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Nitric Oxide Mediates Interleukin-1-Induced Gene Expression of Matrix Metalloproteinases and Basic Fibroblast Growth Factor in Cultured Rabbit Articular Chondrocytes[1]

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We recently reported that nitric oxide (NO), which is produced by chondrocytes treated with interleukin-1β (IL-1), releases basic fibroblast growth factor (bFGF) stored in the matrix of articular chondrocytes. To clarify the mechanism of the IL-1-induced bFGF release, we investigated the production and gene expression of bFGF, matrix metalloproteinases (MMPs), syndecan 3, and inducible NO synthase (iNOS) by IL-1-treated rabbit articular chondrocytes. IL-1 stimulated not only the release of bFGF but also the production of it. Gelatin and casein zymography revealed that IL-1 stimulated the production of not only MMP-9 but also MMP-3. The increase in the production of these MMPs preceded the IL-1-stimulated bFGF release. An MMP inhibitor partially suppressed the release of bFGF, indicating that matrix degradation is at least partially involved in the IL-1-stimulated bFGF release even if increased production of bFGF is related to the release. IL-1 sequentially stimulated mRNA expression of iNOS, membrane type 1-MMP, MMP-9 and -3, and bFGF, in that order. N^G-Monomethyl-L-arginine, an inhibitor of NO production, inhibited gene expression of MMP-9 and bFGF. These findings suggest that elevation of the NO level *via* iNOS mRNA expression stimulated by IL-1 mediates gene expression and production of MMPs and bFGF, resulting in the release of bFGF, and also reveal molecular mechanisms implicating the degradation of articular cartilage followed by angiogenesis in the synovium in arthritic joints.

Key words: basic fibroblast growth factor, chondrocytes, interleukin-1β, matrix metalloproteinase, nitric oxide.

Based on the results of *in vitro* and *in vivo* studies, it has been proposed that inflammatory cytokines such as interleukin-1β (IL-1) play an important role in mediating cartilage degradation (*1-3*). For example, increased levels of IL-1 in the synovial fluid of rheumatoid arthritic (RA) and osteoarthritic (OA) joints have been demonstrated (*4, 5*), and intraarticular injection of IL-1 causes the production of proteoglycan-degrading enzymes, such as matrix metalloproteinase (MMP)-9 and MMP-3 by chondrocytes (*6*). Moreover, IL-1 causes chondrocytes to synthesize proteoglycan-degrading enzymes, such as MMP-9 and MMP-3 *in vitro* (*7*). More recently, membrane type 1-MMP (MT1-MMP), which acts on type I collagen synergistically with MMP-2, also has been shown to digest cartilage proteoglycan (*8*). These findings suggest that MT1-MMP also participates in the cartilage degradation in OA and RA.

Nitric oxide (NO) is a gaseous free radical synthesized from L-arginine by NO synthases (NOS); inducible NOS (iNOS), endothelial NOS, and neuronal NOS (*9*). The inducible form of nitric oxide synthase releases high levels of NO in response to inflammatory mediators, such as IL-1 and tumor necrosis factor-α (TNF-α) in articular chondrocytes (*10, 11*). Indeed, it has been reported that the concentration of nitrite, which represents the local NO production, is increased in synovial fluid and sera from patients with inflammatory diseases such as OA and RA (*12, 13*).

RA is a chronic inflammatory disease of unknown etiology. In the synovial fluid and synovium in RA, various cytokines derived from macrophages and/or fibroblasts, such as IL-1, IL-6, and TNF-α, are readily detected (*14-*

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Abbreviations: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IL-1, interleukin-1β; MT1-MMP, membrane type 1-matrix metalloproteinase; NOS, nitric oxide synthase; NMA, N^G-monomethyl-L-arginine; OA, osteoarthritis; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; RAC, rabbit articular chondrocytes; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor-α.

17). In the OA synovium, the profile of cytokines produced is comparable with that in RA (18, 19). Although articular cartilage is avascular under normal circumstances, in OA and RA patients, invasion of blood vessels into the cartilage and proliferation of the synovium with neovascularity are often seen.

