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Requirement of mitogen-activated protein kinase for collagenase production by the fibronectin fragment in human articular chondrocytes in culture

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Abstract Fibronectin fragments have been shown to up-regulate matrix metalloproteinase production in chondrocytes. We investigated the roles of mitogen-activated protein kinase (MAPK) pathways activated by the COOH-terminal heparin-binding fibronectin fragment (HBFN-f) in collagenase production by human chondrocytes in culture. In articular cartilage explant culture, HBFN-f stimulated type II collagen cleavage by collagenase in association with increased secretion of MMP-1 and MMP-13. In human articular chondrocytes, HBFN-f induced the collagenases with activation of the extracellular signal-regulated kinase (ERK), p38, and the c-Jun NH_2-terminal kinase (JNK). PD98059 that inhibits the ERK pathway blocked HBFN-f-stimulated production of MMP-1 and MMP-13 in explant culture. SB203580 at 1 μM, the concentration that inhibits p38 only, partially suppressed HBFN-f-induced collagenase production, whereas at 10 μM, the inhibitor that blocks both p38 and JNK almost completely inhibited collagenase induction. PD98059 and SB203580 individually blocked HBFN-f-increased cleavage of type II collagen in the explant culture, although 10 μM SB203580 strongly inhibited the collagen cleavage compared with 1 μM of the inhibitor. These results indicate that collagenase production leading to type II collagen cleavage in cartilage explants requires ERK, p38, and JNK.

Key words Chondrocyte · Collagenase · Fibronectin fragment · Mitogen-activated protein kinase (MAPK) · Type II collagen

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Introduction

Fibronectin is a component of normal cartilage matrix.[1] Fibronectin consists predominantly of three types of homologous repeating segments (designated I, II, and III). Fibronectin contains amino (NH_2)-terminal heparin-, gelatin-, cell-, and carboxyl (COOH)-terminal heparin-binding domains. Elevated levels of fibronectin are found in osteoarthritic cartilage,[2–4] and in both synovial fluid and plasma of patients with osteoarthritis (OA) and rheumatoid arthritis (RA).[5,6]

Fibronectin fragments are generated by the proteolysis of native fibronectin,[4] and increase in OA synovial fluid and plasma.[5] The central cell-, NH_2-terminal heparin-, and NH_2-terminal gelatin-binding fragments of fibronectin have been shown to stimulate proteoglycan breakdown[7] and the release of catabolic cytokines[8] in cultured articular cartilage explants. In addition to those fibronectin fragments, we have recently found that a 40-kDa COOH-terminal heparin-binding fibronectin fragment containing both the III12-14 and IIICS domains (HBFN-f) can stimulate type II collagen cleavage by collagenase following proteoglycan degradation in association with enhanced production of matrix metalloproteinase (MMP)-13 and MMP-3 in bovine articular cartilage explant culture.[9] Thus, increased levels of fibronectin fragments are thought to be involved in cartilage destruction in OA and RA through the induction of cytokines and MMPs.

MMPs are a family of zinc-dependent enzymes that mediate the turnover of extracellular matrix proteins. The up-regulation of MMPs has been implicated in numerous pathological processes, including OA and RA. Of the MMPs, collagenases are particularly important because of their ability to cleave fibrillar collagen, the most abundant component of the extracellular matrix.[10] MMP-1 (collagenase 1) is expressed ubiquitously and is found in various cells, including chondrocytes.[10] MMP-8 (collagenase 2) is expressed mainly in neutrophils and may be expressed in OA chondrocytes.[11] Of all the collagenases, MMP-13 (collagenase 3) exhibits the highest activity against type II col-

lagen, which is the predominant collagen in cartilage.[12] While MMP-13 expression is restricted to bone development and bone maintenance under normal physiologic conditions,[13,14] it is up-regulated in OA chondrocytes.[11]

Activator protein-1 (AP-1), which includes members of the Jun and Fos families, is a pivotal transcriptional factor that regulates the production of cytokines and MMPs. The upstream regulatory regions of MMP genes, including MMP-1[15] and MMP-13,[16] contain the AP-1 recognition site. AP-1 can be activated by protein kinases that phosphorylate specific amino acid residues, and especially by mitogen-activated protein kinase (MAPK) families.[17] Three major MAPK families have been identified: extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK).[18,19] In response to interleukin-1 (IL-1), all three MAPKs are activated in chondrocytes.[20]

