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Induction of CD44 and MMP Expression by Hyaluronidase Treatment of Articular Chondrocytes

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In this study, the effects of fragmentation of the glycosoaminoglycans of the cell-associated matrix by hyaluronidase (HAase) on the expression of CD44 receptor and matrix metalloproteinase (MMP) mRNAs in cultured articular chondrocytes were examined. Chondrocytes, isolated from rabbit and bovine articular cartilage, were treated with bovine testicular HAase (0–200 units/ml) in the presence or absence of an antibody for CD44. The mRNA levels of CD44, CD44 variant (CD44v), MMPs (MMP-1, -3 and -9), and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) were determined by RT-PCR. The treatment of cultured chondrocytes with HAase resulted in the production of low molecular weight fragments of hyaluronan (HA). The expression of CD44, CD44v and MMP (MMP-1, -3 and -9) mRNAs, but not TIMP-1 or TIMP-2 mRNA, was up-regulated in the cultures treated with HAase, whereas this expression was not affected by treatment with purified HA of 1.0 × 10^5 Da. Furthermore, the induction of CD44 and MMPs on treatment with HAase was suppressed by an anti-CD44 antibody. The results suggest that the fragmentation of HA may lead to cartilage destruction in terms of the enhanced expression of MMPs as well as the upregulation of CD44.

Key words: CD44, chondrocytes, hyaluronan, hyaluronidase, matrix metalloproteinase (MMP).

Abbreviations: HA, hyaluronan; HAase, hyaluronidase; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; OA, osteoarthritis; RA, rheumatoid arthritis; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; HAS3, hyaluronan synthase3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CM, conditioned medium; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; HPLC, high performance liquid chromatography; FACE, fluorophore-assisted carbohydrate electrophoresis; AMAC, 2-aminoacridone.

Cartilage consists of a few cells, chondrocytes, within an extensive extracellular matrix composed of such macromolecules as proteoglycans, glycosaminoglycans (*1*), and collagens (types II, IX, and XI) (*2*). The cartilage proteoglycan aggregates consist of a core filament of hyaluronan (HA) to which aggrecan monomers are bound (*1*). These molecules contribute to the flexibility of cartilage, and protect joint components against various mechanical forces such as compression, shearing and tensile loads. Also, HA plays some essential roles in joint movements. Due to its large size and high negative charge, HA absorbs large amounts of hydration water and functions as a cushion for the mechanical forces on articular cartilage. Furthermore, the viscosity of HA contributes to the lubrication function of the synovial fluid. In addition to these biomechanical functions, HA plays various important roles in cell adhesion, proliferation and differentiation mediated by various HA binding proteins and cell surface receptors (*3–5*). HA can form a pericellular coat around chondrocytes by binding to the cell surface receptor CD44 (*5*).

Under pathological conditions such as osteoarthritis (OA) and rheumatoid arthritis (RA), HA fragmentation is induced, which increases the amount of low molecular weight-HA in the synovial fluid (*6*). This increase in low molecular weight-HA leads to a reduction of the viscosity of the synovial fluid. Furthermore, low molecular weight-HA accelerates the inflammatory responses mainly mediated by signal transduction *via* the HA receptor CD44 (*7*).

An interesting finding is that the expression of CD44 is up-regulated in synovial fibroblasts or chondrocytes in pathological conditions (*8–10*). It was previously demonstrated that inflammatory cytokines such as IL-1β and TNF-α up-regulate the expression of CD44 (*11*, *12*). These findings suggested that CD44 is a key determinant for the pathological proces of arthritic diseases. However, the mechanism undelying CD44 up-regulation in pathological conditions has not been fully clarified.

Previous studies demonstrated that inflammatory cytokines enhance the expression of hyaluronan syn-

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thase3 (HAS3), which can synthesize lower molecular weight-HA (~3.0×10^5 Da) (*13*), and the expression of HAases (HYALs), which have the ability to degrade HA (*14*, *15*). These findings could adequately explain the increase in fragmented HA in pathological conditions. HA catabolism is mainly accomplished through internalization *via* CD44 (*16*). It is, thus, suggested that the expression of CD44 is modulated by the fragmented low molecular weight-HA, and that the catabolic action of chondrocytes is mediated by the HA-CD44 pathway, as in the case of macrophages. To substantiate the above suggestions or hypothesis, we examined the effects of fragmentation of HA by HAase treatment on the expression of CD44, its variant and matrix metalloproteinase (MMP) mRNAs in cultured articular chondrocytes.

MATERIALS AND METHODS

Cell Isolation and Culture—Rabbit chondrocyte cultures: Chondrocytes were isolated from the superficial and middle zones of the knee joint cartilage of 4-week-old male Japanese white rabbits by digestion with 0.1% trypsin/EDTA in phosphate buffererd saline (PBS, pH 7.4) and 0.15% collagenase, as described previously (*17*). The cells were seeded at 2×10^5 cells *per* 100 mm culture dish, and maintained in 10 ml of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo) containing 10% fetal bovine serum (FBS; Mitsubishi-Kasei, Tokyo) (Medium A), 32 units/ml of penicillin-G (Sigma Chemical, St. Louis, MO), and 60 μg/ml of kanamycin (Meiji-Seika, Tokyo). The cultures were incubated under an atmosphere of 5% CO_2 in a humidified incubator at 37°C. When the cultures reached confluence, the experiments were initiated.

