant differences in body weight among the 4 groups of mice, and articular cartilage in the 4-MU–treated mice was microscopically normal (data not shown).

Histologic assessment of arthritis severity. Histologic signs of inflammation and/or joint destruction were observed in 63% of mice in the control group (19 of 30), 60% of mice receiving 0.5 mg/gm of 4-MU (6 of 10), 40% of mice receiving 1.5 mg/gm of 4-MU (4 of 10), and 33% of mice receiving 3 mg/gm of 4-MU (10 of 30). The knee joints of control mice showed marked pathologic changes, including synovial hyperplasia with a large number of infiltrating inflammatory cells, pannus formation, and severe cartilage and bone destruction (Figure

2A). The knee joints of mice treated with 3 mg/gm of 4-MU showed only mild synovitis, without any obvious damage to cartilage or bone. The histologic score was lower in mice treated with 4-MU than in control mice and decreased in a dose-dependent manner. Synovial inflammation was significantly less severe in the mice treated with 1.5 mg/gm or 3 mg/gm of 4-MU compared with control mice ($P < 0.05$ and $P < 0.01$, respectively) (Figure 2B).

Immunohistochemical analysis findings. The expression of MMP-3 and MMP-13 in joint cartilage was markedly increased in the control group and was suppressed by 4-MU (Figures 3A and B, top panels). The numbers of cells positive for MMP-3 and MMP-13 were significantly lower in the 4-MU group than in the control group ($P < 0.01$) (Figures 3A and B, bottom panels). Expression of MMP-3 and MMP-13 at the sites of synovial invasion and synovial tissue was also markedly

increased in control mice with CIA. In contrast, expression of MMPs in synovial tissue was suppressed in 4-MU-treated mice with CIA (Figures 3C and D).

The sites of synovial invasion into bone showed an abnormal accumulation of HA in control mice with CIA, while proliferation of HA-positive synovial tissue was absent in 4-MU-treated mice with CIA. HA positivity in synovial cells was decreased in 4-MU-treated mice with CIA and normal mice. The stainability of HA in the chondrocytes of mice with CIA receiving 4-MU treatment was similar to that in the chondrocytes of normal mice (Figure 4A).

Serum HA levels in mice with CIA. The mean \pm SEM serum levels of HA were 696.2 ± 335.2 ng/ml, $1,264.5 \pm 438.7$ ng/ml, and 539.4 ± 191.6 ng/ml in the groups of normal mice (without CIA), control mice with CIA, and mice with CIA treated with 3 mg/gm of 4-MU, respectively. The serum HA levels in 4-MU-treated mice with CIA were almost the same as those in normal mice (P not significant) but were significantly lower than those in untreated (control) mice with CIA ($P < 0.01$). The serum HA levels in untreated mice with CIA were significantly increased compared with those in normal mice ($P < 0.01$) (Figure 4B).

Effect of 4-MU on mRNA and protein expression of MMP-1 and MMP-3 in FLS. MMPs are factors in the destruction of joint cartilage and are mainly secreted by RA FLS (17). Real-time reverse transcription-PCR and Western blot analysis with densitometric evaluation revealed that treatment with 4-MU decreased the mRNA and protein expression of MMP-1 and MMP-3 in FLS in a dose-dependent manner (Figures 5A and B). However, after washout of 4-MU, the expression of *MMP1* and *MMP3* mRNA in FLS increased again to similar levels (1.37-fold and 1.12-fold, respectively) compared with those in $\text{TNF}\alpha$ -stimulated FLS without 4-MU. Hyaluronidase treatment also significantly decreased MMP mRNA expression in $\text{TNF}\alpha$ -stimulated FLS (Figure 5A). Exogenously added HA did not cancel the effects of 4-MU on MMP expression in FLS (Figure 5A). The mRNA expression of the $\text{TNF}\alpha$ gene in LPS-stimulated FLS was not decreased by 4-MU treatment (data not shown).

