Increased Expression of CD44 in Bovine Articular Chondrocytes by Catabolic Cellular Mediators*

Geraldine Chow, Cheryl B. Knudson‡, Gene Homandberg, and Warren Knudson§

From the Departments of Biochemistry and Pathology, Rush Arthritis and Orthopedics Institute, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612

Bovine articular chondrocytes cultured in alginate beads were used to study the effect of catabolic cellular mediators on CD44 expression. Treatment with either the 29-kDa fragment of fibronectin or interleukin-1α results in a time- and dose-dependent inhibition of proteoglycan synthesis as well as a stimulation in the expression of CD44 mRNA level as determined by semiquantitative polymerase chain reaction following reverse transcription. No noticeable effect at 6 h was observed. By 24 h, the major CD44 product (CD44H) from fibronectin fragment-treated cultures showed an 8-fold increase; CD44H from interleukin-1α-treated cultures showed a 6-fold increase as compared to control cultures. In addition, a minor band, determined to be an isoform of CD44, was also shown to be up-regulated by both mediators. Stimulation of CD44 mRNA via interleukin-1α was also evident by in situ hybridization studies of bovine as well as human articular cartilage in organ culture. The increased in CD44 mRNA is matched by an increase at the protein level as determined by Western blot analysis. The Western blot reveals a doublet protein band at 80–90 kDa that corresponds to the molecular mass of CD44H. Cultures incubated with fibronectin fragments for 24 h had an 8.0-fold increase in CD44, while a 6.6-fold was observed for interleukin-1α. Flow-rescine-conjugated hyaluronan binding and internalization studies indicate that the increase in CD44 protein, induced by interleukin-1α, closely correlates with an increase in functional hyaluronan receptors present at the chondrocyte cell surface. Taken together these results indicate that conditions that up-regulate chondrocyte catabolism also up-regulate the expression of CD44, a cell surface hyaluronan receptor involved in hyaluronan endocytosis.

Articular cartilage is a specialized tissue that covers the ends of bones to provide smooth articulation of the joints during load-bearing and physical activities. It is composed of a small number of living resident cells, the chondrocytes, embedded in an extensive extracellular matrix. The chondrocytes maintain the composition of this extracellular matrix by regulation of the synthesis and degradation of the matrix components. The two major components of cartilage are type II collagen (1) and aggrecan, the cartilage-specific proteoglycan (PG) (2–4). Aggrecan molecules, often as many as 50, interact with single filaments of hyaluronan (HA) to form PG aggregates (2, 5–8) with molecular mass between 10^7 and 10^9 daltons (5–7, 9), and this interaction is further stabilized by link protein (2, 10). It is the presence of the PG aggregates within the collagenous network that gives cartilage its unique ability to resist compression. The aggrecan-rich matrix is assembled and retained at the cell surface of chondrocytes via the interaction of HA with CD44/HA receptors (11). Thus, HA plays a pivotal role in the organization and retention of aggrecan molecules within cartilage extracellular matrix. Understanding the mechanism(s) involved in HA turnover are therefore critical to a complete understanding of cartilage turnover as a whole.

Aggrecan catabolism is thought to occur extracellularly and involve the proteolytic cleavage between the G1 and G2 domain of the core protein of PG; the resulting degradation products are rapidly lost from the matrix of cartilage (12–14). Although it has been shown that the turnover of PG and HA is coordinately regulated, having similar half-lives in the range of 13–25 days, no HA degradation products could be detected either in the medium or within the extracellular matrix of the tissue (15, 16). Furthermore, no enzymes for extracellular breakdown of cartilage HA have been documented (17). However, a previous study (18) from our laboratory has demonstrated that chondrocytes do have the capacity to internalize and degrade HA to small oligosaccharides within a low pH lysosomal compartment. This endocytosis mechanism, resulting in the catabolism of HA, has also been shown to be mediated via cell surface CD44/HA receptors (18).

A variety of agents, including the inflammatory cytokine interleukin-1 (IL-1) elicit an enhanced catabolic state of cartilage tissue (14, 19–22) and have been used to study matrix turnover (19). The principal effects of IL-1 on chondrocytes are enhanced matrix degradation due to the secretion of metalloproteinases and decreased PG synthesis. This up-regulation of cartilage matrix catabolism by IL-1 closely mimics the degradation of articular cartilage seen in osteoarthritis and rheumatoid arthritis (19). Other potential cellular mediators with the capacity to modulate chondrocyte metabolism are fragments of the adhesive glycoprotein, fibronectin (FN-f). Addition of the 29-kDa FN-f to explant cultures of articular cartilage causes an increase in expression of extracellular metalloproteinases resulting in PG degradation and release from the cartilage as well as the release of chondrocyte-derived IL-1 (23, 24). Furthermore, PG synthesis is also inhibited. Similar fragments of FN have been identified in the synovial fluid of osteoarthritis.

