Comparison of hyaluronan effects among normal, osteoarthritis, and rheumatoid arthritis cartilages stimulated with fibronectin fragment

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ABSTRACT

High molecular weight hyaluronan (HA) is widely used in the treatment of osteoarthritis (OA) and rheumatoid arthritis (RA) by intra-articular injection. However, comparative studies of HA actions on catalytically activated cartilages in different pathologic conditions have rarely been investigated. This study was aimed to compare the inhibitory effects of HA on nitric oxide (NO) production by COOH-terminal heparin-binding fibronectin fragment (HBFN-f) between normal and diseased cartilages. When articular cartilage explants from normal, OA, or RA joints were incubated with HBFN-f, the RA and OA cartilages produced higher levels of NO compared with normal cartilage. Pretreatment with 2700 kDa HA resulted in significant suppression of HBFN-f-stimulated NO production in OA and RA cartilages. While CD44 was up-regulated in OA and RA cartilages, anti-CD44 antibody reversed HA inhibition of HBFN-f action in those cartilages. The present results clearly demonstrated that HA blocked HBFN-f actions in OA and RA cartilages through interaction with CD44. HA, which targets CD44 highly expressed on OA and RA chondrocytes, could suppress catabolic actions by fibronectin fragments like HBFN-f in diseased cartilage.

Fibronectin (FN) is a component of normal cartilage matrix. FN consists predominantly of three types of homologous repeating segments (designated I, II, and III). While FN is encoded by a single gene, significant protein heterogeneity results from alternative RNA splicing at three sites, termed Extra type III Domain A (ED-A), Extra type III Domain B (ED-B), and the variable (V) region. The V region is also referred to as the connecting segment between III14–15 (IIICS). There are 10 principal isoforms predicted by RNA splicing patterns, each of which contains amino (NH₂)-terminal heparin-, gelatin-, cell- and carboxyl (COOH)-terminal heparin-binding domains. Elevated levels of FN are found in osteoarthritic cartilage (12, 21) and in both synovial fluid and plasma of osteoarthritis (OA) and rheumatoid arthritis (RA) (29, 34). FN is readily degraded into fragments by proteinases. In OA and RA, activation of extracellular proteolysis may lead to the fragmentation of FN. Indeed, increased levels of 30–200 kDa fibronectin fragments (FN-fs) are found in cartilage and synovial fluid from patients with OA and RA (9, 34). Native FN has no catabolic effect on cartilage. Once FN is fragmented, those proteolytic fragments acquire catalytic activities whereby increased FN-fs are thought to cause cartilage destruction in OA and RA (35). Of FN-fs, the fragments with the central cell-, NH₂-terminal heparin- and NH₂-terminal gelatin-binding domains have been extensively investigated. Those FN-fs can stimulate proteoglycan breakdown in cultured articular cartilage explants (8). We have recently found that another FN-f, 40 kDa COOH-terminal heparin-binding fragment containing both the III12–14 and IIICS domains (HBFN-f), enhances type II collagen cleavage by collagenase in association with enhanced production
of matrix metalloproteinases (MMPs) in articular cartilage explant cultures (40, 41).

Nitric oxide (NO) is produced by a variety of cells including chondrocytes (31). NO has a number of functions in various physiologic and pathologic processes in the body (22). NO acts both regulatory and cytotoxic effects in the inflammatory reaction. NO acts principally as a proinflammatory and destructive mediator. The nitrite levels are elevated in RA synovial fluid (6). The pathogenetic role of NO in arthritis is supported by the observation that inhibitors of NO synthase suppress the development of disease in animal models such as adjuvant arthritis and streptococcal cell wall arthritis (10). Of FN-fs, HBFN-f has been shown to stimulate NO production in cartilage (38).

High molecular weight hyaluronan (HA) is widely used in the treatment of OA and RA by intra-articular injection. Although the inhibitory mechanism of HA is not totally elucidated, HA has been shown to suppress NO production by HBFN-f-stimulated RA chondrocytes through down-regulation of nuclear factor-κB (NF-κB) (39). HA associates with several cell surface proteins such as CD44 (3) and intercellular adhesion molecule-1 (ICAM-1) (7), which are constitutively expressed in chondrocytes (5, 14). There is an increasing body of evidence that HA action is mediated through its receptors (7, 13, 14, 36). Exogenous FN-fs can penetrate articular cartilage and accumulate around chondrocytes (15). Because FN binds several integrins and other cell surface protein ligands, exogenous FN-fs could act through the cell surface receptors. While HBFN-f is known to bind CD44 (11), anti-CD44 antibody blocks HBFN-f-induced MMP production in human cartilage explant culture (41). Therefore, the receptor that could mediate HBFN-f action is CD44. Currently, however, there is no evidence that HA inhibits HBFN-f action via interaction with CD44.