There is evidence that some fibronectin fragments can activate MAPK in articular chondrocytes. A 120-kDa central cell-binding fragment of fibronectin activates all three pathways of ERK, p38, and JNK, and increases the production of MMP-13 and gelatinases by human chondrocytes.[21] Another fibronectin fragment, a 29-kDa NH_2-terminal heparin-binding fibronectin fragment, stimulates nitric oxide production in association with the activation of ERK, p38, and JNK in human chondrocytes.[22] At present, however, the role of MAPK pathways in collagenase induction leading to type II collagen breakdown by other fibronectin fragments, including HBFN-f, remains to be clarified. In this study, we attempted to investigate which MAPK pathways activated by HBFN-f containing COOH-terminal heparin-binding domains are responsible for type II collagen breakdown with collagenase production in human articular cartilage explant cultures.

Materials and methods

Materials

Antihuman MMP-1 that reacts with 53-kDa and 51-kDa bands of proenzyme, and antihuman MMP-13 that recognizes the latent proenzyme at 60 kDa, were obtained from Sigma (St. Louis, MO, USA). Anti-ERK, -p38, and -β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiphospho-ERK, -phospho-p38, -JNK, and -phospho-JNK were obtained from Cell Signaling Technology (Beverly, MA, USA). Alkaline phosphatase-conjugated goat antirabbit IgG and rabbit antimouse IgG were purchased from Southern Biotechnology Associates (Birmingham, AL, USA) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), respectively. A 40-kDa COOH-terminal heparin-binding fragment of human plasma fibronectin (HBFN-f) containing type III12-14 segments and IIICS generated with α-chymotrypsin digestion, and human plasma fibronectin (FN) were obtained from GIBCO BRL (Rockville, MD, USA). The purity of the protein preparations, especially HBFN-f, was checked as reported previously.[9] Recombinant human IL-1-β was purchased from R&D Systems (Minneapolis, MN, USA). PD98059 and SB203580 were from Wako Pure Chemical Industries (Osaka, Japan). The FN and HBFN-f were tested for endotoxin levels with an endotoxin assay kit (Sigma) prior to use, and were found to be free of detectable endotoxin.

Articular cartilage explant culture

Adult human articular cartilage was obtained at replacement surgery from femoral heads of four female patients with femoral neck fracture (ages at operation, 66, 68, 69, and 72 years). No significant arthritic change, such as fibrillation, was found in the articular cartilage by macroscopic examination. The cartilage was assigned to 24-well plates (ca. 80 mg/well) and kept in 1.5 ml serum-free Dulbecco's modified Eagle's medium (DMEM) containing 100 μg/ml penicillin, 100 units/ml streptomycin, and 10 mM HEPES (all from GIBCO BRL) in a humidified 5% CO_2 atmosphere at 37°C. The cartilage was precultured for 2 days and the medium was changed at day 0. Thereafter, the medium was replaced every 4 days. HBFN-f, FN, or IL-1-β was freshly added from day 0 at each medium change. In some experiments, following preincubation with PD98059 or SB203580 for 1 h, articular cartilage was co-incubated with HBFN-f from day 0. Control cultures had no additives. In this study, a serum-free cartilage explant culture system was employed because chondrocytes in cartilage explants under serum-free conditions are still viable 40 days after treatment with fibronectin fragments,[23] and serum supplementation decreases the chondrolytic activity of fibronectin fragments.[7] The cartilage explant and conditioned media were harvested on days 4 and 8, and stored at −20°C.

Articular chondrocyte monolayer culture and cell lysate preparation

Adult human articular chondrocytes were separated by collagenase digestion from articular cartilage obtained from femoral heads, as described above. The cells were kept in DMEM containing 10% fetal bovine serum, grown to confluence in 6-well plates, washed with phosphate-buffered saline (PBS), and precultured for 2 days in 2 ml serum-free DMEM. Confluent primary chondrocytes were incubated with HBFN-f or IL-1-β under serum-free conditions for 72 h.

