Hyaluronan Inhibits Matrix Metalloproteinase-1 Production by Rheumatoid Synovial Fibroblasts Stimulated by Proinflammatory Cytokines

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ABSTRACT. Objective. To study the inhibitory effects of hyaluronan (HA) on the production of matrix metalloproteinase-1 (MMP-1) by rheumatoid synovial fibroblasts (RSF) stimulated by proinflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β).

Methods. HA of various sizes at various concentrations was added to monolayer cultures of RSF in the presence of TNF-α or IL-1β, with or without pretreatment with a monoclonal antibody against CD44, OS/37. Concentrations of MMP-1 in cell lysates and conditioned media and of CD44 on RSF were assayed by immunoblotting. MMP-1 expression was analyzed by reverse transcriptase-polymerase chain reaction. Binding of HA to RSF was evaluated by confocal microscopy using fluorescein-conjugated HA and OS/37.

Results. Treatment with HA (0.3–3.0 mg/ml) resulted in a significant decrease in the production of MMP-1 induced by TNF-α and IL-1β, in a dose-dependent manner. HA of 250−2300 kDa at 3 mg/ml was found to suppress the induction of MMP-1 by TNF-α. HA decreased the cytokine-induced MMP-1 synthesis in RSF at mRNA and protein levels. The monoclonal antibody, which showed abundant expression of CD44 on RSF by immunofluorescence cytochemistry, partially blocked the binding of fluorescein-conjugated HA to RSF. Pretreatment with OS/37 reversed the inhibition of MMP-1 production in TNF-α or IL-1β-stimulated RSF caused by HA.

Conclusion. HA suppresses the production of MMP-1 by TNF-α or IL-1β-stimulated RSF. Based on data from anti-CD44 treatment, HA binding to CD44 is directly involved in the suppression of MMP-1 production. Those results provide the rationale for a therapeutic role of HA in treatment of rheumatoid joints. (J Rheumatol 2003;30:1164–72)

Key Indexing Terms: HYALURONAN MATRIX METALLOPROTEINASE-1 RHEUMATOID ARTHRITIS

Hyaluronan (HA), a large glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetylglucosamine, is one of the major components of joint fluid and connective tissues. In the joint cavity, it plays an important role in joint lubrication and protects articular cartilage from damage. While HA acts as a lubricative agent, it has direct effects on chondrocytes. HA enhances proteoglycan synthesis1-3 and suppresses matrix metalloproteinase (MMP) expression1 with enhanced production of tissue inhibitor of metalloproteinase-1 production4 in chondrocytes. It also protects chondrocytes from apoptosis5. In addition to the effects on chondrocytes, HA has anti-catabolic effects on osteoarthritic (OA) synovial cells by inhibiting the production of prostaglandin-E26, by downregulating MMP-3 and interleukin 1β (IL-1β) expression7, and by suppressing the activities of urokinase-type plasminogen activator (u-PA)8. Based on those findings, HA is now widely used for the treatment of osteoarthritic knee joints. Clinically, intraarticular injection of HA into the knee joints in patients with rheumatoid arthritis (RA) improves their symptoms9. Thus, HA may have antiinflammatory effects on rheumatoid synovial fibroblasts (RSF). Indeed, similar suppression of u-PA and IL-1β by HA has been observed in RSF8,9.