RA cartilage produces higher amounts of NO than normal cartilage in response to HBFN-f. The strong response to HBFN-f correlates with high levels of CD44 expression in RA cartilage (38). At present, it remains unclear how HBFN-f works in OA cartilage compared with normal. The differential effects of HBFN-f between normal and RA cartilages indicate that HA may act differentially among HBFN-f-stimulated cartilages obtained from normal, OA, and RA joints. In spite of the clinical application of HA to OA and RA joints, comparative studies of HA actions on catalytically activated cartilages in different pathologic conditions have rarely been investigated. Thus, this study was conducted to compare HA effects on HBFN-f actions among human articular cartilages obtained from normal, OA, and RA joints. The present study also attempted to explore the role of CD44 in HA inhibition of NO production by HBFN-f in OA and RA cartilages. The data shown herein suggest that HA effectively inhibits NO production by HBFN-f via highly expressed CD44 in OA and RA cartilages.

MATERIALS AND METHODS

Reagents. Human plasma FN and HBFN-f generated with α-chymotrypsin digestion of human plasma FN were purchased from GIBCO BRL (Rockville, MD, USA). The purity of the protein preparation was confirmed using the same method as used in our previous studies (40). The FN and HBFN-f were found to be free of detectable endotoxin. Anti-CD44 antibody and subclass-matched control mouse IgG were obtained from eBioscience (SanDiego, CA, USA). Monoclonal mouse anti-CD44 antibody conjugated with fluorescein isothiocyanate (FITC) was from Seikagaku Corporation (Tokyo, Japan). FITC-conjugated non-specific mouse IgG was obtained from ICN (Aurora, OH, USA).

Articular cartilage explant culture. RA and OA cartilage specimens were obtained from the distal femur from patients undergoing total knee replacement surgery who were diagnosed as having RA and OA based on the American College of Rheumatology 1987 revised criteria (2) and on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA (1), respectively. Normal articular cartilage without significant arthritic changes like fibrillation was taken from femoral neck at replacement surgery for patients with femoral neck fracture. All the samples were obtained under each patient’s consensus, and the experimental design was approved by the local ethical committee. The cartilage was assigned to 24-well plate (ca. 80 mg/well) and kept in 1.5 mL serum-free Dulbecco’s modified Eagle’s medium containing 100 μg/mL penicillin, 100 units/mL streptomycin, and 10 mM HEPES (DMEM, all from GIBCO BRL) in a humidified 5% CO₂ atmosphere at 37°C. The cartilage was precultured for 2 days and medium was changed at day 0. Following preincubation with or without 2700 kDa HA for 1 h, articular cartilage was coincubated in the presence or absence of HBFN-f or FN from day 0. In another set of experiments, following preincubation with anti-CD44 antibody or non-specific IgG for 1 h, articular cartilage was in-
cubated with or without 2700 kDa HA at 1 mg/mL for 1 h, followed by coincubation with 100 nM HBFN-f from day 0. Control cultures had no additives. The cartilage explants and conditioned media were harvested at day 3, and stored at −20°C.

**Analysis of NO release.** NO release was measured by estimating the stable NO metabolite, nitrite, in conditioned media using a spectrophotometric method based on the Griess reaction as described previously (38, 39). Following culture of the cartilage explants for 72 h, 100 μL of the culture supernatants or sodium nitrite standard dilutions were mixed with 100 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 1.25% H₃PO₄) and incubated for 10 min at room temperature. Nitrite concentrations were determined by measuring absorbance at 550 nm.

**Assay of DNA.** DNA content was measured with the proteinase K digests of articular cartilage explants as described previously (37, 38).