Bovine chondrocyte cultures: Metacarpophalangeal joints from 18-month-old steers were obtained from a local slaughterhouse. Full-thickness slices of articular cartilage were dissected for sequential pronase/collagenase digestion (Calbiochem, San Diego, CA; Boehringer Mannheim, Indianapolis, IN, respectively) to liberate chondrocytes. Following isolation, the chondrocytes were plated as high-density monolayers (6.2×10^5 cells/100 mm^2) in DMEM (Life Technologies, Grand Island, NY) containing 10% FBS (Summit Biotechnology, Fort Collis, CO) (Medium A), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C under an atmosphere containing 5% CO_2 for 24 h culture prior to the start of the experiment.

Experimental Design—Both the bovine and rabbit chondrocyte cultures were treated with 10 μg/ml cycloheximide (Sigma) in Medium A for 2 h. After pretreatment with cycloheximide, the medium was changed to serum-free DMEM containing 1 μg/ml cycloheximide (Medium B), and then the cultures were treated with bovine testicular hyaluronidase (HAase) (Wako Pure Chemical Industries, Osaka, and Sigma) (0–200 units/ml) for 0–24 h. In the remaining experiments, the rabbit chondrocytes were treated with 100 units/ml of HAase in Medium B in the presence (0–7.5 μg/ml) or absence of an antibody for CD44 (clone MCA806; Cosmo Bio, Tokyo). This antibody specifically recognizes the rabbit cell surface CD44 antigen (*18*). Other rabbit chondrocytes were treated with purified HA of molecular weight 1.0×10^5 Da (Seikagaku Corporation, Tokyo) (0–100 μg/ml) for 12 h in Medium B.

High Performance Liquid Chromatography (HPLC)—HA in conditioned medium (CM) from cultured chondrocytes with or without HAase treatment was purified by a solid phase extraction method. CM (3 ml) was applied first to a column of Bond Elute SCX[R] (GL Science, Tokyo), and then to a second column of Bond Elute SAX[R] (GL Science). After washing the second column with distilled water and methanol, 3 ml hydrochloric methanol (50 mM) was finally applied to the column to elute the HA. The effluent was dried, dissolved in 0.1 M NaCl (final volume, 1 ml), and then filtered through a 0.45 μm filter (Millipore, Billerica, MA).

HPLC was performed (*19*) with a Waters 600E Multisolvent Delivery System[R] (Waters, Milford, MA). 25 μl purified HA with a molecular weight of 1.0×10^5 Da (2 mg/ml., Seikagaku Corporation) or the same volume of a prepared sample was applied to the analytical column (OHpak SB-804HQ, 300 mm × 8.0 mm I.D; Showa Denko, Tokyo), which can detect polymers with molecular weights equal to or less than 1.0×10^5 Da. The column was eluted with 0.1 M NaCl buffer at the flow-rate of 1.0 ml/min, with back pressure values of 240–260 psi. The column effluent was monitored with an RI Detector (RI504R; GL Science).

FACE (fluorophore-assisted carbohydrate electrophoresis) Analysis—For detection of fragmentation of HA, chondrocyte cultures were incubated in PBS or DMEM containing HAase (100 units/ml) for 12 h. Aliquots (50 μl) were incubated with 10 units/ml chondroitinase ABC (Seikagaku Corporation) for 4 h at 37°C. The resulting samples were boiled for 10 min and then clarified by centrifugation. The liquid samples were concentrated to dryness with a speed vac, and them resuspended in 40 μl 12.5 mM 2-aminoacridone (AMAC) (Molecular Probes, Eugene, OR), dissolved in 85% dimethylsulfoxide/15% glacial acetic acid, and incubated for 15 min at room temperature. Next, 40 μl of 1.25 M sodium cyanoborohydride in ultrapure water was added and the samples were incubated overnight at 37°C for fluorophore conjugation (*20*). After cooling, 20 μl glycerol was added to each sample, followed by thorough vortexing. Standards (Std), *i.e.* ΔDi-hyaluronan, ΔDi-chondroitin, ΔDi-chondroitin-6-sulfate and ΔDi-chondroitin-4-sulfate (Sigma), were also conjugated with AMAC by the same procedure. 16% bis-acrylamide gels (19:1 acrylamide:bis-acrylamide; Bio-Rad, Hercules, CA) were cast in TAE (Tris-Acetate EDTA) buffer (Bio-Rad). Five microliter aliquots of conjugated samples were subjected to electrophoresis at 800 V and 10°C for 90 min using 0.1 M Tris, 0.1 M borate, pH 8.5, running buffer. Following electrophoresis, the gels were visualized with a Fluor-S Imager (Bio-Rad) using the UV setting for ethidium bromide, and then the fluorescent disaccharide bands were quantified using Quantity One 4.1.1 software (Bio-Rad).