MMP can degrade essentially all components of the extracellular matrix. RSF produce massive amounts of these enzymes10. The levels of MMP are higher in RA joints compared with osteoarthritic (OA)10,11. Of MMP, MMP-1 (collagenase 1) is thought to contribute significantly to joint destruction in RA. MMP-1 degrades type I collagen in bones and ligaments, and type II collagen in articular cartilage. The invasion of cartilage by synovial pannus is a characteristic feature of RA12. Studies have shown that IL-1β-stimulated invasion of articular cartilage by RSF requires collagenase activity in vitro13. Thus, suppression of
MMP-1 production by RSF is of great concern in RA treatment. CD44 is a cell surface receptor for HA\textsuperscript{16}. CD44 is highly expressed in RA synovial cells\textsuperscript{17}. The expression of CD44 in various types of cells is upregulated by proinflammatory cytokines, e.g., IL-1\textalpha, IL-18, tumor necrosis factor-\alpha (TNF-\alpha), which are all increased in RA joints\textsuperscript{18–20}. As an adhesion molecule, CD44 contributes to the recruitment of leukocytes to the sites of inflammation\textsuperscript{21}. Through signal transduction, CD44 induces chemokine gene expression in macrophages\textsuperscript{22}, and augments IL-6 production by RSF\textsuperscript{23}. Anti-CD44 treatment using monoclonal antibodies has been reported to suppress joint swelling in a murine arthritis model induced by proteoglycan\textsuperscript{24}, and to inhibit cartilage destruction by RSF invasion \textit{in vitro}\textsuperscript{25}. These data suggest that CD44 mediates inflammatory processes and joint destruction in RA.

We investigated the inhibitory effects of HA on the production of MMP-1 by RSF stimulated with TNF-\alpha and IL-1\textbeta, which are increased in RA joints\textsuperscript{26,27}. We observed that the interaction between HA and CD44 on RSF is involved in the suppression of MMP-1 production by TNF-\alpha and IL-1\textbeta-stimulated RSF.

MATERIALS AND METHODS

\textit{Materials.} HA of 250, 500, 800, 1250, and 2300 kDa [HA(250), HA(500), HA(800), HA(1250), and HA(2300), respectively] was a gift from Seikagaku Co. (Tokyo, Japan). HA of 2000 kDa [HA(2000)] was a gift from Chugai Co. (Tokyo, Japan). OS/37, a monoclonal anti-human CD44 antibody, was obtained from Seikagaku. Fluorescent HA of 720 kDa labeled with 5-aminofluorescein and fluorescein isothiocyanate (FITC)-conjugated OS/37 were also from Seikagaku. Recombinant TNF-\alpha and IL-1\textbeta were purchased from Pepro Tech (London, UK) and R\&D Systems (Minneapolis, MN, USA), respectively.

\textit{Cell culture.} RA synovial tissues in knee joints were obtained from 6 patients with RA (5 women, one man; ages at operation 47, 52, 52, 55, 62, and 65 yrs) who fulfilled the American College of Rheumatology 1987 revised criteria at the time of total knee arthroplasty. OA synovial tissues were also obtained at the time of total knee arthroplasty from patients who were diagnosed as having OA based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA. The tissues were minced into small pieces and digested with 2 mg/ml collagenase (Wako Pure Chemical, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Grand Island, NY, USA) containing 10 mM HEPES buffer (Gibco BRL), 100 units/ml of penicillin, 100 units/ml of streptomycin (Gibco BRL), and 3.7 g/l NaHCO\textsubscript{3} (DMEM) at 37°C for 2 h. After incubation with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) for 30 min, the cell suspension was poured through nylon mesh and centrifuged for 5 min at 1500 rpm. The cell pellet was suspended in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) (ICN, Aurora, OH, USA), plated on 75 cm\textsuperscript{2} dishes (Iwaki, Minneapolis, MN, USA), and cultured at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}/95% air. At confluence after 3–7 passages in 6-well plates (Corning Ltd., Corning, NY, USA), RSF were washed with phosphate buffered saline (PBS) 3 times and cultured with TNF-\alpha (5 ng/ml) or IL-1\textbeta (2 ng/ml) in serum-free DMEM in the presence or absence of HA at 37°C for 48 or 24 h with TNF-\alpha or IL-1\textbeta, respectively. In some experiments, the cells were pretreated with OS/37 (200 µg/ml) or subclass-matched nonspecific mouse IgG\textsubscript{\textalpha} (ICN) (200 µg/ml) for 1 h before cytokine or HA treatment. The conditioned media were collected and stored at –80°C prior to assay. The cultured cells were microscopically fibroblast-like cells (data not shown).