hyaluronan; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CD44, cluster of differentiation 44; CD44v10, cluster of differentiation 44 variant 10; PCR, polymerase chain reaction; RT, reverse transcription; fl-HA, fluorescein-conjugated hyaluronan; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium.
Increased CD44 Expression in Chondrocytes

and rheumatoid arthritis patients, bathing the underlying articular cartilage with these potent mediators (25). Since HA and PG turnover are co-ordinately controlled (15, 16), it was postulated that conditions that up-regulate PG turnover would also stimulate the mechanisms involved in the turnover of HA. Furthermore, if CD44-mediated endocytosis is the primary pathway leading to the catabolism of HA, CD44 expression would be similarly regulated by these cell mediator signaling pathways.

In the present study, it was demonstrated that treatment of chondrocytes with either IL-1α or the 29-kDa FN-f results in an inhibition of PG synthesis as well as an increase in CD44 mRNA expression. Furthermore, this increase in CD44 mRNA is matched by an increase in CD44 at the protein level. Fluorescein-HA binding studies indicate that the IL-1α-induced increase in CD44 protein is closely correlated with an increase in functional HA receptors present at the chondrocyte cell surface. Thus, conditions that up-regulate chondrocyte metabolism, including the elevation of extracellular matrix-degrading enzymes, also up-regulate the expression of a cell surface HA receptor involved in HA endocytosis.

**EXPERIMENTAL PROCEDURES**

Materials—Dulbecco’s modified Eagle’s medium/Ham’s F-12 and ter-
dermal deoxyribonucleotidedtyryltransferase were obtained from Life Technolo-
gies, Inc. Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). Recombinant human interleukin-1α (IL-1α) was obtained from Genzyme (Cambridge, MA). The 29-kDa amino-terminal fragments of fibronectin were purified as described previously and shown to be free of endotoxin contamination (26). Alginate was a kind gift of Merck. Pronase and collagenase P used in dissociation of tissue were purchased from Calbiochem (San Diego, CA) and Boehr-
gringer Mannheim, respectively. GeneAmp RNA PCR kit for reverse trans-
transparent gelatinase reaction was purchased from Perkin-
Elmer. Specific primers for CD44 were custom made by Research Ge-
etics (Huntsville, AL). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were purchased from Clontech Lab Inc. (Palo Alto, CA). Restriction enzyme, ApaI was purchased from New England Biols, Inc. (Beverly, MA). A CD8 plasmid containing the human CD44E insert was a kind gift of Dr. E. Bartnik (Wiesbaden, Germany). Glycogen (20 mg/ml) was purchased from Boehringer Mannheim. Ni
trocellulose membrane for immunoblot and peroxidase-conjugated goat anti-rat IgG were purchased from Pierce. The monoclonal antibodies IM 7.8.1 and KM 201 were purified from hybridoma cultures obtained from ATCC (Rockville, MD). Rat isotype control, IgG2a, was from Pharmin-
gen (San Diego, CA). Radioactive [35S]ATP ([35S]ATP (1000 Ci/
mole) was obtained from Amersham Corp. Radioactive 5'-[γ-32P]ATP was from DuPont NEN. Agarase was from FMC BioProducts (Rockland, ME). All other enzymes and chemicals, either molecular biology grade or reagent grade materials, were purchased from Sigma.

**Tissue Culture—**Metacarpophalangeal joints from 18-month-old steers were obtained from a local slaughterhouse. Full thickness articular cartilage slices were dissected under aseptic conditions and then subjected to sequential Pronase/collagenase digestion to liberate chondrocytes from the tissue as described previously by Aydelotte et al. (20). Isolated chondrocytes were cultured in alginate beads as described by Häuselmann et al. (27). Cultures were allowed to recover for 5 days with daily medium changes. After 5 days in culture, the chondrocytes were treated with recombinant human IL-1α or 29-kDa FN-f for a period of up to 3 days.

**Reverse Transcription—**An equivalent aliquot of total bovine chon-
drocyte RNA from experimental condition was reverse transcribed (RT) at 42 °C for 30 min using CD44-specific antisense primer (0.75 μM), reverse transcriptase, Moloney murine leukemia virus (2.5 units/μl), and PCR reagents (5 μM magnesium chloride, 50 μM potassium chloride, 10 μM Tris-HCl, and 1 μM dNTP). The cDNA generated was then diluted 5-fold with sterile distilled water and subjected to either conventional PCR as described above or semi-quantitative PCR (see below).

