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Upregulation of CD44 expression on bovine articular chondrocytes induced by synthetic lipid A

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Abstract The purpose of the study reported in this article was to investigate effects of synthetic lipid A on the expression of adhesion molecule CD44 on bovine articular chondrocytes. Full-thickness bovine articular cartilage was dissected from the carpometacarpal joints of 24 cows. Cartilage pieces were enzymatically digested to liberate chondrocytes. The chondrocytes were incubated in the presence of synthetic lipid A in suspension culture. Cell characteristics and binding of monoclonal antihuman CD44 antibodies were assessed with a flow cytometer. The expression of CD44 mRNA in chondrocytes was detected by reverse transcription-polymerase chain reaction (RT-PCR) technique. PCR products were quantified with a charge-coupled device image sensor. The percentage of CD44-positive chondrocytes was 42.2% ± 12.0%, 51.7% ± 6.8%, and 51.1% ± 5.0%, in the presence of lipid A at 0.25 μg/ml, 2.5 μg/ml, and 25 μg/ml, respectively, whereas it was 39.2% ± 8.9% in the absence of lipid A. In flow cytometry, two subpopulations of chondrocytes were found in each of five separate experiments, one with smaller number of forward scatter (FS) and the other with larger number of FS. The percentage of CD44-positive cells was 24.8% ± 8.5% in the subpopulation with smaller number of FS and 31.9% ± 6.4% in the subpopulation with larger number of FS at time 24 h after incubation. The bacterial component, lipid A, upregulated expression of CD44 on articular chondrocytes.

Key words CD44 · Chondrocyte · Lipid A · Suspension culture

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Introduction

In the chronic synovitis of rheumatoid arthritis (RA), proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) were produced in large amounts by the inflamed synovium and have been detected in synovial fluid.[1–4] IL-1 and TNF-α were considered to be major contributors to the degradation of articular cartilage.[5,6] These cytokines inhibited the production of cartilage matrix constituents such as collagen and proteoglycan by chondrocytes.[5,7–10] They also stimulated synovial cells and chondrocytes to produce proteolytic enzymes that caused cartilage matrix degradation.[5,9] Recent study suggested that superficial-layer and deep-layer chondrocytes differ not only morphologically but also phenotypically and functionally.[11] It was important to examine whether there were differences in expression of cell-surface marker between two populations. CD44, a polymorphic transmembrane glycoprotein expressed on a variety of cells, was recognized as a receptor for hyaluronan, fibronectin, and collagen and as a member of the lymphocyte activation and homing receptor family.[12,13] It was reported to be upregulated in rheumatoid synovial tissue and chondrocytes[14–16] and considered to play an important role in the inflammatory process. Lipid A, the center of the bioactive moiety of lypopolysaccharide (LPS), is a structural component of the outer membrane of all gram-negative bacteria.[17,18] We focused on its various effects on articular tissues, in the course of joint destruction, including chondrocyte activation.[19]

In the present study, the effects of synthetic lipid A on the expression of adhesion molecule CD44 on bovine articular chondrocytes were investigated.

Materials and methods

Chondrocyte isolation

Bovine articular cartilage was aseptically removed with a scalpel from the 24 carpometacarpal joints of 24 cows within

6 h after slaughter. Each piece was diced into 2 × 2 × 2 mm cubes and digested with 1 mg/ml testicular hyaluronidase (Sigma, St. Louis, MO, USA) in Roswell Park Memorial Institute's medium 1640 (RPMI 1640; Gibco, Grand Island, NY, USA) for 45 min at room temperature. After being washed, the pieces were incubated with 0.25% trypsin in phosphate-buffered saline (PBS) containing 1 mM ethylenediamine-*N*,*N*,*N*,*N*-tetraacetic acid (EDTA; Gibco) for 20 min. After being washed, the pieces were further incubated with clostridial collagenase (type I; Sigma), at 1 mg/ml (150–200 units/mg) in RPMI with 10% fetal bovine serum (FBS Qualified; Gibco), overnight at 37°C in 5% CO_2.