Based on these findings, we recently demonstrated that high levels of NO induced by IL-1β cause the release of basic fibroblast growth factor (bFGF), a strong angiogenesis factor, from the cell layer of rabbit articular chondrocytes (RAC) in culture (20). We also suggested that increased production of MMP-9 may mediate the release of bFGF because IL-1-stimulated MMP-9 production preceded the release of bFGF. In the present study, to clarify the mechanism of the IL-1-stimulated bFGF release, we investigated the production and gene expression of bFGF, MMPs, syndecan 3, which is a heparan sulfate proteoglycan, and iNOS in IL-1-treated articular chondrocytes.

MATERIALS AND METHODS

Materials—Recombinant human IL-1β (mol wt., 17,376; isoelectric point, 6.9; purity, >95%; lymphocyte-activating factor activity, 2×10^7 U/mg protein) was donated by Otsuka Pharmaceutical (Tokushima). MMP inhibitor/BAY 12-9566 (mol wt., 410.9; inhibition activity toward MMP-2: $K_i = 11$ nM, MMP-3: $K_i = 134$ nM, and MMP-9: $K_i = 301$ nM) was donated by Bayer Yakuhin (Osaka). Recombinant human bFGF (mol wt., 16,000; purity, >97%) was purchased from R&D Systems (Minneapolis, MI). N^G-Monomethyl-L-arginine (NMA) was purchased from Sigma Chemical (St. Louis, MO). The enzyme immunoassay system used to determine the bFGF concentration was purchased from Amersham International (Buckinghamshire, UK).

Cell Culture—Chondrocytes were isolated from the articular cartilage of the knees of young male New Zealand rabbits, weighing 300-500 g, as described (21, 22). The cartilage specimens were minced and treated with 0.1% EDTA (w/v) in phosphate-buffered saline (PBS) containing 0.2% glucose (w/v). Chondrocytes were then released by digestion with 0.15% collagenases (Wako, Osaka) (w/v) in PBS containing 0.2% glucose. These cells were seeded at a density of 2×10^4/cm^2 into 6-cm diameter dishes (Sumitomo Bakelite, Tokyo) for preparation of conditioned medium, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Cansera International, Rexclale, Ont., Canada) and 50 μg/ml kanamycin at 37°C under an atmosphere of 5% CO_2 and air. Only primary and confluent cultures were used for the experiments.

Determination of the bFGF Concentration—Confluent chondrocytes were cultured for appropriate periods with or without IL-1, or cultured for 48 h with or without IL-1, in the presence or absence of an MMP inhibitor (1 μM) in DMEM containing 0.5% FBS. bFGF in the cell layer was extracted with PBS containing 3 M NaCl. The amounts of bFGF in the conditioned culture medium (CM) and the cell layer were determined with an enzyme immunoassay kit according to its instruction manual.

Gelatin and Casein Zymography—For measurement of the activity of gelatinases and caseinase, we carried out gelatin- and casein-substrate zymography, respectively, using the CM of RAC treated for appropriate periods with or without IL-1, or treated for 48 h with IL-1 in the presence or absence of bFGF in DMEM containing 0.5% FBS, as described previously (23, 24). Five milliliters of this CM from a 6-cm diameter plate was lyophilized and then reconstituted in 0.5 ml of distilled water. Then, 15 μl of this solution was electrophoresed at 4°C in a 10% polyacrylamide gel containing 0.1% gelatin or casein, respectively. After the electrophoresis, the gels were washed with 10 mM Tris-HCl (pH 8.0) containing 2.5% Triton X-100. Then the gels for gelatinases or caseinase were incubated in 50 mM Tris-HCl (pH 8.0) containing 0.2 M NaCl and 5 mM $CaCl_2$ at 37°C for 48 or 96 h, respectively. The gels were then stained with 1% Coomassie Brilliant Blue and destained. The visualized bands disappeared when the enzyme reaction was performed in the presence of EDTA, indicating that they were those of MMPs. In some experiments, photographs of the stained gels were taken and analyzed semiquantitatively with a Foto/Analyst Image Analysis System (Fotodyne, Hartland, WI).