**Semi-quantitative PCR**—An aliquot of the diluted bovine cDNA sample was used for semi-quantitative PCR whereby a constant amount of target cDNA for CD44 was co-amplified with a 10-fold serial dilutions of the purified internal standard (CD44E) and determined by densi-
tometry. The amplified products were resolved on 4% polyacrylamide gels and visualized by ethidium bromide staining. Following electrophoresis, the bands corres-
denting to the 600-bp target bovine CD44 and 872-bp CD44E were excised and quantified by scintillation counting.

**Quantitative PCR—**An aliquot of the diluted bovine cDNA sample was used for semi-quantitative PCR whereby a constant amount of target cDNA for CD44 was co-amplified with a 10-fold serial dilutions of the purified internal standard (CD44E) and determined by densi-
tometry. The amplified products were resolved on 4% polyacrylamide gels and visualized by ethidium bromide staining. Following electrophoresis, the bands corres-
denting to the 600-bp target bovine CD44 and 872-bp CD44E were excised and quantified by scintillation counting.

**Analysis of CD44 Isomorphs**—Purified isomorphs of CD44 were obtained by using the CD44A amplified products using low melting agarose gel. The bands were excised, treated with 1 unit/μl agarase for 2 h at 45 °C, and precipitated with ethanol. The purified isomorphs of CD44 were digested with ApaI (10 units/μl) in the presence of 50 μM potassium acetate, 20 μM Tris acetate, 10 μM magnesium acetate, 1 μM dithiothreitol, pH 7.9, supplemented with 100 μM acetylated bovine serum albumin for 16 h at 37 °C. The reaction was stopped by addition of a drop of the stopping solution containing 50% glycerol, 50 mM EDTA, pH 8, and 0.05% bromophenol blue. The digestion products were ana-
yzed on 4% polyacrylamide gels in 89 mM Tris, pH 8.0, 89 mM boric
acids, and 0.2 mM EDTA buffer and stained with ethidium bromide to visualize the products. Purified 754-bp CD44 was also subjected to nested PCR using an antisense primer specific for bovine variant 10 exon (sequence determined in our laboratory) in conjunction with the same sense primer. The variant-specific antisense primer sequence is as follows: 5'-AAC TTC AGT AAC TCC AAA AGA CCC-3'. The amplified nested fragment was then digested with restriction enzyme, ApaI, separated on 4% polyacrylamide gel, and stained with ethidium bromide as described above.

Quantification of CD44 Immunoreactive Protein—Protein Extraction—Following various culture conditions, chondrocytes were released from alginate beads using sodium citrate (27) followed by incubation with 3% activity of milk in phosphate-buffered saline, 0.05% Tween 20 for 1 h, followed by incubation with two different rat anti-mouse CD44 monoclonal antibodies; IMM 7.8.1 and KM 201, after a dilution of 1:3000. The blot was then washed extensively with several changes of wash buffer before incubation with peroxidase-conjugated goat anti-rat IgG (1:7000) for 1 h. The blot was once again washed extensively before peroxidase detection using an enhanced chemiluminescence kit. The bands imaged on the x-ray film were then quantified by densitometry. As controls, the blots were probed with either irrelevant rat isotype control IgG2b, or peroxidase-conjugated goat anti-rat IgG (secondary antibody).

In Situ Hybridization of Bovine and Human Cartilage—A 25-mer primer, identical to the antisense primer used in the RT-PCR, was radiolabeled with 32P-dCTP using terminal deoxynucleotidyltransferase (31).

Bovine articular cartilage slices from 18-month-old steer were either fixed immediately following dissection by immersing in 4% paraformaldehyde or cultured in DMEM/Ham's F-12 supplemented with 10% FBS in the absence or presence 1 ng/ml IL-1α for 48 h. Full thickness non-calcified human articular cartilage was removed and the femoral condyle ankle joint of a 54-year-old female donor obtained from the Regional Organ Bank of Illinois, and slices were treated in the same manner except that IL-1β was used in place of IL-1α (32). Following fixation in 4% paraformaldehyde, tissue slices were embedded in paraffin, sectioned, and processed according to the in situ hybridization protocol provided by Sandell et al. (31). Briefly, sections treated with acetic anhydride (0.25% in 0.1 M triethanolamine), dehydrated, delipidated, and air-dried. The 32P-labeled oligosaccharide probe was added to the hybridization buffer containing 25% deionized formamide, 10% dextran sulfate, 300 mM sodium chloride, 10 mM Tris, 1 mM EDTA, 1 X Denhardt's, 0.5 mg of yeast tRNA/ml, and 10 mM dithiothreitol. A 60-μl aliquot containing 2 pmol of probe/ml was applied to each slide. In some instances, a mixture containing equal molar concentration of labeled and unlabeled antisense probes was used in competitive assays. The slides were incubated with probe overnight in a moist chamber at 30.9 °C, washed with four changes of 1 X standard saline citrate for 30 min each time at 47 °C, followed by two 90-min washes with water. The slides were dehydrated through a graded series of alcohols containing 300 mM ammonium acetate, dipped in NTB2 emulsion (Kodak) diluted with 600 mg ammonium acetate, exposed for 4 days, and developed in D-19 developer (Kodak). Sections were counterstained with cresyl violet acetate and were photographed using a Nikon Microphot-FXA microscope. Sections were also viewed under the electron microscope whereby the hybridized probe to mRNA within the cells was observed as grains localized over cells within lacuna.