Stimulation

Synthetic lipid A (Daiichi-Kagaku Yakuhin, Tokyo, Japan) in 0.025% triethylamine solution was used as the stimulus. Chondrocytes were incubated in suspension with slow rotation for 24 h at 37°C in RPMI with 10% FBS in the presence of synthetic lipid A at different concentrations (0.25, 2.5, or 25 μg/ml). As a control, cells were incubated with 0.025% triethylamine solution in the absence of lipid A.

Indirect immunostaining

The cell suspension was filtered through two layers of sterile gauze, then washed three times, and resuspended in PBS. Cell viability, as determined by trypan blue dye exclusion, was more than 90%. Cells were divided into conical tubes (2.0×10^6 cells/tube) for indirect immunostaining with monoclonal antihuman CD44 antibodies (clone J173; Immunotech, Marseilles, France). Briefly, chondrocytes were incubated with 50 μl of monoclonal antibodies (1 μg/ml) for 30 min. After being washed three times with PBS, cells were combined with fluorescein isothiocyanate- (FITC-) conjugated goat antimouse IgG (Southern Biotech. Associates, Birmingham, AL, USA) and incubated for 30 min at 4°C. Mouse IgG (Charles River, Southbridge MA) was used as primary antibodies for negative control staining. The chondrocytes were incubated with 50 μl (1 μg/ml) of the nonspecific mouse IgG.

Flow cytometry

Cell characteristics (size and granularity) and antibody binding of living cells were assessed with a flow cytometer (Epics, Elite Esp; Coulter, CA, USA). Data processing was carried out with an IBM computer using Epics Elite version 4.0. A gate for positive cells was created based on fluorescence intensity (horizontal axis) and cell number (vertical axis) because less than 5% cells were required to be positive in the negative control stained with mouse IgG to exclude the false-positive cells. CD44-positive chondrocytes were defined with more intensive FITC level than the gate.

RNA extraction, cDNA synthesis, and reverse transcription-polymerase chain reaction amplification

To investigate the expression of CD44 mRNA in chondrocytes, reverse transcription-polymerase chain reaction (RT-PCR) was carried out. Freshly isolated chondrocytes in suspension were incubated with or without 2.5 μg/ml lipid A for 12 or 24 h in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/Ham's F-12; Gibco) with 2% FBS with slow rotation. Total RNA was extracted from chondrocytes in the suspension culture using a RNeasy total RNA kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg total RNA primed with random primer and reverse-transcribed using Super Script II (Gibco BRL, Gaithersburg, MD, USA). Sequences of CD44 primers used for PCR were as follows: sense, 5′-GAT CCA CCC CAA TTC CAT CTG TGC-3′ (270 bases downstream of the start codon); antisense, 5′-AAC CGC GAG AAT CAA AGC CAA GGC C-3′ (870 bases downstream of the start codon).[20] PCR mixtures containing 0.5 μl of the primers and 0.5 μl of bovine β-actin primers in a final volume of 50 μl containing 0.25 μl Taq DNA polymerase and 1.0 μl cDNA were coamplified using an AmpliTaq DNA polymerase kit (Perkin-Elmer, Branchburg, NJ, USA). PCR was performed using GeneAmp PCR system 2400 (Perkin-Elmer) for 35 cycles (30 s at 94°C, 30 s at 55°C, 30 s at 72°C). The PCR products were loaded and separated by electrophoresis on 2% agarose gels followed by staining with ethidium bromide to detect a 600-bp band of amplified fragment. The luminescence intensity of the 600-bp target products stained with ethidium bromide was semiquantified by a charge-coupled device (CCD) image sensor (Gel doc 1000; Bio-Rad, IL, USA) and analyzed by Molecular Analyst software version 2.1.2 (Bio-Rad). In our preliminary experiment, we performed PCR from 10 to 44 cycles. Because the ratio of CD44 mRNA to β-actin mRNA was fixed from 28 to 44 cycles, we selected 35 cycles for PCR.