RT-PCR for Quantitation of mRNA Expression—Total RNA was isolated from RAC cultured for appropriate periods with or without IL-1, using guanidine thiocyanate-phenol reagent (Nippon Gene, Tokyo) according to the method of Chomczynski and Sacchi (25), and about 20 μg of

TABLE I. **Nucleotide sequences of the primers used for RT-PCR.**

		Sequence	Expected size of PCR product (bp)
18S rRNA	(S)[a]	5′-GCGAATTCCTGCCAGTAGCATATGCTTG-3′	126
	(AS)[b]	5′-GGAAGCTTAGAGGAGCGAGCGACCAAAGG-3′	
MMP-2	(S)	5′-CTGGCTTCCCTCGGCTCATCGCGGA-3′	497
	(AS)	5′-CTGAGATAGTCCCTGTTGGCTCCGA-3′	
MMP-9	(S)	5′-GCAAGGTGCTGCTGTTCGGCGCGCA-3′	271
	(AS)	5′-GGAAGGACGCCCTAGTCCTCAGGGC-3′	
MMP-3	(S)	5′-TTTAAAGACAGGCACTTTTGGCGC-3′	249
	(AS)	5′-GGC(AT)GCATCGAT(CT)TTC(CT)TCACGGT-3′	
MT1-MMP	(S)	5′-TGGACACGGAGAATTTTGTGCTG-3′	300
	(AS)	5′-GGCCCCGCCAGAACTGGCCAATG-3′	
iNOS	(S)	5′-GATGAGAGTGGCAGCTACTGGGTC-3′	469
	(AS)	5′-TCCGCACAAAGCAGGGCACTGGGTC-3′	
bFGF	(S)	5′-CACTTCAAGGACCCCAAGCGGCT-3′	414
	(AS)	5′-TAGATGTGGCCATTAAAATCAGCTCTT-3′	
Syndecan 3	(S)	5′-ACCCACAGAGGTGGCTCAGACCCC-3′	225
	(AS)	5′-AGGCCAAGGCCTGGGCGGGCACCC-3′	

[a]Sense primer. [b]Antisense primer.

the isolated total RNA was treated with 0.8 U of DNase I (Promega Corp., Madison, WI) at 37°C for 30 min. RT-PCR was performed with a Gene Amp RNA PCR Kit (Perkin Elmer, Branchburg, NJ). Total RNA, 0.2 μg, was reverse transcribed to cDNA using oligo $d(T)_{16}$ and M-MLV reverse transcriptase for 30 min at 42°C in the presence of 46 kBq of [α-^{32}P]dCTP (110 TBq/mmol, Amersham Int.). Then, the cDNAs of bFGF, MMP-2, -9, and -3, and 18S rRNA were amplified with each primer set using 0.5 U of Ampli Taq DNA polymerase, and the cDNAs of iNOS, MT1-MMP, and syndecan 3 were amplified using 0.5 U of Ampli Taq Gold (Perkin Elmer). The sequences of the synthesized primers and the expected sizes of the PCR products are shown in Table I. The amplification conditions were as follows: for 18S rRNA, 94°C (2 min) for 1 cycle, 94°C (1 min)-57°C (1 min)-72°C (2 min) for 19 cycles, and final incubation at 72°C for 5 min; for MMP-2, -9, and -3, and bFGF, 94°C (2 min) for 1 cycle, 94°C (1 min)-57°C (1 min)-72°C (2 min) for 32 cycles, and final incubation at 72°C for 5 min; for iNOS, 95°C (6 min) for 1 cycle, 96°C (1 min)-62°C (2 min) for 30 cycles, and final incubation at 62°C for 10 min; for MT1-MMP, 95°C (6 min) for 1 cycle, 96°C (1 min)-60°C (2 min) for 30 cycles, and final incubation at 60°C for 10 min; and for syndecan 3, 95°C (6 min) for 1 cycle, 96°C (1 min)-63°C (2 min) for 42 cycles, and final incubation at 63°C for 10 min. Five microliters of each reaction mixture was electrophoresed on a polyacrylamide gel (3.5%), and then the gel was dried. The dried gels were exposed to imaging plates and then the amounts of RT-PCR products were determined with a Bioimaging analyzer BAS2000 (Fuji Film, Tokyo). Under these conditions, the ratio of the PCR products to the 18S rRNA gene increased constantly and linearly. Therefore, the amounts of the RT-PCR products were compared with that of 18S rRNA as a standard, and relative expression ratios were obtained.