RT-PCR—Total RNA was isolated from the rabbit chondrocyte cultures according to the instructions of the manufacturer with a Total RNA Extraction Kit[R] (Pharmacia Biotech, Tokyo). The first strand cDNA was synthesized from 1 μg total RNA using a Rever-Tra Ace α[R] (Toyobo, Osaka). PCR analysis was carried out with a Gene Amp PCR System 2400[R] (Perkin-Elmer, Wellesley,

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Table 1. **The nucleotide sequences of primers for RT-PCR.**

	Gene	Base pairs	Primer sequences
Rabbit			
	CD44	321	5′-AATGGTCGCTATAGCATCTC-3′
			5′-TATGGTAATTGGTCCGTCAA-3′
	CD44v	198	5′-TCTACAGATGACGACGTGAG-3′
			5′-TTCAGATCCGTGCGTGGTAT-3′
	MMP-1	537	5′-ACAGCTCCTTTGGCTTCCCT-3′
			5′-TTGAACCAGCTATTAGCTTT-3′
	MMP-3	607	5′-TCAGGATTCTCGAACCTGAG-3′
			5′-CAGTTCATGCTCGAGATTCC-3′
	MMP-9	384	5′-CGCCCAGTTTGTATCCGGCA-3′
			5′-CTAGTCCTCAGGGCAGTGCA-3′
	TIMP-1	305	5′-ACCACCTTATACCAGCGTTA-3′
			5′-AAACAGGGAAACACTGTGCA-3′
	TIMP-2	440	5′-TTCTCTGTGACCCAGTCCAT-3′
			5′-CAGTCGTTGTGGCTCTGGAG-3′
	GAPDH	613	5′-GTCAAGGCTGAGAACGGGAA-3′
			5′-GCTTCACCACCTTCTTGATG-3′
Bovine			
	CD44	221	5′-TATAACCTGCCGATATGCAGG-3′
			5′-CAGCACAGATGGAATTGGG-3′
	GAPDH	142	5′-GTCAACGGATTTGGTCGTATTGGG-3′
			5′-TGCCATGGGTGGAATCATATTGG-3′

MA) under the following conditions; denaturation at 94°C for 30 s and primer extension at 60°C for 2 min. The nucleotide sequences of the primers for CD44, CD44v (*21*), MMP-1, MMP-3, MMP-9 (*22*), and GAPDH are given in Table 1. The PCR products (16–20 cycles for GAPDH, 21–25 cycles for CD44, 21–25 cycles for CD44v, 28–30 cycles for MMP-1, 26–30 cycles for MMP-3, 25–28 cycles for MMP-9, 30 cycles for TIMP-1, and 25 cycles for TIMP-2) were separated on an agarose gel. In the experiment on the time-dependent effects of HAase on CD44 and CD44v mRNA expression, the cycle numbers for amplification were set at 25 for CD44 and 25 for CD44v to detect significant differences in the intensities of the bands between controls and treated cultures. The band densities in the gels were quantified with Scion Image Analysis software (http://www.scioncorp.com/).

Total RNA was isolated from the bovine chondrocyte cultures according to the instructions of the manufacturer with Trizol^{R} Reagent (Gibco BRL, Gaithersburg, MD). Total RNA was reverse transcribed with a GENE Amp RNA PCR kit (Roche Molecular Systems, Pleasanton, CA, USA) and a PTC-100^{TM} Programmable Thermal Controller (MJ Research, Watertown, MA).

For real time PCR, the PCR products were detected with SYBER^{R} Green Nucleic Acid Gel Stain (Molecular Probes). The primer-specific amplification (A) and quantification (Q) cycles were run at the temperatures (T) indicated as AT and QT: GAPDH, AT 57°C, QT 87°C; and CD44, AT, 60°C, QT 90°C. The QTs were set below the melting peaks of individual PCR products. The GAPDH and CD44 (*23*) primers sequences are given in Table 1. Thermal cycling and fluorescence detection were performed with SmartCycler software (Cepheid^{TM}).

The efficiency (E) of the real time PCR was calculated according to the equation proposed by Rasmussen (*24*) as $E = 10^{[-1/\text{slope}]}$ for both CD44 and GAPDH. The slope was determined from the graph of x = ng cDNA input and y = cycle number at the crossing point (CP). CP is the PCR cycle number for the peak of the 2nd derivative curve. The fold increase was calculated as a relative ratio of the target gene (*e.g.*, CD44) to GAPDH, according to the new mathematical model and equation introduced by Pfaffl (*25*, *26*).