**Expression of CD44 evaluated by fluorescence microscopic analysis.** After blocking with 1% bovine serum albumin (BSA) for 24 h, cartilage slices were incubated with 5 μg/mL of FITC-conjugated anti-CD44 antibody or non-specific IgG for 24 h at 37°C to investigate the expression of CD44 on chondrocytes. Cartilage slices were recovered, subjected to cryostat sectioning and slides were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min. After extensive wash with PBS, slides were counterstained with propidium iodide and examined by confocal microscopy (FluoView; Olympus, Tokyo, Japan). On the digital images, immunofluorescence-positive and -negative cells were counted as described previously (38).

**Statistical analysis.** All data are expressed as mean ± SD. Data between two groups and between more than three groups were compared using Student’s t test and one-way analysis of variance (ANOVA), respectively. Significant differences were set at \( P < 0.05 \).

**RESULTS**

**Inhibition by HA of HBFN-f-stimulated NO production in OA and RA cartilages**

The initial attempt was to compare the ability of HBFN-f to induce NO production among cartilage explants from normal, OA, and RA joints (Fig. 1).

![Fig. 1 Inhibition of enhanced nitric oxide (NO) production by carboxyl-terminal heparin-binding fibronectin fragment (HBFN-f) in cartilage explant culture. Articular cartilage was obtained from normal femoral head without arthritic changes or from diseased knee joints with osteoarthritis (OA) or rheumatoid arthritis (RA). Cartilage was incubated in the presence or absence of HBFN-f at 1, 10, or 100 nM or intact plasma fibronectin (FN) at 100 nM for 3 days under serum-free conditions with or without pretreatment with 2700 kDa hyaluronan (HA) at 0.1 or 1 mg/mL for 1 h. Control cultures had no additives. Nitrite levels in conditioned media were determined as described in Materials and Methods. One-way ANOVA confirmed a significant effect of HBFN-f and HA concentrations on resultant NO levels compared with those in control and HBFN-f-treated cultures, respectively (\( P < 0.05 \)). Values are the mean ± SD for four determinations. Three separate experiments were performed with similar results. \(^* P < 0.05 \) vs. control cultures of cartilage from the same origin, and \(^# P < 0.05 \) vs. cultures of the same origin with treatment with 100 nM HBFN-f, by t test.

When normal articular cartilage was incubated with HBFN-f at 1, 10, and 100 nM, 100 nM of HBFN-f significantly increased NO production. In OA cartilage, 100 nM HBFN-f also significantly enhanced NO production. Incubation of RA cartilage with 10 and 100 nM of HBFN-f resulted in a significant increase in NO production. Compared with normal cartilage, OA and RA cartilages produced approximately 3- and 9-fold higher amounts of NO in response to 100 nM HBFN-f, respectively. Intact FN
at 100 nM failed to enhance NO production. The concentrations used (1–100 nM) are within the levels found in OA synovial fluid (34) and cartilage (9).

Next, the ability of HA to suppress HBFN-f-stimulated NO production was compared among cartilage explants from normal, OA, and RA joints (Fig. 1). When normal cartilage was pretreated with 2700 kDa HA at 1 mg/mL, no significant effect was observed in NO production by HBFN-f. In contrast, pretreatment with HA resulted in a dose-dependent inhibition of HBFN-f-stimulated NO production in OA and RA cartilages and the effect of 1 mg/mL HA was found to be significant. The molecular weight (2700 kDa) and the maximal concentration (1 mg/mL) of HA used in the experiment are within a range of physiological molecular weight (2150–4960 kDa) and concentration (< 4 mg/mL) of HA in synovial fluid (4), respectively.

**Correlation of CD44 levels with HBFN-f-stimulated NO production and HA inhibitory effect on the HBFN-f action**

In line with our previous findings (38), anti-CD44 antibody significantly suppressed HBFN-f-stimulated NO production in part in RA cartilage. Similar effect of anti-CD44 on the HBFN-f action was observed in OA cartilage (Fig. 2). These results indicate that HBFN-f acts at least partially via interaction with CD44 in RA and OA chondrocytes.