After incubation with HBFN-f or IL-1-β under serum-free conditions for various periods of time at 37°C, cell lysates were prepared following the harvest of conditioned media for assays. Briefly, cells were washed twice with cold PBS and then lysed in a lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 mM N-ethylmaleimide, and 1% Triton X-100 at 4°C. Total cell lysates were cleared by centrifugation at 16000 *g* for 10 min at 4°C.

Immunoblot analysis

Total cell lysates and conditioned media were heated with SDS–PAGE sample buffer at 80°C for 20 min. Proteins were separated by SDS–PAGE under reducing conditions, and thereafter transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The amount of samples applied was determined on the basis of DNA content in the cartilage explants and chondrocyte cell layers. Membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween 20, and incubated with the first antibody (concentration 1/1000) overnight at 4°C. After incubation with the alkaline phosphatase-conjugated second antibody (concentration 1/1000) for 3 h at room temperature, immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The protein band intensity was evaluated by densitometry using National Institute of Health image software.

Extraction and assay for cleaved type II collagen by collagenase

Cartilage explants were digested to extract cleaved type II collagen as described previously.[9,24] Briefly, the harvested cartilage explant was incubated overnight with 1.0 mg/ml α-chymotrypsin at 37°C to cleave and solubilize denatured collagen. After inhibition of α-chymotrypsin activity with N-tosyl-L-phenylalanine-chloromethyl ketone (Sigma), the samples were centrifuged and the supernatants were removed. The COL2-3/4C$_{short}$ epitope (hereafter referred to as COL2-3/4C) generated by the cleavage of type II collagen by collagenase[24] was measured in α-chymotrypsin extracts by immunoassays. The release of the COL2-3/4C epitope into the media was also measured by immunoassay.[9,25,26] From the measurements of COL2-3/4C in α-chymotrypsin extracts and medium, the total amount of epitope present in both the tissue and the medium for the period between the last medium change and harvest was calculated. The remaining explant residues were digested overnight with 1.0 mg/ml proteinase K at 56°C for DNA assay, as described as below.

Assay for DNA

DNA content was measured with the proteinase K digests of articular cartilage explants and articular chondrocyte cell layers as described previously.[27]

Statistical analysis

Statistical comparisons between two groups were performed using the *t*-test and the Wilcoxon test for parametric and nonparametric analyses, respectively. $P < 0.05$ was considered to be significant.