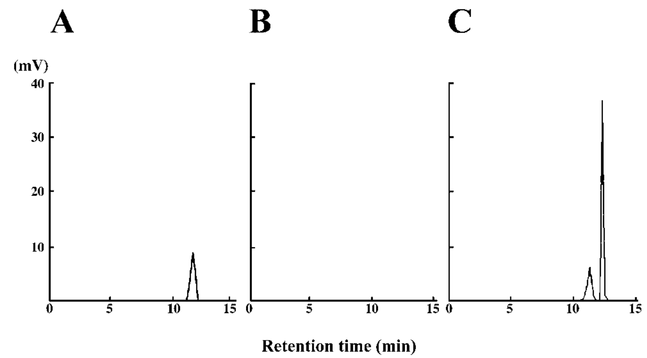

Fig. 1. **HPLC determination of the molecular mass of HA in rabbit chondrocyte cultures treated with bovine testicular HAase.** The retention time for the purified HA standard with a molecular weight of 1.0×10^5 Da was 11 min (A). There was no peak revealing low molecular weight HA of less than 1.0×10^5 Da in the media of normal chondrocyte cultures (B). A low molecular weight-HA (less than 1.0×10^5 Da) was present in the conditioned medium from chondrocyte cultures treated with 100 units/ml of HAase (C).

RESULTS

We investigated whether or not treatment with exogenous HAase causes the degradation of HA in cultured chondrocytes, by HPLC determination of the molecular weight of HA in the CM of chondrocyte cultures treated with bovine testicular HAase. The retention time for the standard purified HA of 1.0×10^5 Da was 11 min (Fig. 1A), and the peaks corresponding to HA of 1.0×10^5 Da and less than 1.0×10^5 Da were detected when the CM of cultures treated with 100 units/ml HAase for 12 h was subjected to HPLC (Fig. 1C). There was no peak showing the existence of low molecular weight-HA in the CM of control cultures without HAase treatment (Fig. 1B).

We also investigated whether or not treatment with exogenous HAase causes the degradation of HA in cultured chondrocytes by FACE analysis. Enzyme digestion in PBS (Fig. 2) and DMEM (data not shown) gave similar profiles, except for the extremely intense glucose band for the DMEM samples. Fragmented oligosaccharides were observed in the samples following HAase treatment (Fig. 2, lane 3), as compared with in the case of control cultures without HAase treatment (Fig. 2, lane 1). These lower molecular weight fragments were sensitive to chondroitinase ABC digestion, as revealed by the increased density of the ΔdiHA band (Fig. 2, lanes 2 and 4). The band intensity following both HAase and chondroitinase treatment was increased 1.3-fold (lane 4) as compared to in the case of control samples treated with only chondroitinase (lane 2).

To determine the effects of HAase treatment on the gene expression of CD44 and its variant, CD44v, the mRNA levels were determined using the total RNA from

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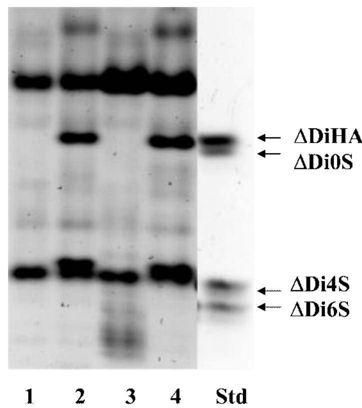

Fig. 2. **FACE analysis for detection of fragmentation of HA.** Bovine chondrocytes were cultured in PBS (lanes 1 and 2) or PBS containing 100 units/ml HAase (lanes 3 and 4) for 12 h. HAase-fragmented oligosaccharides were detected in the samples following HAase treatment (lane 3, lower bands) as compared with samples from untreated chondrocyte cultures (lane 1). Aliquots were treated with chondroitinase ABC (lanes 2 and 4), to generate disaccharides. The oligosaccharides generated with HAase (lane 3) were sensitive to chondroitinase ABC digestion, as revealed by the increased density of the ΔDiHA band (lane 4). Std, AMAC-conjugated ΔDi-hyaluronan (ΔDiHA), ΔDi-chondroitin (ΔDi0S), ΔDi-chondroitin-4-sulfate (ΔDi4S), and ΔDi-chondroitin-6-sulfate (ΔDi6S) standards.

cultured chondrocytes treated with bovine testicular HAase (0–200 units/ml) for 0–24 h. For both rabbit and bovine chondrocytes, dose- and time-dependent responses to HAase treatment resulting in markedly enhanced expression of CD44 mRNA were observed. For rabbit chondrocytes, the major effects of HAase on the induction of the mRNAs of CD44 and CD44v were observed with doses >50 units/ml (Fig. 3A). The induction of CD44 and CD44v mRNAs by HAase was observed at 6 h, and became prominent at 12 h and 24 h, respectively (Fig. 3B). In another experiment, the expression of the CD44 and CD44v mRNAs was almost invariable irrespective of the dose (0–100 μg/ml) of purified HA with a molecular weight of 1.0×10^5 Da (data not shown).

For bovine chondrocytes, the effect of HAase on the induction of CD44 mRNA was dose-dependent and was observed to increase with increasing concentration from 10 to 200 units/ml (Fig. 4A). On real-time RT-PCR, shifts in the curve revealed upregulation of CD44 mRNA after HAase treatment for 6 h, 12 h and 24 h; a typical pattern, as seen at 12 h, is shown in Fig. 4B. Calculations revealed a >4-fold increase in CD44 mRNA at 12 h following HAase treatment as compared to the control, with moderately enhanced CD44 mRNA expression with a treatment time of 6 h or 24 h (Fig. 4C).