The efficiency of single knockdown of *HAS1*, *HAS2*, or *HAS3* was 95% for *HAS1*, 61% for *HAS2*, and 72% for *HAS3*. The efficiency of triple knockdown (*HAS1*, *HAS2*, and *HAS3*) was 98% for *HAS1*, 88% for *HAS2*, and 92% for *HAS3*. Single knockdown of *HAS1*, *HAS2*, or *HAS3* did not show similar inhibitory effects of 4-MU on *MMP1* or *MMP3* mRNA expression, whereas triple knockdown of *HAS1*, *HAS2*, and *HAS3* did (Figure

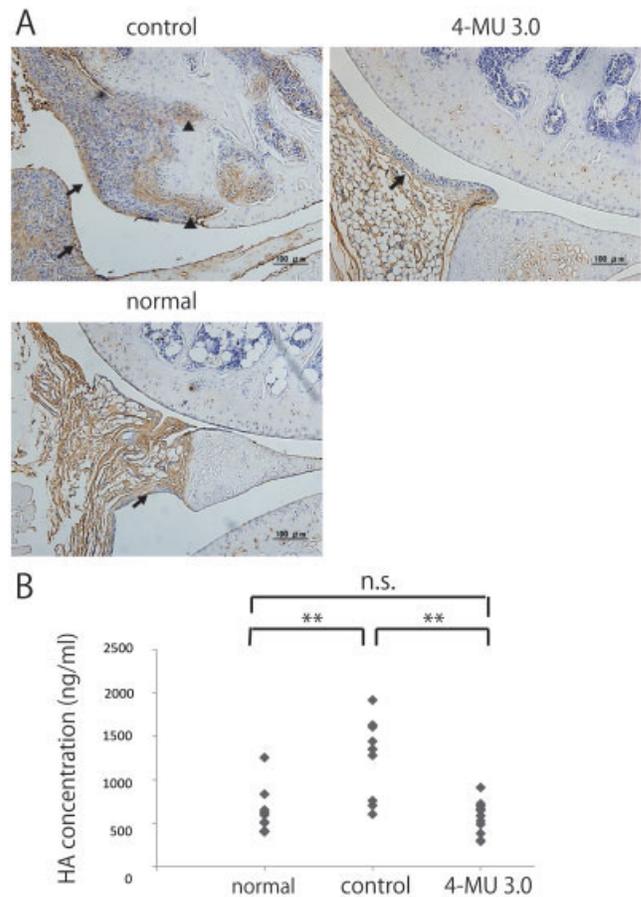


Figure 4. Hyaluronan (HA) expression in synovial tissue and serum concentration of HA in untreated mice with collagen-induced arthritis (CIA; control), mice with CIA treated with 4-methylumbelliferone (4-MU), and normal mice. **A**, HA staining of knee joint sections using a biotinylated HA binding protein. Synovial tissue, particularly in areas of invasion (**arrowheads**) and in superficial layers (**arrows**), showed strong staining. Synovial cells in 4-MU-treated mice with CIA showed weak staining (**arrow**), which was similar to that in control mice. Microscopic findings were identical in 4-MU-treated mice and control mice. **B**, Effect of 4-MU on serum HA levels in mice with CIA. ** = $P < 0.01$, by Dunn's test with Bonferroni adjustment. NS = not significant.

5C). These results suggested that the inhibitory effects of 4-MU on MMP-1 and MMP-3 expression may be mediated by an HA-dependent mechanism.

Suppression of HA accumulation in FLS by 4-MU. The cells stimulated with $\text{TNF}\alpha$ (10 ng/ml) for 12 hours displayed prominent staining for HA, particularly on the cell surface and/or the protruding portion. Staining for HA in the cells stimulated with $\text{TNF}\alpha$ was suppressed by 4-MU treatment. A region thought to be the filopodia was strongly stained with HABP in $\text{TNF}\alpha$ -