\textit{Measurement of HA concentration in the conditioned media.} RSF were cultured 24 h in the presence or absence of 5 ng/ml TNF-\alpha or 2 ng/ml IL-1\textbeta. Each 1 ml of the conditioned media was chromatographed on PD-10 columns (Pharmacia, Uppsala, Sweden). The void volume (2.5 ml) was discarded and the fraction eluted with 3.5 ml of distilled water was collected. This fraction was concentrated by 0.2 ml with a Centrifugal Evaporator centrifuge EC-57C (Sakuma Seisakusyo Ltd., Tokyo, Japan). HPLC analysis of the unsaturated tetrasaccharide of HA (ΔTetra-HA) and the unsaturated hexa-accharide of HA (ΔHexa-HA) was performed according to the method of Takazono, et al\textsuperscript{28} and Shinmei, et al\textsuperscript{29}. The HPLC system used in this study was constructed from 2 pumps (Model 880-PU; Japan-Spectroscopic Co., Ltd., Tokyo, Japan), an auto-sampling injector (Model 851-AS; Japan-Spectroscopic), a stainless steel column packed with polyamine-bound silica (YMC gel PA-120; YMC Ltd., Kyoto, Japan), a dry reaction bath (DB-3; Shimamura Instrument Co., Tokyo, Japan), a fluoro-monitor (Model FP-920; Japan-Spectroscopic), and an integrator (Model 807-IT; Japan-Spectroscopic). The ΔTetra-HA and ΔHexa-HA in each sample were eluted with a gradient of 0–100 mM sodium sulfate for 45 min at a flow rate of 0.5 ml/min. To the eluant from the column was added 100 mM sodium tetraborate buffer (pH 9.0) containing 1% 2-cyanoacetamide, at a flow rate of 0.5 ml/min. The area of each peak corresponding to ΔTetra-HA and ΔHexa-HA was calculated by the integrator and converted to an amount of hyaluronan against the area of standard ΔTetra-HA and ΔHexa-HA (Seikagaku).

\textit{DNA assay.} After the conditioned media were collected, the cells were digested with proteinase K (0.5 mg/ml) (Wako) for 12 h at 37°C. DNA content in proteinase K digest was determined as described\textsuperscript{30}. 

\textit{Immunoblot analysis.} For the detection of MMP-1 secreted into the conditioned media, collected conditioned media were diluted twice with 2 x Laemmli sample buffer, heated at 80°C for 20 min, and separated by electrophoresis on SDS-polyacrylamide gel under reducing conditions. Proteins were transfected onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The amount of sample applied was determined based on DNA content of each sample. The membrane was blocked in PBS with 5% skim milk for 2 h at room temperature, and then incubated with 0.5 µg/ml primary antibody for human MMP-1 that reacts with bands at the proactive forms of 53 kDa and 51 kDa as well as the active forms (Sigma Chemical, St. Louis, MO, USA) for 2 h at room temperature. It was incubated with 0.5 µg/ml alkaline phosphatase conjugated anti-rabbit IgG second antibody (Southern Biotechnology, Birmingham, AL, USA) for an additional 2 h at room temperature. Detection was carried out using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate disodium (NBT-BCIP) solution. The presence of HA in the conditioned media had no significant effect on the results of immunoblotting for MMP-1 (data not shown).