Statistical Analysis—Unless otherwise specified, experiments were repeated at least twice and similar results were obtained in the repeated experiments. Statistical analysis was performed by one-way analysis of variance. Data are expressed as means ± the standard deviation (SD). A value of $p < 0.05$ was considered significant.

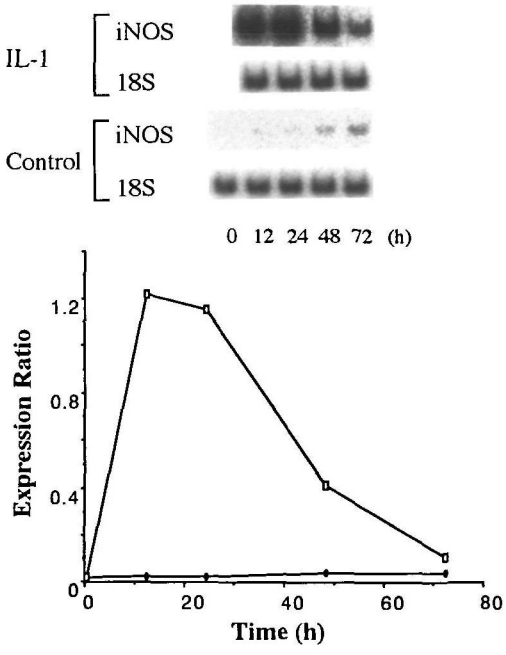

Fig. 1. **Time-dependent effect of IL-1 on the expression of mRNA of iNOS.** When RAC cultured in a 6-cm diameter dish reached confluence, the cells were washed with DMEM containing 0.5% FBS and then further incubated in an aliquot of fresh medium in the presence (□) or absence (●) of IL-1 at 1 ng/ml. Total RNA was isolated at the indicated times. (top) RT-PCR was performed, and the PCR products were subjected to electrophoresis and then exposed to imaging plates as described under "MATERIALS AND METHODS." (bottom) The graph shows the relative expression level compared with the level of 18S rRNA.