We have already shown that exogenous HA penetrates cartilage and binds chondrocytes via CD44 (14). Thus, blocking experiments with anti-CD44 antibody was conducted to investigate whether the inhibitory mechanism of HA involves CD44 in OA and RA cartilage explant cultures stimulated with HBFN-f (Fig. 2). Pretreatment with anti-CD44 antibody at 20 μg/mL for 1 h significantly cancelled the inhibitory effect of 1 mg/mL HA on HBFN-f-stimulated NO production in RA cartilage. In contrast, anti-CD44 antibody blocked HA inhibitory effect in OA cartilage. The proportion of CD44-positive chondrocytes was evaluated by fluorescence microscopy, the proportion of CD44-positive chondrocytes was higher in OA (~60%) and RA (~90%) knee cartilages than that in normal femoral head cartilage (~35%) (Fig. 3). Therefore, it is likely that enhanced NO production in response to HBFN-f and effective HA inhibition of HBFN-f actions in OA and RA cartilage explant cultures (Fig. 1) are associated with up-regulation of CD44.

**DISCUSSION**

This is the first study that compared HA effect on FN-f action among cartilages from normal, OA, and RA joints. The present study clearly demonstrated that diseased cartilage produced higher amounts of NO in response to HBFN-f compared with normal, while HA significantly inhibited HBFN-f actions in OA and RA cartilages with little effect on normal cartilage (Fig. 1). FN isoforms with III12–14 and IIICS are present in human cartilage (28) and in RA synovial lining layer (23). Thus, it seems likely that increased FN-fs with the COOH-terminal heparin-binding region play a role in NO production in arthritic cartilage. Because increased FN-fs contribute to cartilage destruction in OA and RA through their catabolic activities (35), inhibition of the FN-f ac-
HA inhibition of FN-f in OA and RA

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Suppression of HBFN-f-stimulated NO production by anti-CD44 antibody (Fig. 2) suggests that HBFN-f exerts its catabolic activities via interaction with CD44, which is compatible with the previous findings (38, 41). Cancellation of HA inhibitory effect on HBFN-f action by anti-CD44 antibody (Fig. 2) also indicates that HA effect is mediated through CD44. Thus, a possible inhibitory mechanism of HA against HBFN-f is competition between HA and HBFN-f for CD44.

The present study demonstrated that the proportion of CD44-positive chondrocytes in RA and OA cartilages was significantly higher than that in normal (Fig. 3). This observation is consistent with the previous findings that the principal hyaluronan receptor is up-regulated in chondrocytes in articular cartilage from patients with OA (26) and RA (32, 38). Although the mechanism of CD44 up-regulation in diseased cartilage is not entirely clear, proinflammatory cytokines increased in OA and RA joints could up-regulate CD44 (16, 25, 30, 33). In OA and RA joints, CD44-binding catalysts like HBFN-f may work through binding to up-regulated CD44 because stronger NO production by HBFN-f in OA and RA cartilage explant cultures is likely to be associated with higher levels of CD44 on chondrocytes in those cultures, compared with normal cartilage with less CD44 expression. The effective inhibition of HBFN-f action by HA in OA and RA cartilage explant cultures in the present study suggests that treatment with HA that targets up-regulated CD44 blocks CD44-binding catabolic stimulators. The role of CD44 in the pathogenesis of inflammation is indicated by the findings that anti-CD44 treatment reduces joint swelling and leukocyte infiltration in murine arthritis model (20) and cartilage destruction by RA synovial fibroblasts (24). The interaction between HA and CD44 has been shown to reduce anti-Fas-induced apoptosis of chondrocytes (18). Compared with monovalent ligation of anti-CD44 antibody, polyvalent ligation of HA with CD44 caused more effective inhibition of HBFN-f-stimulated NO production in OA and RA cartilages (Fig. 2). In IL-1β-stimulated normal and OA cartilages, HA inhibitory effect on enhanced MMP production is stronger than that of anti-CD44 antibody (14). Therefore, it is likely that anti-CD44 treatment with HA is a reasonable strategy for cartilage protection in OA and RA joints.

Fig. 3 Comparison of CD44 expression among normal, OA, and RA cartilages. When cartilage samples obtained at replacement surgery were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD44 antibody at 5 μg/mL for 48 h, there was fluorescence on the plasma membrane of chondrocytes. Compared with normal (A), CD44 signal was intense in OA (B) and RA (C) cartilage samples. There was no signal around chondrocytes in the sample incubated with FITC-conjugated non-specific IgG at 5 μg/mL (D). Bar = 50 μm.
events by HBFN-f via α4β1 integrin or other receptor(s), which is under investigation.

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REFERENCES