The effects of HAase treatment on the expression of matrix metalloproteinase (MMP-1, MMP-3 and MMP-9) and tissue inhibitor of metalloproteinases (TIMP-1 and TIMP-2) mRNAs was examined under the same conditions as described above. 12 h-treatment with HAase resulted in enhanced expression of MMP-1, MMP-3 and MMP-9 mRNA in cultured rabbit chondrocytes, whereas the mRNA levels of TIMP-1 and TIMP-2 revealed almost unchanged (Fig. 5).

The induction of the CD44 and CD44v mRNAs by treatment with HAase was decreased by an anti-CD44 antibody; 2.5 or 5 μg/ml antibody completely inhibited the induction of the CD44 and CD44v mRNAs in rabbit chondrocytes (Fig. 6A). When the ratio of CD44/GAPDH or CD44v/GAPDH under the control conditions was set as one, it can be seen that the antibody treatment reduced the expression of CD44 or CD44v mRNA and abrogated the induction by HAase (Fig. 6B).

The induction of MMP mRNA expression by HAase (100 units/ml) was also inhibited by the antibody for CD44 (Fig. 7A). When the induced expression of MMP-1, MMP-3 or MMP-9 after HAase treatment with respect to GAPDH expression in the same cultures is set as one, it can be seen that treatment with nearly all doses of the antibody to CD44 decreased the MMP induction, but that MMP expression remained somewhat elevated after HAase treatment (Fig. 7B).

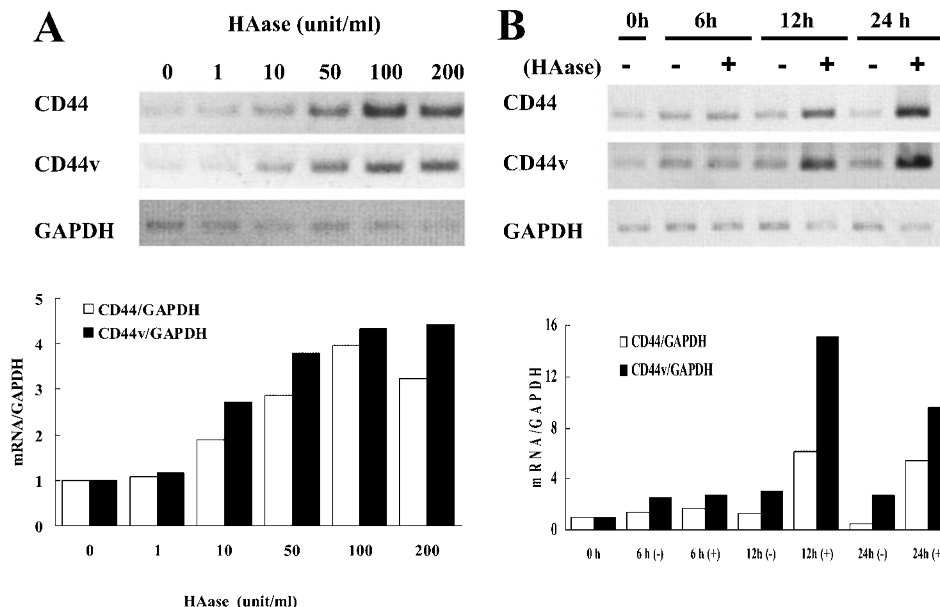

Fig. 3. **The effects of bovine testicular HAase on CD44 and CD44v gene expression in the rabbit articular chondrocytes.** (A) Rabbit chondrocytes were treated with bovine testicular HAase at 0–200 units/ml for 12 h. CD44 and CD44v gene expression was determined by means of RT-PCR. The expression of CD44 and CD44v mRNAs was enhanced in a dose-dependent manner. (B) Rabbit chondrocytes were treated for 0–24 h with HAase (100 units/ml). The induction of CD44 and CD44v mRNA expression was observed at 6 h after the HAase treatment and became prominent at 12 h after the treatment.

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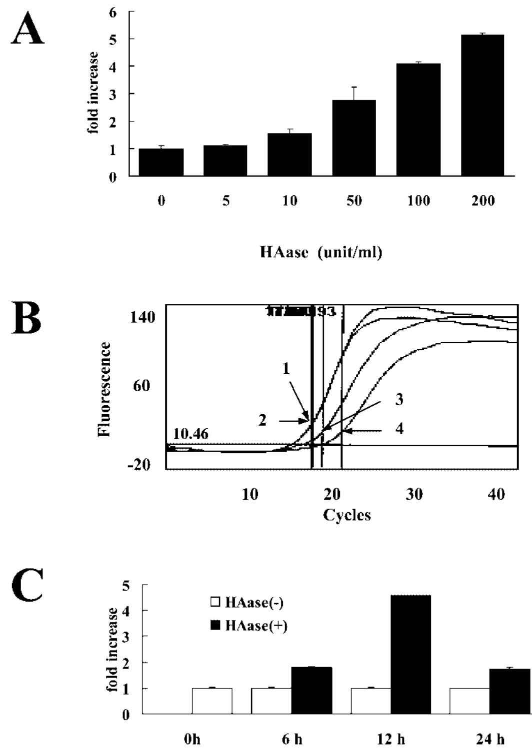