RESULTS

Effect of IL-1 on the Expression Level of mRNA of

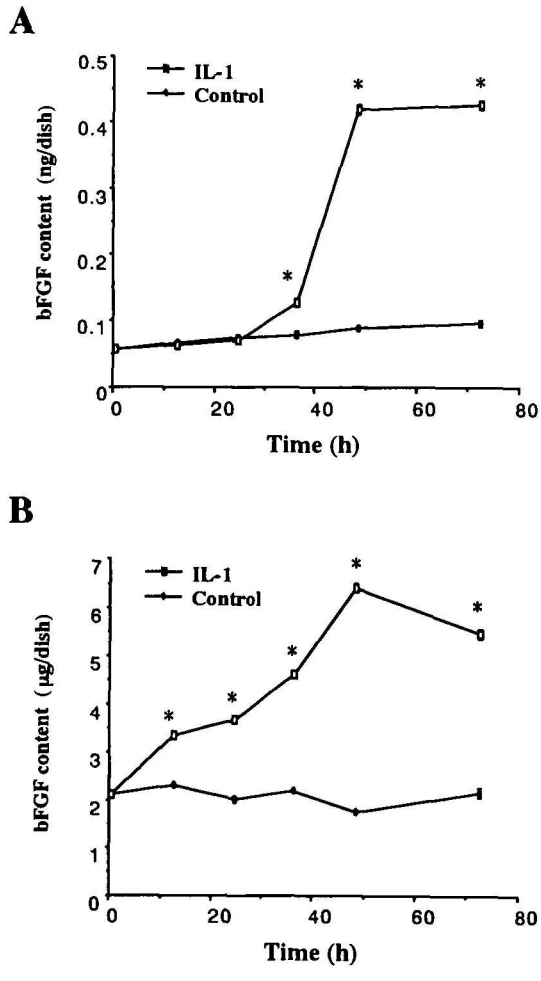

Fig. 2. **Time-dependent effect of IL-1 on the level of bFGF in an RAC culture.** When RAC cultured in a 6-cm diameter dish reached confluence, the cells were washed with DMEM containing 0.5% FBS and then further incubated in an aliquot of fresh medium for the indicated times with IL-1 at 1 ng/ml. PBS was added to control cultures. bFGF in the cell layer was extracted with PBS containing 3 M NaCl. The culture medium and the cell layer were collected at the indicated times and stored at −20°C until use. The bFGF concentrations in the medium (A) and the cell layer (B) were determined as described under "MATERIALS AND METHODS." Points and bars are the means and SD, respectively, for four cultures. *$p < 0.05$, significantly different from the control cultures.

iNOS—We recently reported that IL-1 induced high levels of NO production by RAC in a time-dependent manner (*20*). The nitrite level in the medium started to increase 12 h after the addition of IL-1 and reached a plateau at 48 h. The profile of this time-dependent change in NO production suggests that the production is due to iNOS induction (*26*). To prove this, we first investigated the expression of mRNA of iNOS in RAC treated with or without IL-1 at 1 ng/ml (Fig. 1). The top photograph shows the result of polyacrylamide-electrophoresis of the radiolabeled RT-PCR products. The bottom chart shows the relative radioactivity of the bands that were analyzed using a Bioimaging analyzer BAS2000 and corrected as to 18S rRNA as the standard. The relative mRNA level of iNOS increased as early as 12 h after the stimulation by IL-1, and then decreased gradually. In contrast, little expression of iNOS was observed in a control culture without IL-1. These characteristics of the induction of NO production suggest that iNOS is involved in the increase of NO production by IL-1-treated chondrocytes.

Effect of IL-1 on the Level of bFGF in an RAC Culture—As reported previously (*20*), the bFGF concentration in the CM of RAC treated with 1 ng/ml of IL-1 began to increase after 36 h, and reached a plateau at 48 h (Fig. 2A). In addition to the level of bFGF in the CM, the amount of bFGF in the cell layer also increased after the addition of IL-1. The increase in bFGF was observable as early as 12 h, the maximum level being reached after 48 h (Fig. 2B), suggesting that IL-1 stimulates not only the release of bFGF but also its production.

Effect of IL-1 on the Production of MMP-2, -9, and -3 by RAC—In an RAC culture, IL-1-stimulated production of MMP-9 preceded IL-1-stimulated release of bFGF (*20*). To investigate the involvement of another gelatinase, MMP-2, and stromelysin (MMP-3), which is known to degrade proteoglycan, one of the two major matrix components of cartilage, the CM from RAC treated with IL-1 at 1