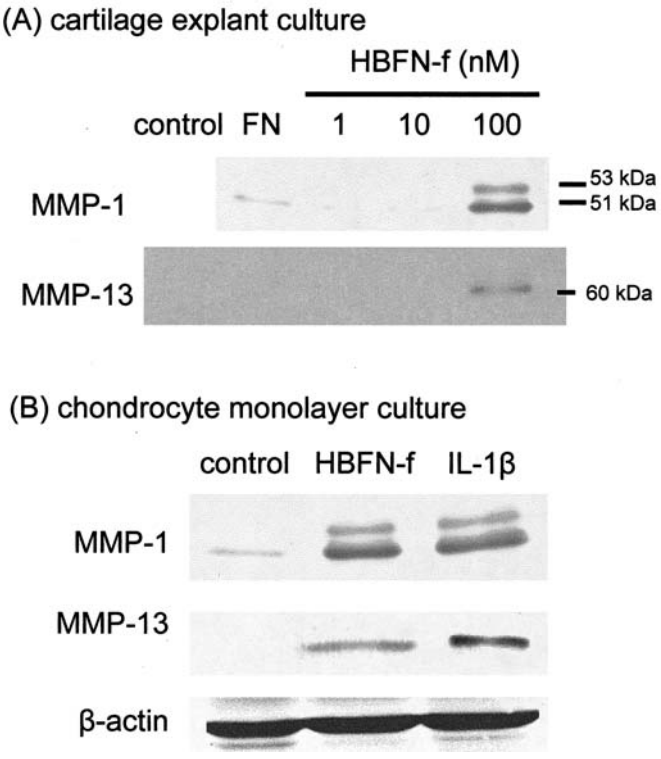

Fig. 1. Collagenase production by the COOH-terminal heparin-binding fibronectin fragment (*HBFN-f*). **A** Following preculture for 2 days under serum-free conditions, articular cartilage in explant culture was incubated with intact human plasma fibronectin (*FN*) at 100 nM or HBFN-f at 1, 10, or 100 nM under serum-free conditions during days 0–4. **B** Following preculture for 2 days under serum-free conditions, articular chondrocytes in monolayer culture were incubated with 100 nM HBFN-f or 2 ng/ml interleukin-1β (IL-1β) under serum-free conditions for 3 days. Secreted levels of matrix metalloproteinase (*MMP*)-1 and MMP-13 in conditioned media were detected by immunoblotting. To verify the equal loading of each sample, the cell lysate was subjected to immunoblotting using anti-β-actin antibody (**B**). The amount of sample applied was determined on the basis of the DNA content of each sample. Control cultures were without any additives. Molecular sizes are indicated on the right-hand side. Three separate experiments were performed with similar results

Results

HBFN-f-induced production of collagenases by human articular chondrocytes in culture

When human articular cartilage was incubated with HBFN-f at 1 and 10 nM or FN at 100 nM during days 0–4 in explant culture after preculture for 2 days under serum-free conditions, immunoblot analyses showed barely detectable levels of MMP-1 and no detectable MMP-13 in conditioned media. In contrast, HBFN-f at 100 nM stimulated the secretion of the collagenases during days 0–4 under serum-free conditions (Fig. 1A). Similarly, treatment with 100 nM HBFN-f for 3 days under serum-free conditions resulted in increased secretion of MMP-1 and MMP-13 in primary articular chondrocyte monolayer culture following preculture for 2 days under serum-free conditions (Fig. 1B). IL-1-β at 2 ng/ml also induced collagenase production in both cartilage explant (data not shown) and chondrocyte monolayer

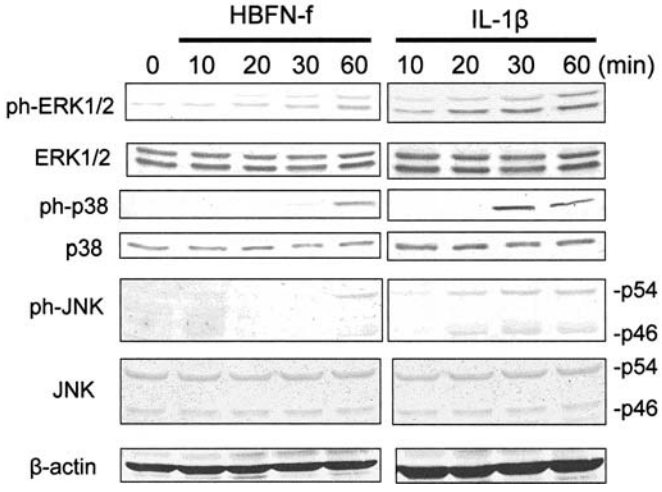

Fig. 2. Activation of mitogen-activated protein kinases (MAPKs) by HBFN-f in articular chondrocyte monolayer culture. After treatment with 100 nM HBFN-f or 2 ng/ml IL-1β under serum-free conditions for the time-periods indicated, chondrocytes were lysed and subjected to immunoblotting with antiextracellular signal-regulated kinase (*ERK*), antiphospho (ph)-ERK, anti-p38 MAPK, anti-ph-p38, anti-c-Jun NH_2-terminal kinase (*JNK*), and anti-ph-JNK antibodies. To verify the equal loading of each sample, the cell lysate was subjected to immunoblotting using anti-β-actin antibody. The amount of sample applied was determined based on the DNA content of each sample. IL-1β was used as a positive control. Three separate experiments were performed with similar results

(Fig. 1B) cultures under serum-free conditions. Immunoblot analysis for β-actin verified the equal loading of each sample on the basis of the DNA content. We found that HBFN-f at 1 and 10 nM or FN at 100 nM failed to increase collagenase secretion in chondrocyte monolayer culture (data not shown).

In OA synovial fluids, approximately 1 μM of 100–200 kDa fibronectin fragments have been found.[5] Since the levels of fibronectin fragments in OA cartilage have been suggested to be similar to those in OA synovial fluids,[4] the contents of fibronectin fragments may reach 100 nM in OA cartilage, which is comparable with the concentration used in the present study.