Statistical analysis

The significance of the difference of means between values was obtained with the Student's *t* test and χ^2 test. A P value less than 0.05 was considered significant.

Results

Expression of CD44

A histogram of flow cytometry of bovine chondrocytes showed significant binding of anti-CD44 antibodies to that cells compared with binding of mouse IgG (Fig. 1). The percentage of CD44-positive chondrocytes was 10.1% ± 2.7% at time 0 after isolation with collagenase, and it increased to 26.3% ± 12.3% after 24 h incubation ($n = 4$ joints). Further incubation with 0.25% trypsin for 20 min reduced the percentage of CD44-positive chondrocytes

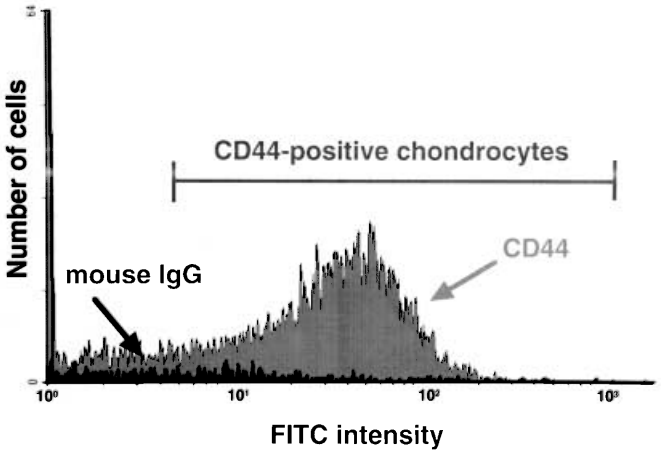

Fig. 1. Flow cytometric analysis of bovine chondrocytes stained with antihuman CD44 and developed with fluorescein isothiocyanate-(FITC-) conjugated mouse IgG. Abscissa shows FITC intensity on a logarithmic scale. Approximately 60% of bovine chondrocytes stained positively with anti-CD44 antibodies after 24 h incubation with 2.5 μg/ml of lipid A in suspension culture

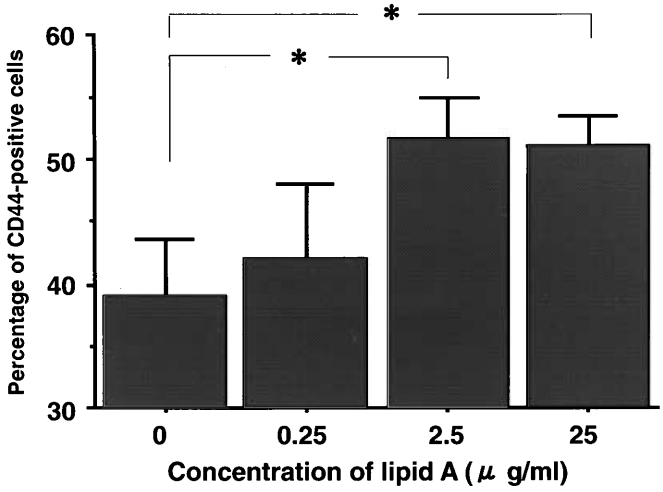

Fig. 2. Percentage of CD44-positive bovine chondrocytes after 24 h incubation with various synthetic lipid A concentrations in suspension culture. Note the increase of CD44-positive cells in a dose-dependent manner. The percentage of positive cells incubated with lipid A at concentrations of 2.5 and 25 μg/ml was higher than that of cells incubated without lipid A. Values are shown as mean and SE of five separate experiments. $*P < 0.05$ (without lipid A versus 2.5 and 25 μg/ml lipid A)

after 24 h incubation with the medium from 33.6% ± 8.7% to 4.3% ± 0.5% ($n = 4$ joints).