Binding of Fluorescein-hyaluronan to Bovine Chondrocytes—Following 5 days of recovery in alginate beads, bovine chondrocytes were treated with 0.2 ng/ml IL-1α for 2 days. The chondrocytes were then digested from alginate and digested with testicular hyaluronidase (30 units/ml) for 60 min at 37 °C in media containing 10% FBS to free the cells of their extracellular matrix. The cells were then plated as nonadherent monolayer cultures (in Petri dishes) in medium containing 100 μg/ml of fluorescein-conjugated hyaluronan (F-HA), in the continued absence or presence of IL-1α for 24 h. F-HA was prepared as described previously by Hua et al. (18). The chondrocytes were detached by trituration, washed twice with phosphate-buffered saline to remove unbound F-HA, and pelleted by centrifugation. Following cell counting, duplicate samples representing whole intact cells were transferred to a fluorescence enzyme-linked immunosorbant assay plate. The remaining cells were treated with 0.25% trypsin for 20 min at 37 °C to remove all cell surface protein, and the cells were collected by centrifugation. The trypsinized cell fraction was diluted in distilled water to induce cell lysis and transferred to the enzyme-linked immunosorbent assay plate. The whole cell and cell pellet fractions from each experimental condition contained an equal number of cells. Relative fluorescence was read using a BioTek FL500 fluorescence plate reader.

RESULTS

Effects of FN-f and IL-1α on Proteoglycan Synthesis—Proteoglycan inhibition studies were performed to determine the approximate concentration range and incubation time required for the 29-kDa FN-f and IL-1α to induce a catabolic state in bovine articular chondrocytes cultured in alginate.

Bovine chondrocytes cultured in alginate beads were treated with a range of concentrations of the 29-kDa FN-f (0.01–0.5 μM) or IL-1α (0.1–5.0 ng/ml) for 3 days. Both of these mediators resulted in a dose-dependent inhibition on proteoglycan synthesis (Fig. 1A). At a concentration of 0.01 μM FN-f, an inhibition of 68% that of control was observed with maximal inhibition of 80% attained at 0.1 μM. A similar inhibition curve was observed with IL-1α cultures whereby proteoglycan synthesis was reduced by 68% at 0.1 ng/ml and peaked at 0.2 ng/ml (75%) as shown in Fig. 1B. For all subsequent experiments, maximal concentrations of either 0.1 μM FN-f or 0.2 ng/ml IL-1α were used to induce the catabolic effects.

In addition, the inhibitory effects on proteoglycan synthesis were time-dependent whereby as early as 24 h, 0.1 μM FN-f and 0.2 ng/ml of IL-1α resulted in approximately 50% inhibition of proteoglycan synthesis (Table I). This inhibition increased to 75% by 48 h, and remained relatively constant thereafter (Table I).

Effects of FN-f and IL-1α on CD44 Receptor Expression at the Message Level—Previous work carried out in our laboratory had implicated CD44 as the primary cell surface receptor responsible for receptor-mediated endocytosis of HA, resulting in its subsequent degradation within the cell. Agents such as FN-f and IL-1α, known to elevate the catabolic state of the chondrocytes, were assessed for their effect on CD44 mRNA level as determined by RT-PCR.

No noticeable effect of these two mediators on CD44 mRNA level was observed at 6 h (Fig. 2A). However, by 24 h, both FN-f and IL-1α-treated cultures showed an increase in the amount of CD44 message level as compared to the control, and this stimulation was maintained at the 48-h and 72-h time points. To ascertain that an equivalent amount of RNA was used in the amplification process, RNA from each experiment was subjected to RT-PCR using primers specific for the “housekeeping gene,” GAPDH. The level of GAPDH product remained nearly constant in the different culture conditions and during the time course of the experiment (Fig. 2B).