Activation of MAPK pathways by HBFN-f in human articular chondrocytes

We next examined whether treatment with HBFN-f results in the activation of ERK, p38, and JNK in primary articular chondrocyte monolayer culture following preculture for 2 days under serum-free conditions. After stimulation with HBFN-f at 100 nM, cell lysates were subjected to immunoblot analyses (Fig. 2). As was found in previous studies,[20] IL-1-β at 2 ng/ml activated all three MAPK pathways in chondrocytes, which served as a positive control. Although low levels of phosphorylated ERK2 were found without any stimulation, the incubation of articular chondrocytes with 100 nM HBFN-f resulted in ERK1/2 activation after 60 min. HBFN-f at 100 nM also activated p38 and p54 JNK at 60 min. Total protein levels of ERK1/2, p38, and JNK (p46 and p54) were unchanged during the whole experimental periods.

MAPK pathways required for collagenase production stimulated with HBFN-f in explant culture

In order to investigate which MAPK pathways were required for collagenase induction by HBFN-f-stimulated articular cartilage, we used selective protein kinase inhibitors: PD98059; a specific MAPK, ERK-kinase (MEK) inhibitor that binds to the inactive forms of MEK-1 and inhibits the ERK signaling cascade;[28] SB203580, which selectively inhibits p38 at low concentrations ($\leq$1 μM) but which inhibits both p38 and JNK at high concentrations ($\geq$10 μM).[22,29] At the medium change on day 0 or day 4 following preincubation with PD98059 or SB203580 for 1 h, articular cartilage was co-incubated with HBFN-f at 100 nM for 4 days. Pretreatment with the MEK inhibitor at 0.5–50 μM resulted in a dose-dependent decrease in MMP-1 and MMP-13 secretion which had been stimulated with 100 nM HBFN-f during days 0–4 and 4–8 (Fig. 3). PD98059 at 50 μM suppressed HBFN-f-stimulated MMP-1 by 88% and 95% during days 0–4 and 4–8, respectively, while it blocked HBFN-f-enhanced MMP-13 by 96% and 92% during days 0–4 and 4–8, respectively. While SB203580 at 0.1–1 μM partially inhibited HBFN-f-induced production of MMP-1 and MMP-13, the inhibitor at 10 μM completely inhibited collagenase production in articular cartilage explant culture during days 0–4 and 4–8 (Fig. 3). SB203580 at 1 μM decreased MMP-1 stimulated with HBFN-f by 55% and 77% during days 0–4 and 4–8, respectively, whereas it reduced HBFN-f-induced MMP-13 by 48% and 73%, respectively. SB203580 at 10 μM blocked HBFN-f-stimulated MMP-1 by 97% and 98% during days 0–4 and 4–8, respectively, while 10 μM of the inhibitor suppressed HBFN-f-enhanced MMP-13 by 98% and 97% during days 0–4 and 4–8, respectively.

Suppression of collagenase-mediated cleavage of type II collagen by MAPK inhibitors in human articular cartilage explant culture

In line with our previous findings in bovine articular cartilage after treatment with HBFN-f,[9] the assay for cleaved type II collagen showed that the fragment at 100 nM stimulated the release of COL2-3/4C epitope generated by collagenase cleavage of type II collagen fibrils in human articular cartilage explant culture (Fig. 4). This release is dependent on the secondary cleavage of the denatured α-chain bearing the epitope that involves MMP activity.[26] When articular cartilage was preincubated with PD98059 at 50 μM for 1 h on day 4 following co-incubation with 100 nM HBFN-f, HBFN-f-activated cleavage of type II collagen was completely blocked to control levels on day 8 (Fig. 4). Co-incubation of cartilage with HBFN-f after pretreatment with SB203580 for 1 h on day 4 also resulted in significant inhibition of type II collagen cleavage stimulated with 100 nM HBFN-f (Fig. 4). Compared with the inhibitor at 1 μM, SB203580 at 10 μM produced stronger and complete