Fig. 4. **The effects of bovine testicular HAase on CD44 gene expression in bovine chondrocytes.** (A) Bovine chondrocytes were treated with HAase at 0–200 units/ml for 12 h. Real-time PCR analysis of CD44 mRNA showed dose-dependent upregulation with HAase. (B) A typical pattern on real time PCR analysis, as seen at 12 h following HAase treatment. Control bovine chondrocytes (1: GAPDH, 4:CD44) or chondrocytes treated with HAase (100 units/ml) (2:GAPDH, 3:CD44) were analyzed. CD44 gene expression was upregulated by HAase treatment. (C) Bovine chondrocytes were treated for 0–24 h with HAase (100 units/ml). The induction of CD44 mRNA expression was observed at 6 h after the HAase treatment and became prominent at 12 h. The data are presented as means ± standard deviation for two different experiments.

DISCUSSION

HA is a glycosaminoglycan that is distributed widely in various tissues and has various functions in the body. HA with a high molecular weight is prominently distributed in the articular cartilage and synovial fluid, and plays crucial roles in buffering mechanical stress and lubrication in the synovial joints. The large size and high negative charge of HA contribute to its physiological features. In addition to its biomechanical functions, HA has important biological functions in both physiological and pathological conditions. Prominent cell-associated matrices are visualized around chondrocytes with a particle exclusion assay. These chondrocyte pericellular matrices can be removed by treatment with a dilute solution of *Streptomyces* or testicular HAase, leading to the hypothesis that HA is the scaffold of the matrix to which other matrix macromolecules are bound. The pericellular matrix can also be displaced by incubation of chondrocytes with HA oligosaccharides, suggesting it is anchored to the chondrocyte cell surface via HA/HA-receptor interactions (*27*). HA has the ability to inhibit cartilage degradation by reducing the release of sulfated glycosaminoglycans from the cartilage (*28*, *29*). In pathological conditions, a reduction of the molecular size of HA is observed in the synovial fluid (*6*).

Given these considerations, in this study we treated cultured chondrocytes with HAase for topical degradation of HA. We have demonstrated that HAase treatment increases the fragmentation of HA in chondrocyte cultures. Furthermore, we have demonstrated that HAase treatment enhances the gene expression of CD44 and its variant in cultured chondrocytes. Previously, a good correlation between the levels of expression of CD44 mRNA and CD44 protein in chondrocytes was determined (*12*). In the present study, we also demonstrated that HAase treatment of cultured chondrocytes enhanced the gene expression of MMP-1, MMP-3 and MMP-9. The addition of exogenous HA of molecular weight 1.0×10^5 Da had no effect on the expression of CD44, CD44v or MMP mRNA, although it was suggested that HA fragmented by treatment with HAase did upregulate chondrocyte expression of CD44 and MMPs. Furthermore, the induction of CD44 and MMP mRNA observed in the present study was suppressed on the addition of an antibody against CD44. These results suggest that the expression of CD44 and MMPs is modulated through the activation of pathways between low molecular weight-HA and CD44.

In previous studies, exogenous HAases was found to negatively modulate the expression of CD44 in cultured skin fibroblasts and tumor cells (*30*, *31*), while topical HA treatment had no significant effect on CD44 expression in an epidermal culture system (*30*). Epithelial or dermal

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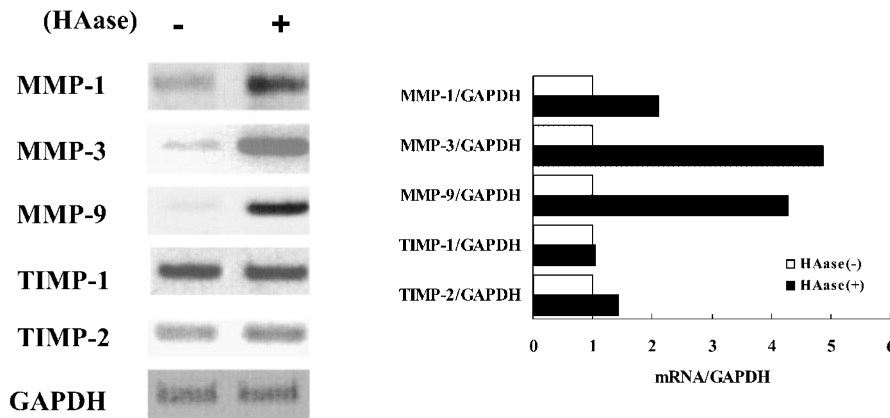

Fig. 5. **RT-PCR analysis of MMP and TIMP expression in cultured rabbit articular chondrocytes treated with bovine testicular HAase.** Rabbit chondrocytes were treated with bovine testicular HAase (100 units/ml) for 12 h. MMP (MMP-1, -3 and -9) and TIMP (TIMP-1 and -2) mRNA levels were examined by RT-PCR. The expression of MMP-1, MMP-3 and MMP-9 mRNAs, but not those of TIMPs, was markedly enhanced in the cultured chondrocytes treated with HAase.