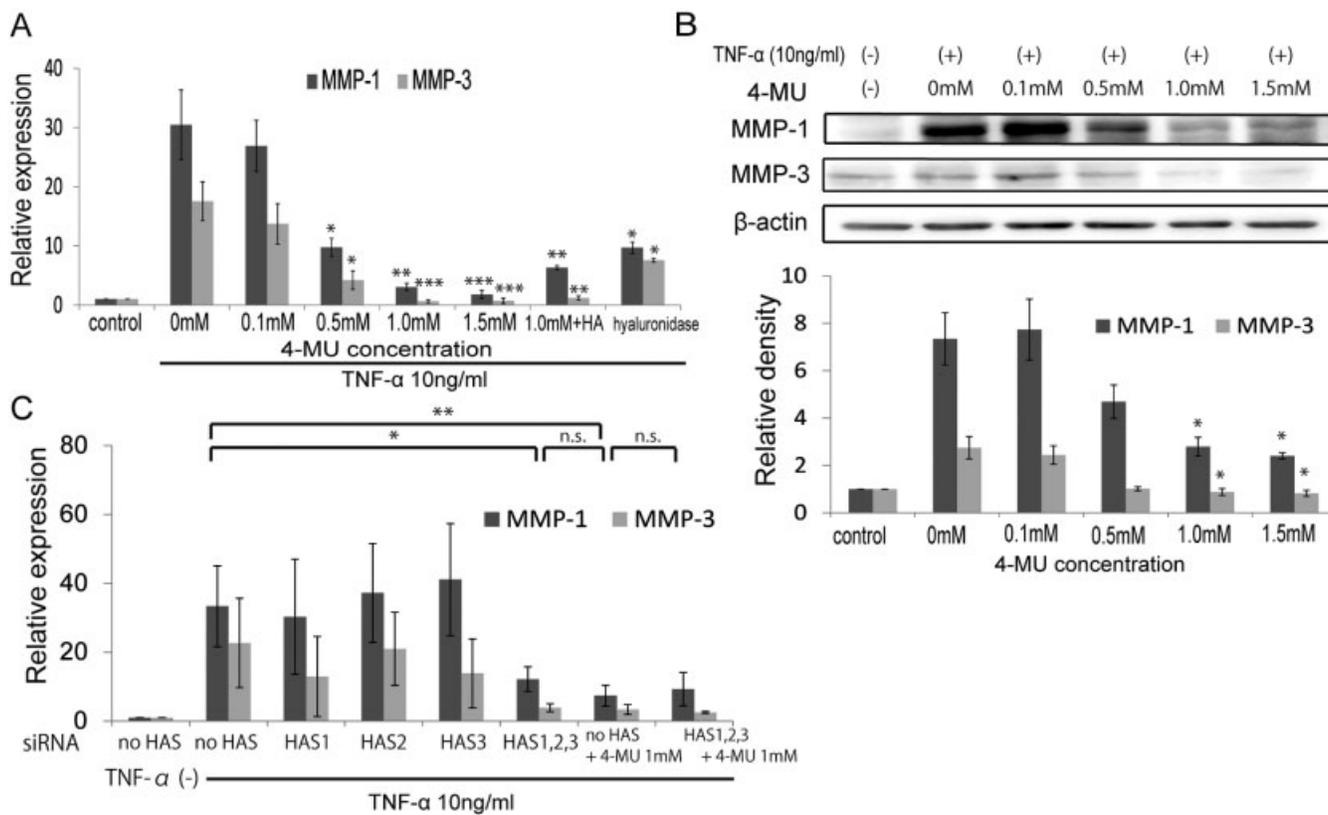


Figure 5. Effect of 4-MU on matrix metalloproteinase 1 (MMP-1) and MMP-3 expression in rheumatoid synovial cells. Fibroblast-like synoviocytes (FLS) were stimulated with tumor necrosis factor α (TNF α) and treated concurrently with 4-MU (0–1.5 mM), hyaluronidase (10 units/ml), or HA (25 μ g/ml) plus 1.0 mM 4-MU for 12 hours. **A**, Relative expression of *MMP1* and *MMP3* mRNA in rheumatoid arthritis FLS stimulated with TNF α , as determined by real-time polymerase chain reaction. Expression of *GAPDH* mRNA was used as a control. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus 4-MU 0 mM, by Dunn's test with Bonferroni adjustment. **B**, Western blot analysis for protein production of MMP-1 and MMP-3 (top) and densities relative to β -actin (bottom). **C**, Expression of *MMP1* and *MMP3* mRNA in TNF α -stimulated FLS following small interfering RNA (siRNA)-mediated knockdown of hyaluronan synthase (HAS). * = $P < 0.05$; ** = $P < 0.01$, by Dunn's test with Bonferroni adjustment. Bars show the mean \pm SEM. See Figure 4 for other definitions.