Effect of lipid A on CD44 expression

In the presence of lipid A at 0.25, 2.5, and 25 μg/ml after 24 h incubation, the percentage of CD44-positive bovine articular chondrocyte was 42.2% ± 12.0%, 51.7% ± 6.8%, and 51.1% ± 5.0%, respectively, whereas it was 39.2% ± 8.9% in the absence of lipid A ($n = 5$ joints) (Fig. 2). After 24 h incubation with lipid A at various concentrations in suspension culture, the percentage of CD44-positive bovine chondrocytes increased in a dose-dependent manner.

Effect of lipid A on CD44 with message level

Electrophoresis of PCR products revealed a increase of CD44 mRNA transcription in chondrocytes cultured for 24 h in DMEM/Ham's F-12 with 2% FBS in the presence of 2.5 μg/ml lipid A ($n = 4$ joints) (Fig. 3). In the analysis of the density of PCR products, the mean ratio of CD44 mRNA to β-actin mRNA at 6, 12, and 24 h after incubation with 2.5 μg/ml lipid A in the four experiments was 0.37, 0.52, and 0.93, respectively, whereas that with 0.025% triethylamine was 0.61, 0.43, and 0.52, respectively (Fig. 4).

Two subpopulations

In flow cytometric analysis with forward scatter and side scatter, the dot plots of bovine articular chondrocytes showed two distributions of the cells in all five separate experiments, one with a smaller number of forward scatter (FS), which reflects cell size, and the other with a larger number of FS (Fig. 5).

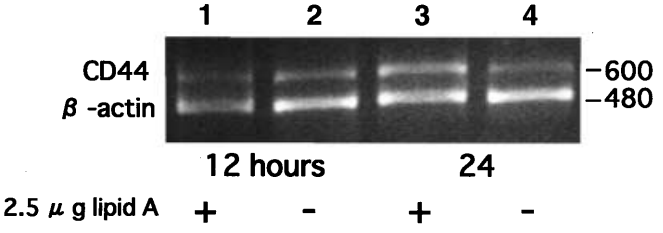

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of inducible CD44 and β-actin messenger RNA stimulated with lipid A. Bovine chondrocytes were stimulated with lipid A (*lanes 1, 3*) or 0.025% triethylamine (*lanes 2, 4*) as control for 12 h (*lanes 1, 2*) or 24 h (*lanes 3, 4*) in Dulbecco's modified Eagle's medium (DMEM) Ham's F12 containing 2% fetal bovine serum (FBS). Total RNA was extracted directly from the chondrocytes and was reverse transcribed. The RT reaction were coamplified with bovine CD44-specific or β-actin-specific primers by PCR method. PCR products of chondrocytes were electrophoresed on 2% agarose gel. The CD44 mRNA band with 600 base pairs and the β-actin mRNA band with 480 base pairs were detected by ethidium bromide staining

Expression of CD44 in the two subpopulations

The percentage of CD44-positive chondrocytes in the subpopulation with a smaller number of FS and the subpopulation with a larger number of FS was 5.4% ± 0.5% and 7.8% ± 0.8% at time 0 after isolation with collagenase, and it increased to 24.8% ± 8.5% and 31.9% ± 6.4% after 24 h of incubation ($n = 5$ joints) (Fig. 6).

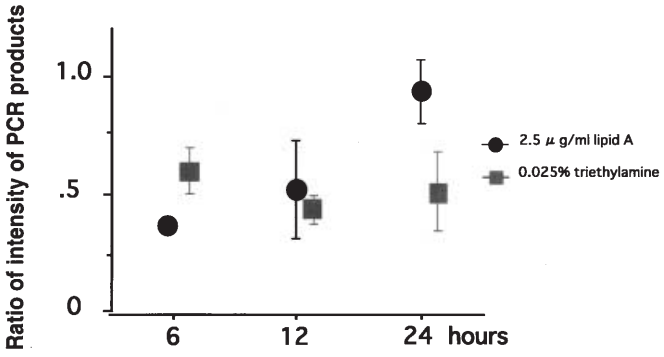

Fig. 4. Semiquantitative PCR analysis of the effects of lipid A on CD44 mRNA. The ratio of PCR product intensity of CD44 to that of β-actin was quantified by a charge-coupled device (CCD) image sensor. The mean ratio of four experiments at 6, 12, and 24 h after incubation with 2.5 μg/ml lipid A (*circles*) was 0.37, 0.52, and 0.93, respectively, whereas that with 0.025% triethylamine (*squares*) was 0.61, 0.43, and 0.52, respectively. The ratio increased at 24 h in chondrocytes cultured with 2.5 μg/ml lipid A.