In addition to the major CD44 product predicted at 600 bp (CD44H, arrow b), another band at 754 bp (arrow a) was detected. The 754-bp band was also up-regulated by the presence of both mediators. The major band at 600 bp was purified, sequenced via cycle sequencing, and found to match the published sequence data for bovine CD44 (data not shown). In addition, DNA sequencing data indicate that the sequence of the 754-bp minor product is identical to the bovine CD44H with the addition of an internal stretch of DNA highly homologous with the alternatively spliced human v10 exon (33, 34). In order to confirm the identity of the 754-bp band as the alternatively...
spliced v10 isoform of CD44, restriction enzyme digestion with ApaLI, predicted to generate one cleavage within either the CD44H or CD44v10 isoforms, was performed. The two CD44 bands were separated by low melting agarose gel electrophoresis and each of the products purified. Following ApaLI restriction enzyme digestion, the 600-bp CD44 product yielded the predicted digestion products: 350-bp and 200-bp (Fig. 3A). These results are consistent with the larger CD44 product (754 bp) being an alternatively spliced v10 isoform of CD44.

The major 600-bp CD44 product was further quantified using purified CD44E as an internal standard in semi-quantitative PCR. It was first important to determine that the amplification efficiencies of the target and the internal standard were similar. To this end, equimolar quantities of target and the internal standard, CD44E were co-amplified in the presence of radiolabeled sense primer. The results shown in Fig. 4 indicate that the amplification efficiencies of the target (600 bp) and the internal standard, CD44E (872 bp) were similar at every PCR cycle. Hence, CD44E can be utilized as an internal standard in assessing the amount of CD44 message by semi-quantitative PCR.

To determine the relative increase in the amount of CD44 mRNA, aliquots of cDNA from control, FN-f, and IL-1α samples were co-amplified with serial dilutions of known amount of internal standard, CD44E. Fig. 5 shows a representation of semi-quantitative PCR carried out on 24-h samples that were co-amplified using 2-fold serial dilutions of the internal standard. The initial amounts of target and the internal standard products are equal in those reactions where the molar ratio of the scanned products are equal. Since the amount of internal standard added to the PCR reaction is known, the initial amount of target can be determined. The results from duplicate samples are that FN-f treatment resulted in an 8-fold increase in CD44E message and IL-1α treatment, a 6-fold increase.

FIG. 1. Effects of different concentrations of FN-f and IL-1α on proteoglycan synthesis. Bovine chondrocytes were treated for 3 days with different concentrations of either FN-f or 0.2 ng/ml IL-1α (Fig. 3A). These results are consistent with the larger CD44 product (754 bp) being an alternatively spliced v10 isoform of CD44.

FIG. 2. Time course of effects of FN-f and IL-1α on CD44 and GAPDH mRNA level. Bovine chondrocytes in alginate beads were incubated with either FN-f (0.1 μM) or IL-1α (0.2 ng/ml) for 6, 24, 48, or 72 h. Total RNA from cultures at each time point was isolated and subjected to RT-PCR with specific primers for CD44 (A) and GAPDH (B). 600-bp isoform of CD44; lane 3, ApaLI digest of 754-bp isoform of CD44. In the experiment depicted in panel B, the purified 754-bp product was subjected to nested PCR using a bovine CD44v10-specific antisense primer. The nested PCR product is depicted in lane 5. This PCR product was again subjected to ApaLI digestion (lane 6). The major CD44 undigested product (arrow b, 600 bp) and the minor CD44 product (arrow a, 754 bp) are indicated.

FIG. 3. Restriction enzyme and nested PCR analysis of CD44 isoforms. The two CD44 PCR products depicted in Fig. 2A were isolated and purified. In the experiment depicted in panel A, each product was digested with ApaLI, the digests separated on 4% polyacrylamide gels, and the products visualized by ethidium bromide staining. Lanes 1 and 4 represent 6X174 DNA/HaeIII markers; lane 2, ApaLI digest of 600-bp isoform of CD44; lane 3, ApaLI digest of 754-bp isoform of CD44. In the experiment depicted in panel B, the purified 754-bp product was subjected to nested PCR using a bovine CD44v10-specific antisense primer. The nested PCR product is depicted in lane 5. This PCR product was again subjected to ApaLI digestion (lane 6). The major CD44 undigested product (arrow b, 600 bp) and the minor CD44 product (arrow a, 754 bp) are indicated.
Increased CD44 Expression in Chondrocytes

Fig. 4. Kinetic amplification of CD44 target cDNA and competitor, CD44E. Equimolar amount (0.1 attomole) of target cDNA (●) and competitor, CD44E (○) were co-amplified in the presence of γ-35S-labeled sense primer. An aliquot was removed from each amplification cycle beginning from cycle 25 to 30. The products were resolved on a 4% polyacrylamide gel. Following gel electrophoresis, the products were excised and the amount of radioactivity determined by scintillation counting.