\textit{Preparation of cell lysate and immunoblotting for CD44, MMP-1, and actin.} To evaluate the expression of CD44 on the cells, the cells were lysed for 30 min at 4°C in a lysis buffer containing Nonidet P-40 (1%), Tris-HCl (50 mM), NaCl (150 mM), EDTA (5 mM), aprotinin (5 µg/ml), phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 µM), NaF (10 mM), Na\textsubscript{3}VO\textsubscript{4} (2 mM), and N-ethylmaleimide (2 mM). After centrifugation, the supernatants were mixed with 2 x Laemmli sample buffer, heated at 80°C for 20 min, and were subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions, followed by transfer onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in PBS...
with 2% skim milk for 2 h, incubated with 1 µg/ml OS/37 overnight at 4°C, and then incubated with 0.5 µg/ml second antibody (goat anti-mouse IgG alkaline phosphatase-conjugated, Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. CD44 protein was visualized with NBT-BCIP. Similarly prepared cell lysates were also subjected to immunoblotting for MMP-1 and β-actin using anti-MMP-1 antibody (Sigma) and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. In some experiments, RSF were stimulated with either TNF-α (5 ng/ml) or IL-1β (2 ng/ml) for 24 h. The ionophore monensin (Sigma) at 5 µM was added to the cultures for the last 4 h to prevent the secretion of newly synthesized proteins, and the cells were lysed as described above. US National Institutes of Health image software was used to compare protein band intensities after immunoblotting.

Reverse transcriptase-polymerase chain reaction (RT-PCR). RSF cultured as described above were changed to serum-free DMEM and treated with 5 ng/ml TNF-α for 4 h. Total RNA was isolated using the reagent TRIzol (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Then 2 µg of total RNA was reverse transcribed using SuperScript™ II RT-PCR System (Invitrogen) with oligo(dT)12-18 and dNTP Mix. The resulting cDNA samples were amplified by the PCR method. PCR primers for MMP-1 were: sense, 5'-GATCATCGGGA-CAACTCTCCT-3', corresponding to positions 567–587, and anti-sense, 5'-TCCGGGTAGAAGGGATTTGTG-3', corresponding to positions 1971–1992. Amplifications were performed for 27 cycles by denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 10 min. Equal amounts of products were separated by electrophoresis on 2% agarose gels containing ethidium bromide.

Immunofluorescin cytochemistry for CD44 and evaluation of HA binding to CD44. RSF were cultured on collagen-coated cover glasses (Iwaki) in 24-well plates (Corning) with DMEM containing 10% FBS. Experiments were carried out with subconfluent cells. To detect the expression of CD44, after extensive washing with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 30 min. Blocking was performed in PBS with 3% bovine serum albumin (BSA) for 1 h at room temperature. Then the samples were incubated with FITC-conjugated nonspecific mouse IgG, (KPL, Gaithersburg, MD, USA) at 2.5 µg/ml for 12 h at 4°C. After washing, RSF were counterstained with 0.08 µg/ml propidium iodide (KPL) for 5 min. To identify the unoccupied CD44 in the presence of HA(800), RSF were incubated with 3 mg/ml HA(800) in DMEM or in DMEM containing the vehicle of HA(800) (physiologic saline containing small amount of phosphate buffer) for 1 h at 37°C, followed by addition of 2.5 µg/ml FITC-conjugated OS/37 for 1 h at 37°C. In some experiments, instead of FITC-conjugated OS/37, FITC-conjugated anti-vascular cell adhesion molecule (VCAM)-1 monoclonal mouse IgG (Santa Cruz Biotechnology) was added at 2.5 µg/ml in the presence or absence of HA. To investigate the binding of HA to the cells, RSF were incubated with 0.3 µg/ml fluorescent HA in DMEM for 30 min at 4°C, with or without pretreatment with OS/37 (200 µg/ml) for 1 h at 37°C. After propidium iodide staining, all samples were mounted on glass slides with glycerol and subjected to confocal microscopic analysis (Olympus, Tokyo, Japan).

Statistical analysis. The band intensities of protein were statistically analyzed by Wilcoxon test; p < 0.05 was considered significant.

RESULTS

Secretion of HA from cultured RSF. RSF are known to secrete HA. In addition, proinflammatory cytokines like TNF-α and IL-1β may affect the HA secretion from RSF. Thus, secreted concentrations of HA in conditioned media were measured in RSF cultures in the presence or absence of the cytokines. The secreted levels of HA from nontreated RSF for 24 h were at 1.45 ± 0.89 µg/ml (mean ± SD, n = 4). In the presence of 5 ng/ml TNF-α and 2 ng/ml IL-1β for 24 h, the secreted levels of HA from RSF were 2.71 ± 1.22 (n = 4) and 2.49 ± 0.51 (n = 4) µg/ml, respectively. Compared with the concentrations of normal synovial fluid (2–4 mg/ml) and exogenous HA used in our study (3.0 mg/ml), intrinsic HA levels were considered to be significantly low.