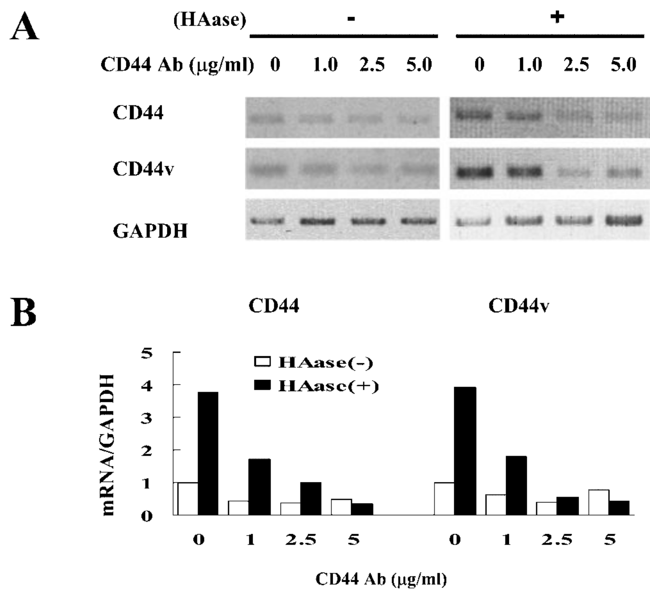

Fig. 6. **The effects of a CD44 antibody on the induction of CD44 and CD44v mRNAs by bovine testicular HAase in cultured rabbit articular chondrocytes.** (A) Control rabbit chondrocytes or rabbit chondrocytes treated for 12 h with bovine testicular HAase (100 units/ml) were incubated in the presence or absence of a CD44 antibody at the concentrations indicated. The CD44 antibody itself did not affect the basal expression levels of CD44 and CD44v mRNA. The CD44 antibody in the presence of HAase suppressed the induction of CD44 and CD44v mRNAs in a dose-dependent manner. (B) A bar graph shows the band intensities, as calculated with Scion Image, for CD44 and CD44v mRNA expression in the presence (black bars) or absence (white bars) of HAase and the CD44 antibody at the concentrations indicated.

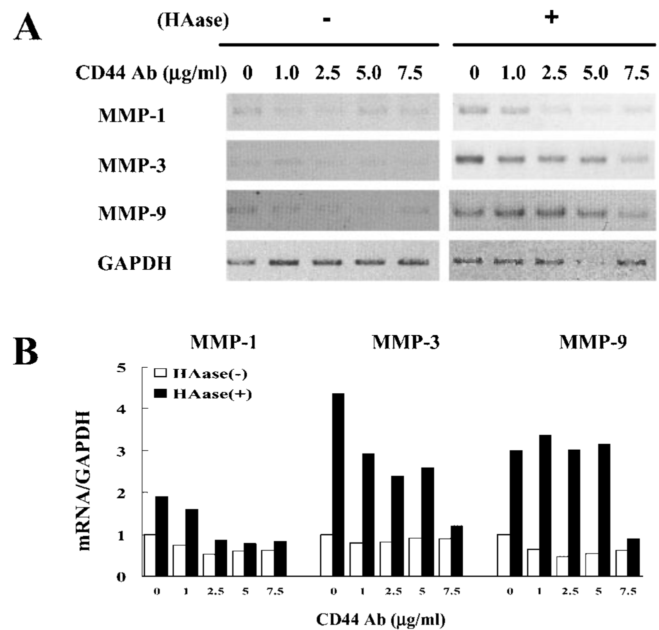

Fig. 7. **(A) The effects of the CD44 antibody on the induction of MMP mRNAs by bovine testicular HAase in cultured rabbit articular chondrocytes.** The CD44 antibody itself did not affect the basal expression levels of MMP mRNAs. The induction of MMP-1, MMP-3 and MMP-9 mRNAs was suppressed by the CD44 antibody in a dose-dependent manner. (B) A bar graph shows the band intensities, as calculated with Scion Image, for MMP-1, MMP-3 and MMP-9 mRNA expression in the presence (black bars) or absence (white bars) of HAase and the CD44 antibody at the concentrations indicated.

cells from adult skin exhibited high expression of CD44 (*32*, *33*). In addition, the expression of CD44 and its variants promoted tumor invasion and metastasis (*34–36*). These findings suggest that the response of CD44 expression to HAase treatment depends on the cell type or the basal level of CD44 expression.