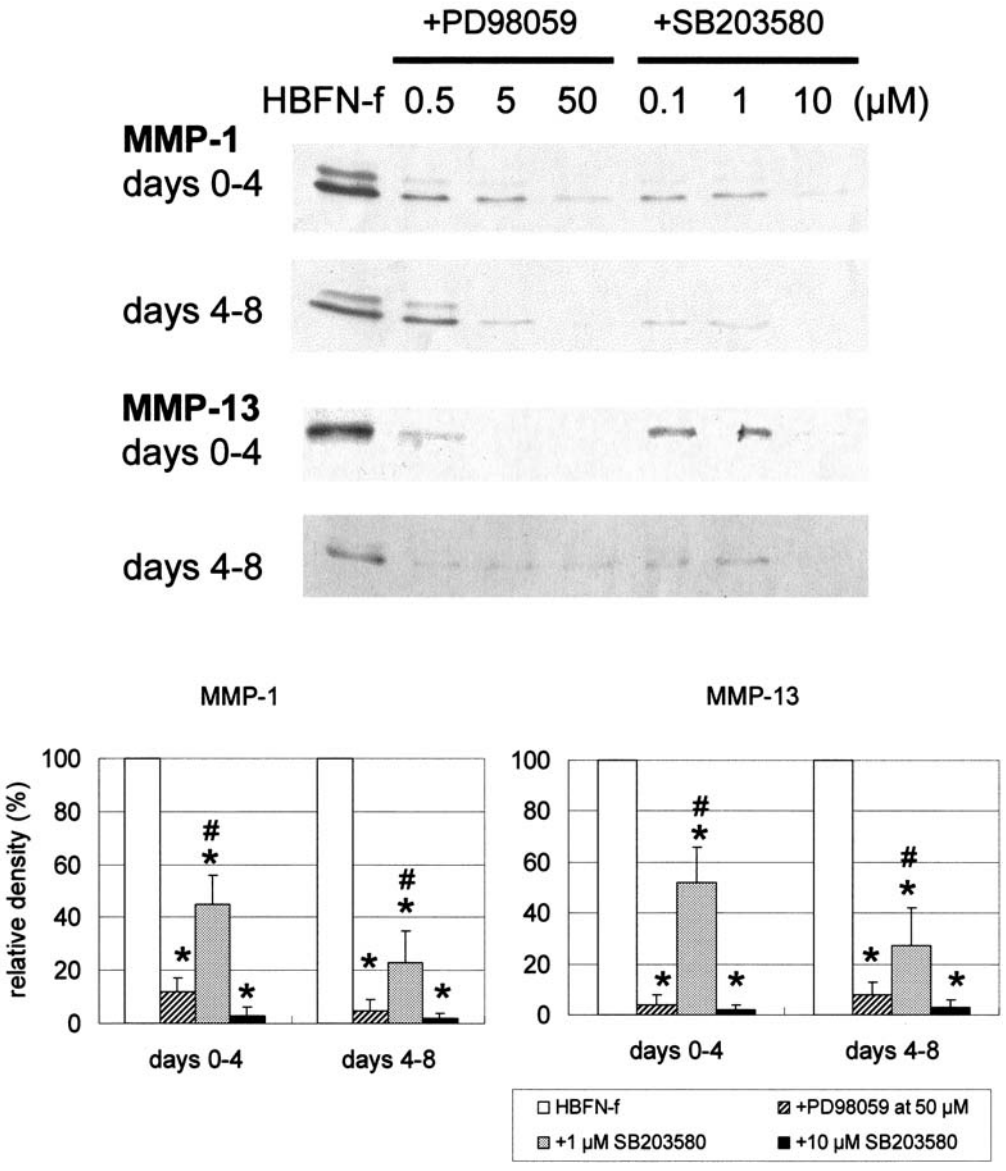

Fig. 3. Effects of MAPK inhibitors on HBFN-f-stimulated production of collagenases in articular cartilage explant culture. After preincubation with or without PD98059 or SB203580 at the concentrations indicated at day 0 or 4, articular cartilage was co-incubated with 100 nM HBFN-f for 4 days. Secreted levels of MMP-1 and MMP-13 in conditioned media during days 0–4 and 4–8 were analyzed by immunoblotting. The amount of sample applied was determined based on the DNA content of each sample. Relative protein band intensities are indicated as mean ± SD from four separate sets of experiments, where the intensity of the HBFN-f-treated culture is defined as 100%. $*P < 0.05$ versus HBFN-f-treated cultures. $\#P < 0.05$ versus 10 μM SB203580-pretreated cultures by the Wilcoxon test for nonparametric analysis with the relative protein band intensities

suppression of the type II collagen cleavage. SB203580 at 1 and 10 μM blocked HBFN-f-increased cleavage of type II collagen by approximately 80% and 100%, respectively. PD98059 and SB203580 also blocked HBFN-f-induced cleavage of type II collagen during days 0–4 (data not shown).