stimulated cells. Treatment with 4-MU inhibited the positivity of HA staining, particularly in this region (Figure 6A). The concentration of medium and pericellular HA of the cultured cells treated with 4-MU was significantly lower than that without 4-MU in a dose-dependent manner (Figure 6B). In contrast, the concentration of intracellular HA in the cells treated with 4-MU was not statistically different from that in cells without 4-MU treatment (data not shown), suggesting that 4-MU inhibited the deposition of HA, which is sensitive to trypsin. Trypsin-sensitive HA may be conjugated with the cell surface receptor of HA, possibly CD44. Results of TUNEL staining and water-soluble tetrazolium salt assay revealed that the effects of 4-MU on apoptotic activity and cell viability were not significant (Figures 6C and D).

DISCUSSION

In this study, we demonstrated that 4-MU, an inhibitor of HA synthesis, inhibited worsening of the clinical arthritis score and the degree of joint inflammation in mice with CIA. Treatment with 4-MU suppressed production of MMPs in mice with CIA in vivo and in RA FLS in vitro. These observations suggest that inhibition of HA synthesis may suppress the inflammatory response wherever inflammation, including synovial inflammation, is present, leading to subsequent inhibition of cartilage destruction in experimental arthritis.

The first point in the current study that needs to be elucidated is whether the inhibitory effects of 4-MU on the inflammatory response are mediated via suppression of HA synthesis or by direct effects. HA is synthe-