HA suppressed MMP-1 production by TNF-α and IL-1β-stimulated rheumatoid synovial cells. Our initial attempt was to confirm the inhibitory effect of HA on MMP-1 production by RSF stimulated with proinflammatory cytokines. When RSF were incubated with 5 ng/ml TNF-α for 48 h or IL-1β for 24 h in the presence or absence of 3.0 mg/ml HA(800), the concentration found in normal synovial fluid, treatment with HA(800) resulted in suppression of TNF-α or IL-1β-induced production of MMP-1 (Figure 1A). Immunoblot analysis for β-actin verified the equal loading of each sample based on the DNA content (Figure 1B). The presence of vehicle of HA had no effect on the cytokine-stimulated MMP-1 by RSF (data not shown).

We next investigated molecular size- and dose-dependent effects of HA on TNF-α-stimulated MMP-1 production. When RSF were incubated with various sizes of HA (250–2300 kDa) at 3.0 mg/ml in the presence of TNF-α at 5 ng/ml, HA concentration and molecular size were correlated with the suppression of MMP-1 production. The concentration of normal synovial fluid (2–4 mg/ml) and exogenous HA used in our study (3.0 mg/ml), intrinsic HA levels were considered to be significantly low.

Figure 1. Effect of hyaluronan (HA) on TNF-α and IL-1β-induced production of MMP-1 by rheumatoid synovial fibroblasts (RSF). RSF were incubated with 5 ng/ml TNF-α for 48 h or with 2 ng/ml IL-1β for 24 h in the presence or absence of 800 kDa HA at 3.0 mg/ml. Secreted levels of MMP-1 in the conditioned media were evaluated by immunoblotting using anti-MMP-1 antibody (A). To verify the equal loading of each sample, the cell lysate was subjected to immunoblotting using anti-β-actin antibody (B). The amount of sample applied was determined based on DNA content of RSF. Control cultures had no additive. Molecular standards are indicated on the left.
ng/ml, immunoblot analyses revealed that HA of 250–2300 kDa caused similar suppression of MMP-1 induced by TNF-α (Figure 2A). HA inhibited TNF-α-induced production of MMP-1 by 40–50% (Figure 2B). When HA(800) or HA(2000) was added at 0.3–3.0 mg/ml, both HA(800) (Figure 3A) and HA(2000) (data not shown) blocked the production of MMP-1 induced by 5 ng/ml TNF-α in a dose-dependent manner. No significant difference of inhibitory power was seen between HA(800) and HA(2000) treatments at the concentrations tested (data not shown). When RSF were incubated with 2.0 ng/ml IL-1β in the presence or absence of HA(800) at 0.3, 1.0, and 3.0 mg/ml, HA(800) also suppressed enhanced MMP-1 production by IL-1β-stimulated RSF in a dose-dependent manner (Figure 3B).

The decrease in cytokine-stimulated MMP-1 in response to HA could reflect decreased MMP-1 synthesis in RSF or decreased release of MMP-1 from matrix-bound stores. To determine whether HA altered MMP-1 synthesis, the enzyme concentrations in cell lysates were evaluated by immunoblotting. While treatment with 5 ng/ml TNF-α or 2 ng/ml IL-1β for 24 h resulted in an increase in MMP-1 synthesis by RSF, HA(800) at 3 mg/ml decreased the MMP-1 levels induced by TNF-α and IL-1β by approximately 80 and 50%, respectively (Figure 4A). In contrast to HA, when RSF were treated with monensin, the ionophore enhanced MMP-1 levels in cell lysates through the intracellular accumulation of newly synthesized MMP-1 (Figure 4B). Immunoblot analysis showed that 2 ng/ml IL-1β stimulated MMP-1 production more effectively than 5 ng/ml TNF-α. In monensin-treated RSF, HA also decreased MMP-1 levels enhanced by TNF-α and IL-1β by about 80 and 45%, respectively (Figure 4B). RT-PCR confirmed that HA reduced TNF-α-induced mRNA levels of MMP-1 (Figure 4C). These results indicate that HA could downregulate the cytokine-induced synthesis of MMP-1 in RSF.