Discussion

Specific sequences have been found in the promoters of MMP genes that mediate the increase in gene expression, including the AP-1 site that binds the Fos and Jun families of transcription factors,[30] and several upstream sequences such as a nuclear factor (NF)-κB like element.[31] The three mammalian MAPK pathways have been characterized in detail: the ERK1/2 pathway (Raf/MEK1, 2/ERK1, 2), the JNK/stress-activated protein kinase pathway (MEK kinase-1-4/MKK4, 7/JNK1-3), and the p38 pathway (MAPK kinase kinase/MKK3, 6/p38α,β).[32] The activation of MAPK and NF-κB has been implicated in proinflammatory cytokine signaling in chondrocytes.[33] In human normal chondrocyte monolayer culture, NH_2-terminal heparin- and central cell-binding fragments of fibronectin[21,22] have been shown individually to activate ERK1/2 and p38, which is compatible with the present data using HBFN-f (see Fig. 2). Those earlier studies also showed that the NH_2-terminal heparin-binding fibronectin fragment activates JNK1,[22] whereas the central cell-binding fragment induces the activation of JNK1/2.[21] In contrast, this study showed that HBFN-f activated JNK2. Thus, different fragments of fibronectin may activate different isoforms of JNK. However, it is not denied that some fibronectin fragments may fail to stimulate MAPK pathways. Although AP-1 activation was not investigated directly in the present study, MAPK families can