The findings in the present study have shown that the induction of mRNA expression of CD44 and MMPs is inhibited by an antibody against CD44. These results suggest that the pathway between CD44 and low molecular weight-HA transmits the signals for modulation of MMP and CD44 expression. The results of the present study are also supportive of the hypothesis that HA oligosaccharides, generated through HAase treatment of chondrocytes, are active elements in the chondrocyte response. The HA oligosaccharides could either signal directly through CD44 or disrupt HA-CD44 interactions. The HAase treatment itself would also disrupt HA-CD44 interactions and the clustering of CD44 on the cell surface. The addition of the anti-CD44 antibody could either block the direct signaling by the HA oligosaccharides, or function to cluster CD44 on the membrane after the HAase treatment restoring chondrocyte homeostasis, and thus abrogating the changes in CD44 and MMP mRNA expression. Also in support of the idea of CD44 clustering is the finding that the addition of HA of 1.0×10^5 Da, which sould have the capacity to cross-bridge at least two CD44 receptors, did not alter mRNA expression for CD44 or MMPs. Disruption of the interaction of other matrix macromolecules with cells has been shown to alter cellular metabolism. In the case of integrin receptors, blocking of the adhesion of fibroblasts to fibronectin induced the expression of MMPs, including collagenase and stromelysin (*37*).

These findings support the finding that cartilage destruction is accelerated in pathologic joints with an increase in low molecular weight-HA. Several functions of low molecular weight-HA in association with inflammation have been demonstrated for various cells. Low molecular weight-HA, but not the high molecular weight form, enhances the expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in murine cortical tubular cells (*38*). The production of chemokines and biologically active IL-12 in human or murine macrophages is also promoted by this type of HA (*39*, *40*). Furthermore, low molecular weight-HA induced nitric oxide synthesis in murine macrophages, which are associated with connective tissue destruction (*41*). Thus, the pathological actions of low molecular weight-HA have been clarified mainly for macrophages. That is, the biological function of low molecular weight-HA in chondrocytes has not been examined extensively.

HA oligosaccharides induce chondrocytic chondrolysis in bovine and human cartilage slices, including elevation of MMP activity (on zymography), increased release of nitric oxide, increased NITEGE epitope staining (indicative of MMP activity that cleaves aggrecan), and promi-

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nent loss of Safranin O staining. The loss of Safranin O staining correlates with proteoglycan accumulation in the media. However, during the same time period, increased incorporation of ^{35}S-sulfate radiolabel is also observed in HA oligosaccharide-treated explants, representing new biosynthesis (*42*). Expression of HAS2 mRNA was stimulated 2.1-fold by HA_{oligos} treatment and aggrecan mRNA was stimulated 1.8-fold by HA_{oligos} treatment of human articular chondrocytes. The GAPDH mRNA copy number per μg total RNA did not change with HA_{oligos} treatment for 7 days (*43*).

The treatment of chondrocytes with hexasaccharides or anti-CD44 antibodies specifically blocked HA binding and endocytosis (*44*). Furthermore, the CD44-mediated endocytosis pathway for the degradation of HA was confirmed in terms of co-localization of internalized CD44 and fluorescein-labeled HA in intracellular vesicles (*16*). CD44 antisense transgenic mice exhibited substantial over-accumulation of HA within the superficial dermis and corneal stroma (*45*). HA accumulation was also observed in the superficial dermis of patients with lichen sclerosis et atrophicus, a human genetic disorder resulting in skin lesions, accompanied by a marked reduction in CD44 expression (*46*). Thus, it could be reasonably assumed that CD44 plays an important role in HA catabolism. In addition, the smaller HA fragments were more rapidly internalized and degraded than high molecular weight-HA (*47*). The present findings, taken together with previous observations, suggest that fragmented HA in cartilage and synovial fluid within pathologic joints may be actively catabolized by chondrocytes through endocytosis via CD44.

MMPs are capable of degrading the macromolecules of connective tissue matrices at neutral pH (*48*), and thus are regarded as major factors in the pathologic destruction of cartilage (*49–51*). In particular, an imbalance between MMPs and their inhibitors, TIMPs, is understood to be highly responsible for the pathogenic sequence of cartilage degradation (*52*). In the present study, HAase treatment had no effect on the expression of TIMPs in cultured chondrocytes, although MMP-1, MMP-3 and MMP-9 were significantly up-regulated. These MMPs are major catabolic factors that exist at high levels in the cartilage or synovial fluid within pathologic joints (*53–56*). Although in this study MMP mRNA was measured, it has been demonstrated in other systems that mRNA levels show excellent correlation with MMP enzyme activity (*57*). Among the MMPs, MMP-3 (stromelysin) can remove proteoglycan from the HA (*58*). The release of proteoglycans from HA can promote the further degradation of HA by lysosomal enzymes (*59*). Moreover, HA catabolism is an integral part of the overall mechanism of proteoglycan resorption in cartilage (*60*), and it has been demonstrated that the a decrease in the cell surface ligand results in the acceleration of CD44 internalization (*16*). These findings suggest that HA fragmentation leads to the vicious circle of CD44 expression and cartilage destruction.

In conclusion, a new finding in this study was that HAase treatment induced the expression of CD44 and MMPs on articular chondrocytes. The mechanism underlying this may involve CD44 signal transduction, either direct CD44 signaling by the HA fragments or dysregulation of HA-chondrocyte interactions, suggesting that fragmentation of HA accelerates cartilage degradation with enhanced CD44 expression and endocytosis, and up-regulation of MMPs in arthritic joints.

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