Competition between HA and OS/37 for binding to CD44. Immunoblot analyses using the cultured cell lysates revealed that RSF expressed considerable levels of CD44. The levels of CD44 in RSF were almost twice as high as those in OA synovial fibroblasts. Treatment with 10 ng/ml TNF-α and 2 ng/ml IL-1β for 48 h significantly enhanced CD44 levels in RSF. However, HA itself had little effect on
CD44 levels in RSF with or without TNF-α stimulation (data not shown).

In agreement with the observations from immunoblotting and previous findings, immunofluorescein cytochemistry using FITC-conjugated OS/37 showed that RSF constitutively expressed CD44 (Figure 5A). Fluorescent HA also showed similar labeling on RSF (data not shown). When we used subclass-matched FITC-conjugated mouse IgG1 instead of FITC-conjugated OS/37, fluorescent was scarcely seen (Figure 5B). Because OS/37 is known to inhibit the binding of HA to CD44, the cells were preincubated with OS/37 before addition of fluorescent HA in order to investigate whether OS/37 competes with HA for binding to CD44 on RSF. Pretreatment with OS/37 at 200 µg/ml partially blocked the binding of fluorescent HA 0.3 µg/ml to RSF (Figure 5C). In contrast, nonspecific mouse IgG1 as a negative control caused no significant inhibition of HA binding to CD44 (Figure 5D).

Inhibition of TNF-α or IL-1β-stimulated production of MMP-1 by CD44 ligation with HA. We further investigated whether CD44 ligation with HA inhibits MMP-1 production stimulated by TNF-α at 5 ng/ml or IL-1β at 2 ng/ml. In contrast to that with nonspecific mouse IgG1, pretreatment with 200 µg/ml OS/37 cancelled the inhibitory effect of HA(800) at 3.0 mg/ml on MMP-1 production by RSF stimulated with TNF-α (Figure 6) and IL-1β (Figure 7). Preincubation with OS/37 or nonspecific mouse IgG1 itself caused no significant effect on the production of MMP-1 (data not shown). Based on the competition studies shown in Figure 5, OS/37 was considered to antagonize with HA on CD44. Therefore, these data indicate that HA suppresses the MMP-1 production in TNF-α and IL-1β-stimulated RSF by binding to CD44.

DISCUSSION

HA is widely used in the treatment of OA by intraarticular injection and has recently been employed for RA treatment. As shown here, HA levels were significantly low (~0.01 mg/ml) in RSF cultures. An increase in HA levels up to 3.0 mg/ml (the concentration found in normal synovial fluid) by exogenous addition of HA resulted in an inhibition of MMP-1 production by TNF-α and IL-1β-stimulated RSF.

Several studies have described that the levels of HA in synovial fluid from patients with RA are significantly low compared with those from healthy individuals (2~4 mg/ml). Thus, restoration of HA to normal levels in RA synovial fluid may decrease enhanced MMP-1 levels in RA joints. Since MMP are thought to play a crucial role in RA joint destruction, suppression of MMP is of special interest in RA treatment. MMP-1 acts at a committed step in the progression of RA, degrading type II collagen in cartilage because cartilage invasion by IL-1-stimulated RSF has been shown to require collagenase activity. Those enzymes are upregulated by proinflammatory cytokines like TNF-α and IL-1β.
in RA joints. Overall, our data support the clinical utility of HA for prevention of joint damage in RA through the inhibition of MMP-1 production by RSF.