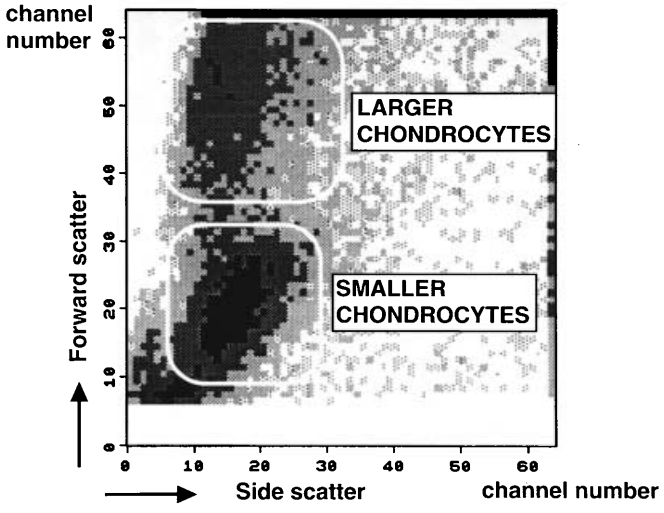

Fig. 5. A representative dot plot in flow cytometric analysis of bovine chondrocytes isolated 24 h after incubation. Value of forward scatter (FS) in the ordinate reflects cell size and value of side scatter in the abscissa reflects cell granularity. Note the two subpopulations of chondrocytes existing separately, one of larger chondrocytes with a higher FS number (*top*) and the other of smaller cells with a lower FS number (*bottom*)

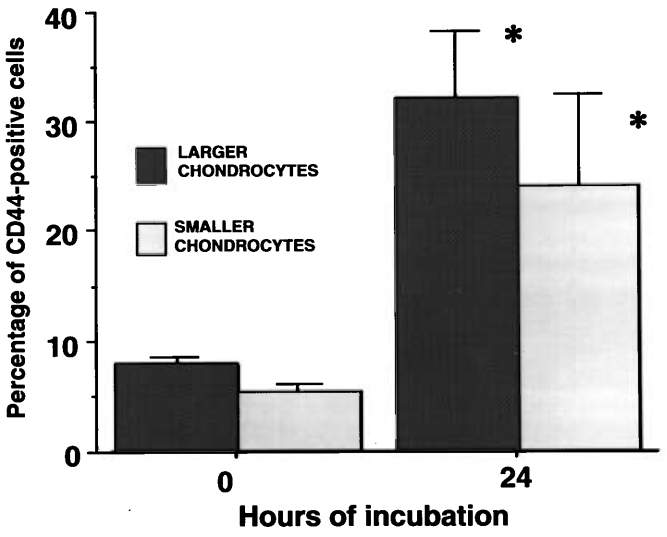

Fig. 6. Flow cytometric analysis of CD44-positive chondrocytes. Percentage of CD44-positive cells in both subpopulations of larger (*black bars*) and smaller (*white bars*) number of FS increased after 24 h incubation in suspension culture containing 10% FBS. Values are means with SE of four separate experiments. $*P < 0.05$ (24 h versus 0 h)

Discussion

In this study, quantification of the percentage of cell-surface protein CD44 with flow cytometry was a key issue. For this measurement, suspension culture of chondrocytes had certain advantages over monolayer culture, a method in which isolation of cells requires trypsinization, which destroys cell-surface proteins such as CD44. Further, stimulus could reach the entire surface of the cells in a suspension culture. In our previous report,[21] chondrocytes continued to synthesize type II collagen in the presence of 10% FBS for 5 days of suspension culture, which suggested that cells maintained their phenotype. Semiquantitative PCR is an established method.[22] In our PCR procedure, we chose 35 cycles of amplication, in which the coamplified PCR products of CD44 mRNA and β-actin mRNA were in linear proportion.