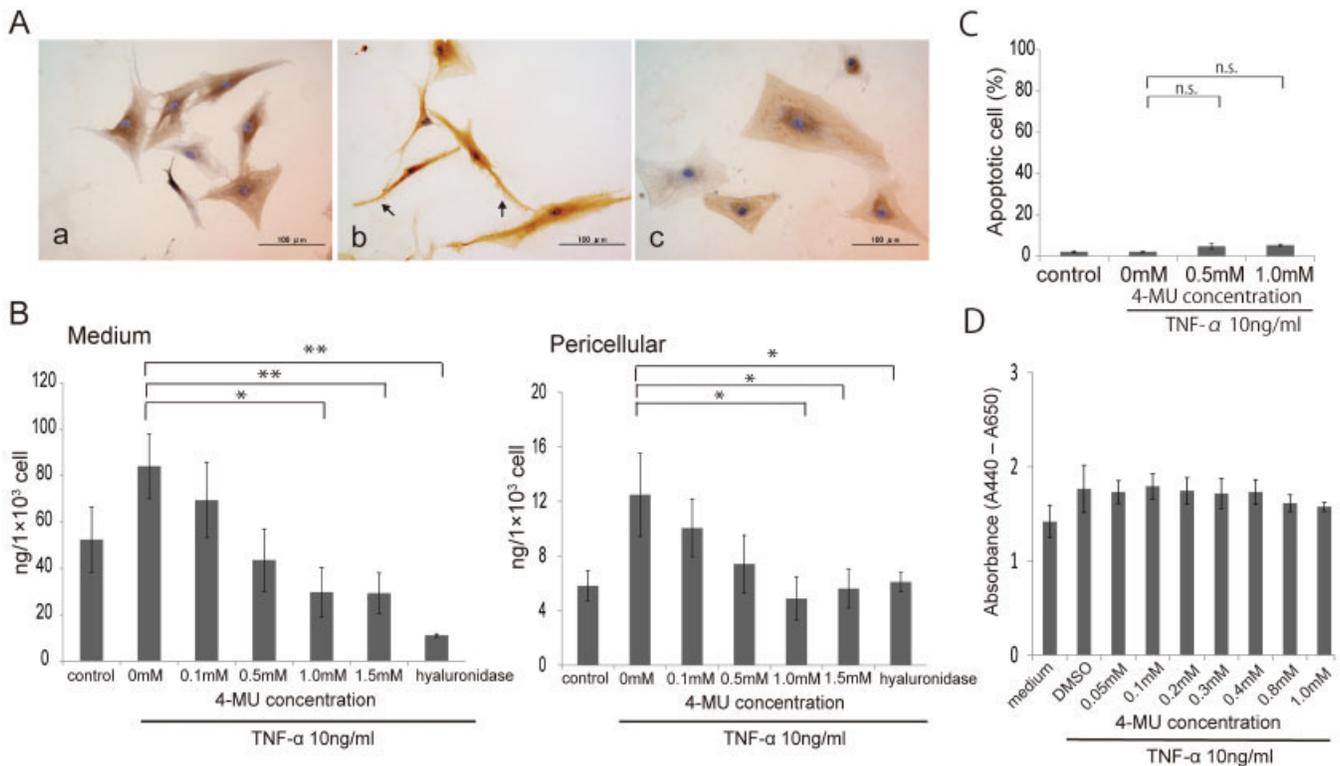


Figure 6. Alteration of HA distribution in rheumatoid arthritis fibroblast-like synoviocytes (FLS) by 4-MU. **A**, HA binding protein staining of FLS. **a**, Untreated FLS. **b**, Tumor necrosis factor α (TNF α)-treated FLS without 4-MU. **c**, TNF α -stimulated FLS with 4-MU treatment. Cells stimulated with TNF α showed prominent staining for HA, particularly on the cell surface at sites of protrusion (arrows). Positivity for HA was suppressed by 4-MU treatment, especially in the region thought to be filopodia. **B**, Medium and pericellular HA concentrations in FLS stimulated with TNF α , with or without 4-MU treatment, as measured with an HA binding assay. **C** and **D**, Effects of 4-MU on cellular apoptotic activity (**C**) and viability (**D**) in TNF α -stimulated FLS, as evaluated by TUNEL staining and water-soluble tetrazolium salt assay, respectively. The proportion of apoptotic cells was estimated by counting the number of TUNEL-positive and TUNEL-negative cells. Bars show the mean \pm SEM. * = $P < 0.05$; ** = $P < 0.01$, by Dunn's test with Bonferroni adjustment. See Figure 4 for other definitions.

sized by membrane-bound synthases on the inner surface of the plasma membrane (18). Three human HAS genes (*HAS1*, *HAS2*, and *HAS3*) were cloned and observed to synthesize HA of different molecular weights (19). In the current study, single knockdown of *HAS1*, *HAS2*, or *HAS3* by siRNA did not inhibit the expression of *MMP1* and *MMP3* mRNA, whereas triple knockdown of *HAS1*, *HAS2*, and *HAS3* significantly inhibited MMP expression (Figure 5C). These results indicate that HA synthesized by *HAS1*, *HAS2*, or *HAS3* plays pivotal roles in the development and progression of arthritis. Triple knockdown of *HAS1*, *HAS2*, and *HAS3* shows similar inhibitory effects of 4-MU on protease expression, indicating that suppression of MMP-1 and MMP-3 expression by 4-MU is possibly mediated by an HA-dependent route. This was also supported by the results showing that the expression of MMPs in TNF α -stimulated RA FLS was decreased by hyaluronidase treatment.

Another possible explanation for the inhibitory effects of 4-MU is induction of apoptotic activity in synovial cells. Recent studies suggest that a variety of cytokines such as interleukin-15, transforming growth factor β 1, and TNF α have antiapoptotic activity on the synovial cells of patients with RA (20–22). In this study, apoptotic activity in RA FLS was not altered by 4-MU treatment, and the response of FLS to TNF α stimulation was recovered after washout of 4-MU, indicating that the effects of 4-MU are unlikely to be mediated by apoptosis.

The mechanism that underlies the induction of arthritis by HA is unknown. However, previous reports have implicated the CD44–HA interaction in the inflammatory process of RA. CD44 is involved in T cell activation, monocyte cytokine release, fibroblast locomotion, and lymphocyte binding to high endothelial venules (23,24). Western blot analysis showed that the

levels of CD44 in the synovial tissue of patients with RA are higher than those in the synovial tissue of patients with osteoarthritis or joint trauma, and that the high level of CD44 is related to the degree of inflammation (25).