The mechanism of HA action on RSF is not fully understood. In this study, OS/37, the monoclonal antibody to CD44, blocked the binding of HA to RSF (Figure 5),

Figure 5. Competition between HA and OS/37 for binding to CD44 on RSF. (A and B) After fixation with paraformaldehyde and blocking with BSA, RSF were stained by FITC-conjugated OS/37 (A) or FITC-conjugated nonspecific mouse IgG (B) at 2.5 µg/ml for 12 h at 4°C. (C and D) After preincubation with OS/37 at 200 µg/ml (C) or nonspecific mouse IgG at 200 µg/ml (D) at 37°C for 1 h, RSF were incubated with fluorescent HA labeled with 5-aminofluorescein at 0.3 µg/ml for 30 min at 4°C. (E and F) RSF were incubated with FITC-OS/37 at 2.5 µg/ml in the absence (E) or presence (F) of 800 kDa HA at 3.0 mg/ml at 37°C for 1 h. (G and H) FITC-conjugated anti-VCAM-1 antibody (2.5 µg/ml) was incubated for 1 h in the absence (G) or presence (H) of HA. Bar indicates 100 µm.
suggesting that HA binds to RSF via CD44. To investigate whether the mechanism of HA is biologically mediated by CD44 on RSF, we performed HA binding inhibition assays using the monoclonal antibody for CD44. Anti-CD44 treatment with OS/37 resulted in a significant block of the inhibitory effects of HA on MMP-1 production by TNF-α-stimulated RSF (Figures 6 and 7), indicating a direct involvement of CD44 in the mechanism of HA action. The observation that ligation of CD44 with HA failed to block the binding of anti-VCAM-1 antibody to VCAM-1 on RSF (Figure 5) suggests that the mechanism of HA action is not a barrier effect. Molecular size-independent inhibition of cytokine-stimulated MMP-1 by HA (Figure 2) also reflects a minor role of the barrier effect in HA action, because an increase in HA molecular weight leads to an increase in HA viscosity that could provide a stronger barrier effect. Since monovalent binding of OS/37 to CD44 had no effect (Figure 6), HA inhibition of cytokine-stimulated MMP-1 seems to require polyvalent ligation with CD44.

CD44 is a transmembrane glycoprotein widely distributed on T cells, granulocytes, monocytes, fibroblasts, keratinocytes, and epithelial cells. CD44 plays a major role in multiple physiological and pathological functions, including cell-cell adhesion, cell-substrate interaction, lymphocyte recruitment to inflammatory sites, and tumor metastasis. The hyaluronan receptor is overexpressed in RA synovial tissues in proportion to the severity of synovial inflammation, and is upregulated by proinflammatory cytokines like TNF-α and IL-1β as shown in this and previous studies. Recently, the function of CD44 as a signaling molecule has been described. Stimulation of CD44 transmits the signal into the cells, leading to activation of T cells and release of cytokine or chemokine from monocytes and RSF. Those findings implicate the role of CD44 in the pathogenesis of inflammation. Indeed, anti-CD44 treatment reduces joint swelling and cartilage destruction by RSF both in vivo and in vitro. Since HA caused no significant alteration in CD44 levels upregulated by TNF-α in RSF (data not shown), it is unlikely that HA exerts its effect by downregulation of CD44 levels. Although our results have clearly shown that HA downreg-
ulates cytokine-enhanced MMP-1 synthesis in RSF, intra-
cellular events after HA binding to CD44 remain to be eluci-
dated. Further, other receptors for HA such as intercellular
adhesion molecule-141 may involve HA action, because
treatment with OS/37 resulted in partial reduction of HA
binding to RSF and HA inhibition of MMP-1. In addition to
HA, ligands for CD44 include collagens, fibronectin,
 laminin, chondroitin sulfate, and osteopontin42. Thus, there
may be another mechanism of HA action that could suppress
proinflammatory cytokine-induced MMP-1 in RSF through
the interference of CD44 ligation with other ligands.

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