Fig. 5. Semi-quantitative PCR analysis of the effects of FN-f and IL-1α in CD44 mRNA. Total RNA (0.5 μg) from 24 h cultures was reverse transcribed and the resultant cDNA diluted. An equivalent aliquot of each diluted cDNA sample was co-amplified with 2-fold serial dilutions of CD44E (0.00625–0.08 attomole) for 30 cycles. The products were separated on 1% agarose and visualized by ethidium bromide staining. Lanes 1-5 represent control cultures; lanes 6-10, FN-f-treated cultures; lanes 11-15, IL-1α-treated cultures. The CD44 products (arrow a, 872 bp; arrow b, 754 bp; arrow c, 600 bp) are indicated.

of CD44 receptor at the protein level was observed at 6 h (data not shown). However, the staining intensity was increased by approximately 8.0-fold at 24 h for FN-f and 6.6-fold for IL-1α

donor, were processed for in situ hybridization as described for bovine tissue. Similar staining and localized of grain development was observed in sections of human cartilage tissue (Fig. 7D), as compared to the bovine tissue (Fig. 7, A–C). This suggests that human chondrocytes also express CD44 mRNA.

Fluorescein-Hyaluronan Binding to Bovine Chondrocytes—To investigate whether the increases in immunoreactive CD44 protein observed following IL-1α treatment (Fig. 6), resulted in an increase in functional HA receptor activity, IL-1α-treated and untreated bovine chondrocytes were incubated with fluorescein-conjugated hyaluronan (fl-HA) (18). Following treatment with IL-1α, the amount of fl-HA bound at the cell surface was 3.2-fold higher than that of control cultures (Table II). In addition, an approximately 2.3-fold increase in the amount of accumulated, trypsin-insensitive, intracellular fl-HA was detected in the IL-1α-treated cultures as compared to control cultures. These findings suggest that the IL-1α-induced increase in CD44 protein is closely correlated with an increase in functional HA receptors, present at the chondrocyte cell surface.

DISCUSSION

Previous studies demonstrated that the HA- and PG-rich pericellular matrix of chondrocytes is bound or tethered to the cell surface via interaction with specific HA binding sites, termed HA receptors (11, 36). The HA receptors expressed on chondrocytes have properties similar to HA receptors present on many transformed cell types such as the human bladder carcinoma cell line, HCV-29T (37, 38). In more recent studies, it has become evident that the HA receptors expressed on many tumor cells are identical to the lymphocyte homing receptor, CD44 (38–40). In an effort to better correlate CD44 with chondrocyte function, we demonstrated that COS-7 cells transfected with a plasmid containing the gene for CD44 gain the capacity to assemble chondrocyte-like pericellular matrices in the presence of HA and chondrocyte-derived aggregating PG (29). In addition to the role of chondrocyte HA receptors in matrix assembly, these HA receptors also appear to participate in the catabolism of HA, mediated via a receptor-coupled endocytosis mechanism (18). Under some conditions, HA receptor function in catabolism may predominate over its role in matrix assembly.

Furthermore, the binding of HA to the surface of chondrocytes and its subsequent endocytosis can be blocked by anti-CD44 antibodies (18). All of these results provide indirect evidence that on chondrocytes, as with other transformed cell types, CD44 functions as the primary receptor for HA. In the present study we demonstrate that bovine articular chondrocytes transcribe mRNA for CD44, which is then translated into functional CD44/HA receptor proteins at the cell surface. In
were inhibited, both by HA hexasaccharides and by IM 7.8.1. The binding and endocytosis of HA by bovine chondrocytes is a receptor-mediated endocytosis mechanism, resulting in its complete degradation (18). The capacity to internalize HA via receptor-mediated endocytosis and degradation within the chondrocyte itself. Previous work from our laboratory demonstrated that chondrocytes have the capacity to internalize HA via a receptor-mediated endocytosis mechanism, resulting in its complete degradation (18). The binding and endocytosis of HA by bovine chondrocytes were inhibited, both by HA hexasaccharides and by IM 7.8.1.

addition, the expression of CD44/HA receptors is regulated by potent cellular mediators of chondrocyte metabolism. The turnover of newly synthesized HA and PG in radiolabeled explant cultures has been shown to be co-ordinately regulated, with nearly identical half-lives in the range of 13–25 days (15, 16). The turnover of aggregan is believed to involve proteolytic cleavage within the interglobular domain of the core protein via an enzyme termed “aggrecanase,” and/or stromelysin, resulting in the release of the chondroitin sulfate rich domain from the cartilage (12, 14). However, in the HA/PG turnover studies, HA degradation products were neither found in the tissue nor shed into the medium. These results, in addition to the lack of any detectable extracellular enzymatic activity toward HA (17), have led to the hypothesis that HA turnover must occur via another mechanism, such as endocytosis and degradation within the chondrocyte itself. Previous work from our laboratory demonstrated that chondrocytes have the capacity to internalize HA via a receptor-mediated endocytosis mechanism, resulting in its complete degradation (18). The binding and endocytosis of HA by bovine chondrocytes were inhibited, both by HA hexasaccharides and by IM 7.8.1 anti-CD44 monoclonal antibodies. A similar endocytosis mechanism, also inhibited by anti-CD44 antibodies, has been demonstrated in the catabolism of HA by macrophages (41).