Previous studies in experimental models of CIA and proteoglycan-induced arthritis showed that treatment with anti-CD44 monoclonal antibodies markedly reduced the synovial inflammatory cellular response and consequent joint damage (26,27). Another study produced controversial results, showing that joint inflammation in CIA was more aggravated in CD44-knockout mice than in wild-type mice, with more HA accumulated in the absence of CD44 (28); these findings suggested that dysfunction of CD44 worsens the inflammatory response. In either case, HA accumulation plays aggravating roles in the development of arthritis. However, only a few studies have demonstrated an effective modality to suppress HA synthesis or accumulation using hyaluronidase or HAS siRNA in an arthritis model (29,30). However, manipulation of HA with hyaluronidase or HAS siRNA is difficult for clinical use due to the associated ethical issues and problems associated with drug delivery. In contrast, 4-MU is readily available for clinical use with fewer complications and has already been validated as a choleric drug.

In fact, the serum HA concentration in patients with RA was greater than that in healthy controls and was significantly decreased by treatment with corticosteroids (6). Compared with the erythrocyte sedimentation rate or the C-reactive protein level, the levels of HA in the serum of patients with RA are better correlated with clinical disease activity (31). Considering that HA is a sensitive disease marker for RA, the fact that 4-MU treatment directly reduced serum HA levels in this study may be significant in terms of devising new therapeutic strategies.

Not only the inflammatory response but also cell behavior, including proliferation and motility, were affected by HA. Several previous studies demonstrated that HA plays important roles in the invasion of various types of cells into surrounding tissue (32,33). Moreover, HA stabilizes focal adhesions, filopodia, and the proliferative phenotype in esophageal squamous carcinoma cells (34). Arthritic FLS form a tumor-like phenotype through the activation of NF- κ B (35), and RA FLS invade and attach to normal human cartilage when coengrafted into appropriate animal strains (36). These findings suggested that HA was possibly involved in the invasion of cartilage by RA FLS (so-called pannus formation or pannus invasion).

The results of the current study revealed that proliferating HA-positive synovial tissue invaded articular cartilage and bone in control mice with CIA but not in 4-MU-treated mice with CIA. Results of an in vitro study showed that HA positivity in the structures thought to be the filopodia of the cells stimulated with TNF α was suppressed by 4-MU treatment. Taken together, these findings suggest that inhibition of HA synthesis by 4-MU may inhibit the invasion of synovial tissue into the surrounding bone and cartilage. Alternatively, the addition of exogenous HA did not cancel the inhibitory effects of 4-MU on MMP expression in RA FLS, suggesting that cell-associated endogenous HA, rather than exogenous HA, may have significant roles in the pathogenesis of arthritis.

In general, inflammation and immune reactions require leukocyte adhesion and chemotaxis. Therefore, 4-MU-induced suppression of inflammation may result from repression of leukocyte adhesion and chemotaxis via inhibition of the CD44-HA interaction. In addition to its function in inflammation and immune reactions, HA forms an HA-rich pericellular matrix around synovial cells. Anti-CD44 antibody inhibits the formation of HA-rich pericellular matrix in vitro and rapidly reduces joint edema (15), which is a major symptom of patients with RA. Direct suppression of HA by 4-MU may have the potential to directly reduce joint edema via reduction of HA-bound water in the extracellular matrix.

This study has several limitations. First, the timing of 4-MU administration will have an impact on its efficacy. In the current study, 4-MU was administered early after disease onset. Whether 4-MU can control disease during the active phase of arthritis remains to be investigated. Second, the expression and roles of CD44 were not analyzed in the current study. However, previous studies have demonstrated the crucial roles of CD44 in CIA or proteoglycan-induced arthritis (26,27). Third, the optimal duration of administration and the durability of the 4-MU effects require clarification, and the occurrence of any adverse effects during long-term treatment must be documented.

In conclusion, we demonstrated that treatment with 4-MU suppressed the development of arthritis in mice with CIA and suppressed the production of MMPs in RA FLS in vitro, supporting the possibility that the inhibitory mechanism of 4-MU is mediated by suppression of HA synthesis. Given that 4-MU is a natural compound and is abundant in many edible plants, including parsley and celery (37), and, moreover, that it has already been clinically used as an oral choleric

agent in Japan, 4-MU can be readily used in patients with arthritis.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nishida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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