Many studies on synthetic lipid A support the concept that it is the biologically active moiety of LPS; that is, almost all biological responses of LPS were embedded in lipid A.[17,23] One of the main functions of lipid A was the activation of monocytes or macrophages to release mediators such as interleukins and TNF-α,[18,24,25] which were considered to play important roles in promoting synovitis and cartilage degeneration.[2] In this context, it would be of great interest to examine the effect of LPS on chondrocyte activation.

Aoki et al.[26] reported that hyperimmunization of rabbits with heat-killed *Escherichia coli* O:14 developed chronic polyarthritis resembling RA with an elevated level of rheumatoid factor-like substance. When Noyori et al. immunized rats with LPS extracted from *E. coli* O:14, the rats developed polyarthritis with serum rheumatoid factor-like substance.[19] Clinically, patients with RA had higher levels of anti-*E. coli* O:14 antibodies measured with enzyme-linked immunosorbent assay (ELISA) than healthy volunteers (unpublished data). Additional clinical data on Crohn's disease and ulcerative colitis showing the relevance of enterobacterial antigens to arthritis were abundant.[27] These findings suggested that LPS from enterobacteria could be a triggering agent of rheumatoid inflammation.

CD44, a polymorphic transmembrane glycoprotein expressed on a variety of cells, is recognized as a cell adhesion

molecule.[12,13] It has been reported to be upregulated in rheumatoid synovial tissue,[14–16] lymphocytes,[28] and chondrocytes[16] and is considered to play an important role in the arthritic process.[14,15,25] In RA, IL-1 and TNF-α were found to be upregulated in synovial fluid.[1–4] IL-1 was reported to increase the expression of CD44 on bovine articular chondrocytes cultured in alginate beads.[20] Anti-CD44 antibodies activated mononuclear cells to release IL-1.[29] We showed the possibility that lipid A upregulated CD44 expression through IL-1 production in the lining cells of the rat air pouch model.[30] CD44 and IL-1 were considered to play an important role in the RA and inflammatory process. Moreover, CD44 was found to modify the second messenger to act in signal transduction in T lymphocytes and monocytes to produce proinflammatory cytokines.[14,31]

Our suspension culture data revealed that stimulation of lipid A induced upregulation of CD44 expression on bovine chondrocytes at the message and expression levels. These findings suggested that the upregulation of CD44 on bovine chondrocytes resulted from the effect of lipid A in this culture condition. These findings suggested that lipid A could promote cartilage degeneration through CD44 expression in an autocrine or paracrine manner.

Recent observations revealed that there might be profound phenotypic differences between the superficial articular chondrocytes subpopulation and deep chondrocytes, in addition to the obvious morphological disparity.[32–35] Superficial chondrocytes secreted greater amounts of metalloproteinase[36] and NO[34] compared to chondrocytes obtained from the deeper layer of cartilage. Fibronectin synthesis by superficial chondrocytes was less than that by deep chondrocytes, and superficial chondrocytes had protective ability in the early phase of inflammatory arthritis.[33] In this study, when the cells were analyzed by size and granularity (forward scatter versus side scatter on flow cytometric studies), the dot plot of the bovine chondrocytes obtained from full-thickness cartilage slices showed two distinct subpopulations: one of larger cells with a larger number of FS and one of smaller cells with a smaller number of FS. The smaller chondrocytes consisted of a superficial layer of articular cartilage and the larger consisted of a deeper layer of articular cartilage.[11,36] The chondrocytes in both subpopulations expressed CD44 in suspension culture.

A significant difference between the two subpopulations in the manner of CD44 expression was not detected after enzymatic isolation of chondrocytes; however, flow cytometric analysis of bovine chondrocytes from the metacarpocarpal joint showed some ability to detect phenotypic differences in the superficial chondrocytes, which may play an important role in the early stage of cartilage destruction.

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