Cellular mediators such as IL-1 trigger a cascade of intracellular signaling events that result in an enhanced catabolic state of chondrocytes (42). This enhanced catabolic state is characterized by inhibition of synthesis of matrix macromolecules, coupled with an increase in matrix turnover. In this study it was demonstrated that IL-1 causes a time- and dose-dependent inhibition of PG synthesis. These results are in agreement with the work of other investigators (43, 44). IL-1 also stimulates the expression of proteases such as the matrix metalloproteinases stromelysin (MMP-3) and vertebrate collagenase (MMP-1), as well as tissue plasminogen activator (45, 46), resulting in enhanced degradation of matrix macromolecules (47). Inhibition of PG synthesis was also observed previously in bovine explant cultures treated with specific concentration range of 29-kDa FN-f (48). In the present study, alginate cultures of isolated bovine articular chondrocytes exhibit inhibition of PG synthesis upon treatment with 29-kDa FN-f with a similar concentration range. Other effects of FN-f on chondrocytes mirror those observed for IL-1-induced catabolism, including the expression of metalloproteinases and the resultant degradation and release of PG from the cartilages (26). It has been postulated that some of FN-f catabolic effects are mediated via endogenous IL-1, as FN-f have been shown to induce the release of IL-1 from chondrocytes (24). Thus, both IL-1α and FN-f are potent cellular mediators and were used in this study to determine the effect of elevating the catabolic state of chondrocytes on the expression of CD44/HA receptors.

We have developed a semi-quantitative PCR procedure to better quantify relative changes in CD44 mRNA expression. With this procedure, it was demonstrated that chondrocytes cultured with FN-f had an 8-fold increase in CD44 mRNA,
while the message was augmented by 6-fold in IL-1α-treated chondrocytes. Nevertheless, it should be noted that this method quantifies only the amount of cDNA present in a given sample. If the efficiency of reverse transcription is less than 100%, this would result in an underestimate of the total amount of mRNA present. Reverse transcription-PCR of GAPDH mRNA was used to verify that the relative amount of mRNA in each sample was equivalent. However, even though little change in GAPDH product was observed from sample to sample (Fig. 2B), we cannot rule out the possibility that IL-1 or FN-f also affect the expression of GAPDH mRNA.

In addition to the standard isoform of CD44, termed CD44H, additional isoforms of CD44 exist that are generated by the alternative splicing of 10 variant exons (33). In our chondrocyte culture system, we have detected a minor CD44 product that is 154 bp larger than the major 600-bp product, CD44H, and identified this product as CD44v10. Interestingly, the mRNA for this 754-bp minor product was also stimulated by both cellular mediators, IL-1α and FN-f. Restriction enzyme analysis helped to confirm the minor band as an isoform of CD44 with the 154-bp additional sequence at the predicted site for insertion of variant exons (33) (i.e. within the 250-bp CD44H restriction fragment; Fig. 3). Although variant isoform expression is observed in some normal tissues (49, 50), variant isoform expression is most often associated with tumor progression, particularly increase in metastatic potential (51, 52). Nonetheless, little is known about the function or regulation of any of the variant CD44 isoforms (33). The role of this CD44v10 isoform expressed by chondrocytes will be the focus of further investigations.

By in situ hybridization, unamplified CD44 mRNA was detected within bovine as well as human articular cartilage. Detection in bovine cartilage immediately fixed following dissection indicates that CD44 mRNA is being continuously expressed in normal adult tissue. Nonetheless, organ culture of cartilage tissue slices in medium containing only FBS resulted in a substantial increase of CD44 message (Fig. 7B). This expression was further elevated by the addition of IL-1α to the organ cultures (Fig. 7C). These results document the presence of CD44 mRNA in chondrocytes within their native matrix environment. The observations are consistent with, and support, our results obtained by RT-PCR of RNA derived from cultured bovine articular chondrocytes. In addition, the in situ hybridization results suggest that CD44 mRNA expression by chondrocytes is not uniform within the tissue. This was most evident in the example of human articular cartilage shown in Fig. 7D. The expression appears to be enhanced in the cells of the superficial layer as compared to cells within deeper layers. This may reflect the differential role of superficial chondrocytes in matrix catabolism, differential responsiveness of superficial cells to cellular mediators, or inherent differences in chondrocyte metabolism within the different layers (20).