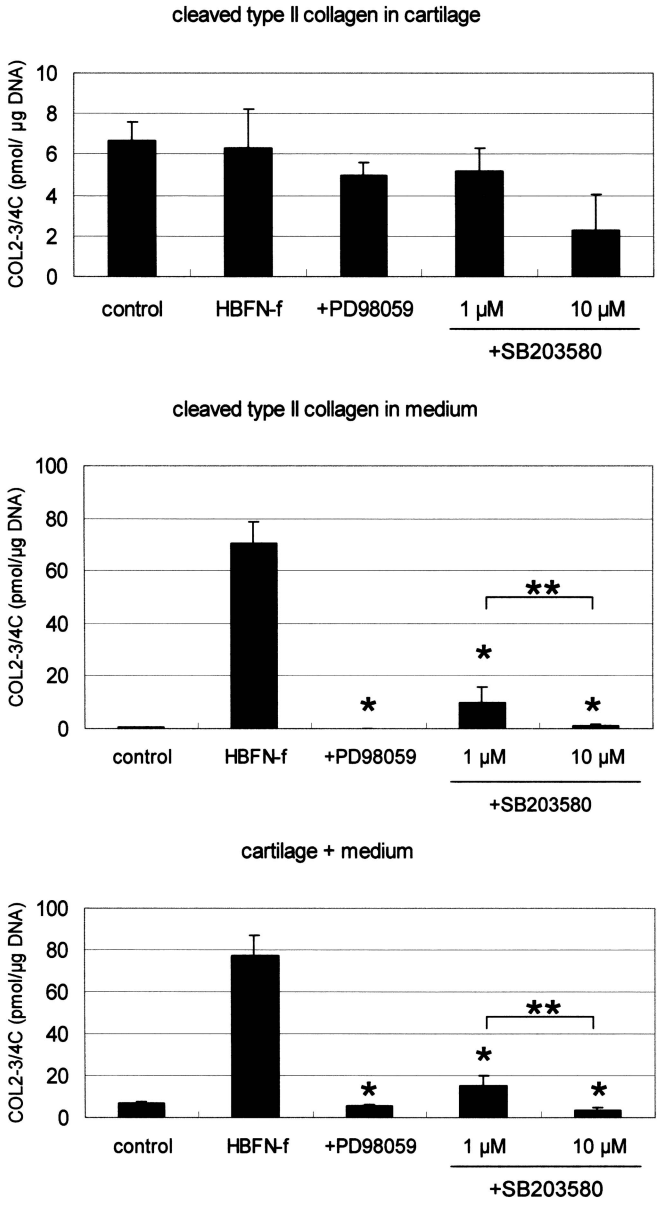

Fig. 4. Suppression of HBFN-f-induced cleavage of type II collagen by collagenase with MAPK inhibitors in articular cartilage explant culture. After preincubation with or without PD98059 at 50 μM or SB203580 at 1 or 10 μM at day 4, articular cartilage was co-incubated with 100 nM HBFN-f for 4 days. Control cultures had no additives. Collagenase-generated epitope of cleaved type II collagen (COL2-3/4C) was measured by enzyme-linked immunosorbent assay. The results on day 8 are shown. Data represent mean ± SD for four determinations. $*P < 0.05$ versus HBFN-f-treated cultures. $**P < 0.05$ between the two groups indicated by the *t*-test. Three separate experiments were performed with similar results

activate AP-1,[17] which could induce collagenase in chondrocytes. Upstream events in the activation of MAPK pathways by HBFN-f remain to be clarified.

In human articular cartilage explant culture, p38 probably plays a role in HBFN-f-stimulated induction of MMP-1 and MMP-13 because SB203580 at 1 μM significantly blocked MMP production (see Fig. 3). In line with this observation, the p38 inhibitor significantly reduced HBFN-f-increased levels of collagenase-generated epitope of cleaved type II collagen (see Fig. 4). In addition to p38, collagenase induction by HBFN-f could involve ERK and JNK because PD98059 and a higher concentration (10 μM) of SB203580 individually caused significant suppression of HBFN-f-stimulated cleavage of type II collagen (see Fig. 4) as well as collagenase (see Fig. 3) production. Thus, all the three MAPK pathways could be responsible for HBFN-f-stimulated collagenase production in human articular cartilage explant cultures. Previous studies using specific MAPK inhibitors have shown that ERK and JNK mediate MMP-13 induction in response to the 120-kDa central cell-binding fragment of fibronectin in human chondrocyte monolayer cultures, although the involvement of p38 in MMP-13 induced by the fragment remains unclear because low concentrations (<1 μM) of SB203580 have not been tested. In the same studies, integrin α5β1, which is recognized by the arginine – glycine – aspartic acid (RGD) sequence in the central cell-binding fragment of fibronectin, has been shown to activate those three MAPK pathways,[21] indicating that integrin α5β1 mediates the action of the fibronectin fragment. Although HBFN-f without the RGD sequence can bind integrin α4β1,[34] chondrocytes in normal cartilage barely express the integrin.[35] Currently, the receptor for HBFN-f on chondrocytes is still unknown. The phenotypic stability of articular chondrocytes and the effects of exogenous reagents on chondrocyte metabolism are found to be dependent on culture conditions.[36,37] Isolation and primary culture of chondrocytes could radically alter their normal tissue environment and may influence their responses to stimulation.[38] Therefore, data from studies with chondrocytes cultured in monolayers may not be directly applicable to cartilage kept in explant cultures. In addition, chondrocytes obtained from donors of different ages may respond differently to fibronectin fragments. Further investigation may be necessary to determine whether different fragments of fibronectin act through the same or different MAPK pathways for collagenase induction by chondrocytes under established experimental conditions.

Degradation products of fibronectin are recognized as one of the amplifiers or catalysts in diseased joints, including RA and OA,[39] because of their ability to stimulate MMP induction and cartilage destruction.[7,9] In contrast to intact fibronectin, HBFN-f can stimulate type II collagen cleavage by collagenase in association with enhanced production of collagenases in human articular cartilage explant culture (see Fig. 1). The observation that the injection of fibronectin fragments into rabbit knee joints induces a depletion of cartilage proteoglycan supports the pathophysiological significance of the fragments.[40] From these findings, it seems that increased fibronectin fragments could play an important role in cartilage destruction in arthritis. Control of ERK, p38, and JNK pathways in concert may contribute to the prevention of cartilage destruction by fibronectin fragments in OA and RA.

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