Based on the nucleotide sequence of CD44, a transmembrane protein of 37 kDa is predicted. However, because the protein contains a high degree of N- and O-linked carbohydrate substitution, its apparent molecular mass is typically estimated as approximately 85 kDa, depending on the cell type studied (53, 54). By Western blot analysis, doublet bands, within the range of 80–90 kDa, were detected using either of the anti-CD44 monoclonal antibodies IM 7.8.1 or KM 201. Both of these antibodies were raised against murine lymphocyte CD44 but have been shown to block the putative CD44-mediated functions on bovine chondrocytes (18). In a recent study, Mizeck et al. determined the epitope on murine CD44 recognized by the IM 7.8.1 antibody (55). This 13-amino acid epitope is also present in the predicted bovine CD44 sequence with three conservative amino acid substitutions. It is therefore likely that this antibody does, in fact, specifically recognize bovine CD44. The nature of the doublet CD44 bands observed in extracts of bovine chondrocytes remains to be determined. The bands may represent identical gene products that vary in post-translational glycosylation or processing. Alternatively, they may represent expression of two different CD44 mRNAs (e.g. expression of CD44H and CD44v10). On the other hand, no higher molecular mass CD44 bands indicative of glycosaminoglycan chain addition were apparent in the detergent extracts of bovine chondrocytes, as has been observed in other cell types (51, 56). Treatment with either FN-f or IL-1α resulted in a time-dependent increase in the expression of the doublet CD44 bands. Western blot quantification of CD44 immunoreactive protein (Fig. 6) paralleled closely the onset of CD44 mRNA up-regulation by these cellular mediators. These results would suggest that 1) there is little intracellularly stored CD44 protein available to respond rapidly to mediator-induced changes in chondrocyte metabolism, and 2) chondrocytes have little stored mRNA for CD44 protein enhancement. It is therefore likely that transcriptional controls regulate CD44 expression.

Osteoarthritis is a degenerative disease characterized by an imbalance in chondrocyte metabolism (19, 57). Catabolic processes are thought to exceed biosynthesis, resulting in depletion of critical components of the extracellular matrix. Many of these metabolic characteristics can be modelled experimentally by treatment of chondrocytes or intact cartilage with cellular mediators such as FN-f or IL-1. We have suggested in previous work that CD44/HA receptors participate in the turnover and degradation of HA (18). If this hypothesis is correct, elevating the catabolic state of chondrocytes should result in enhanced HA turnover. We would also predict that CD44 expression will be elevated in human osteoarthritic cartilage. The application of in situ hybridization techniques to the study of human cartilage will help elucidate this hypothesis. In the present study, we observe that both FN-f and IL-1 treatment results in increased mRNA for CD44, increased CD44 protein and increased HA binding capacity at the surface of chondrocytes. In addition, the amount of fli-HA that accumulated within the treated chondrocytes was also increased (Table II). Additional studies will be required to determine whether expression of CD44 actually affects an increased capacity and/or increased rate of HA endocytosis. Nonetheless, the increased accumulation of fli-HA suggests that more HA is being internalized, destined for degradation within the lysosomal compartment. CD44/HA receptors thus represent part of the delivery mechanism used by chondrocytes to bring extracellular HA into intracellular organelles. In future studies we will investigate whether treatment of chondrocytes with cellular mediators results in changes in the levels of lysosomal hyaluronidase activity involved in the actual degradation of internalized HA.

Acknowledgements—We thank Drs. Ada Cole and Susan Chubinskaya for their guidance and helpful advice with the in situ hybridization procedures. To Dr. Klaus Huch, we extend our appreciation for performing the dissection on human anklejoint. We gratefully acknowledge collaboration with Allan Valdellon of the Regional Organ Bank of Illinois and his staff.

REFERENCES

1. Eyer, D. R., Wu, J. J., and Woods, P. (1992) in Articular Cartilage and Osteoarthritis (Kuettner, K. E., Schleyerbach, R., Peyron, J. G., and Hascll, V. C., eds) pp. 119–131, Raven Press, New York
2. Hascall, V. C. (1988) in Extracellular Matrix Biochemistry (Pizel, K. A., and Reddi, A. H., eds) pp. 277–328, Elsevier Science Publishing Co., Inc., New York
3. Hascll, V. C. (1988) J. Biol. Chem. 263, 189–198
4. Hascall, V. C. (1988) J. Biol. Chem. 263, 189–198
5. Hascll, V. C. (1988) J. Biol. Chem. 263, 189–198
6. Buckwalter, J. A., and Rosenberg, L. C. (1982) J. Biol. Chem. 257, 9830–9839
Increased Expression of CD44 in Bovine Articular Chondrocytes by Catabolic Cellular Mediators
Geraldine Chow, Cheryl B. Knudson, Gene Homandberg and Warren Knudson

J. Biol. Chem. 1995, 270:27734-27741.
doi: 10.1074/jbc.270.46.27734

Access the most updated version of this article at http://www.jbc.org/content/270/46/27734

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 21 of which can be accessed free at http://www.jbc.org/content/270/46/27734.full.html#ref-list-1