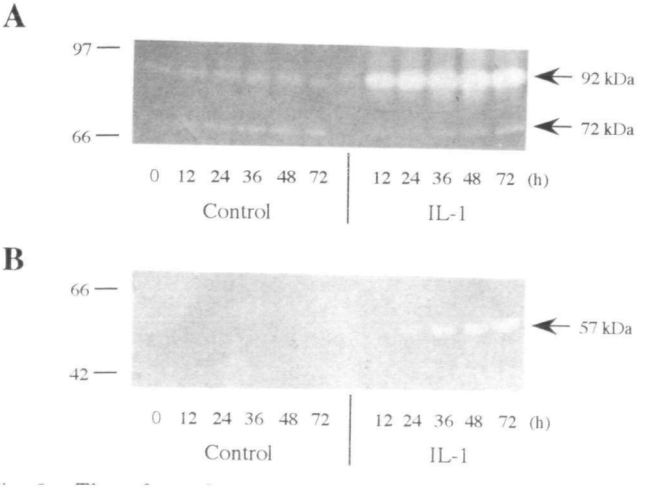

Fig. 3. **Time-dependent effect of IL-1 on the secretion of gelatinases (A) and caseinases (B) to the CM by RAC.** When RAC cultured in a 6-cm diameter dish reached confluence, the cells were washed with DMEM containing 0.5% FBS and then incubated in an aliquot of fresh medium for the indicated times with IL-1 at 1 ng/ml. After the incubation, the culture medium and the cell layer were collected and stored at −20°C until use. Gelatin- and casein-substrate zymographies were performed, respectively, as described under "MATERIALS AND METHODS." On the left margin are indicated the molecular weights of the marker proteins (×10³ Da).

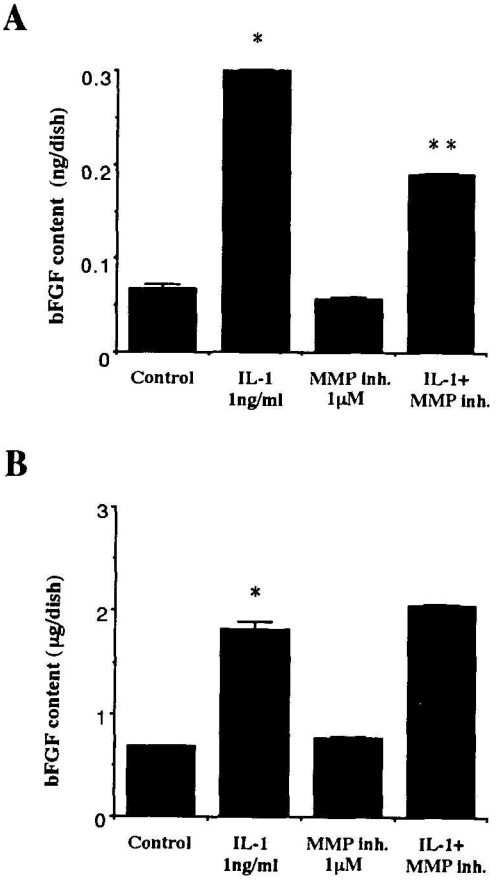

Fig. 4. **Effects of IL-1 and the MMP inhibitor on the level of bFGF in an RAC culture.** When RAC cultured in a 6-cm diameter dish reached confluence, the cells were washed with DMEM containing 0.5% FBS and then incubated in an aliquot of fresh medium for 48 h with IL-1 at 1 ng/ml in the presence or absence of the MMP inhibitor at 1 μM. The experimental procedures were the same as those described in Fig. 2. Columns and bars are the means and SD, respectively, for four cultures. (A) medium, (B) cell layer. $^{*}p<0.05$, significantly different from the control cultures. $^{**}p<0.05$, significantly different from the IL-1-treated cultures.

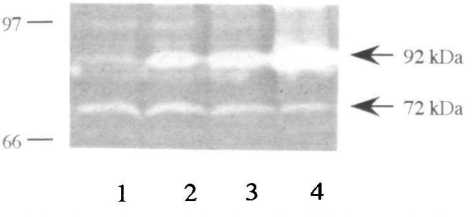

Fig. 5. **Additive effect of IL-1 and bFGF on gelatinases secreted to the CM by RAC.** When RAC cultured in a 6-cm diameter dish reached confluence, the cells were washed with DMEM containing 0.5% FBS and then incubated in an aliquot of fresh medium for 48 h with IL-1 at 1 ng/ml in the presence or absence of bFGF at 1 ng/ml. After the incubation, the CM and the cell layer were collected and stored at −20°C until use. Gelatin-substrate zymography was performed as described under "MATERIALS AND METHODS." On the left margin are indicated the molecular weights of the marker proteins (×10³ Da). Lane 1, control; lane 2, IL-1 (1 ng/ml); lane 3, bFGF (1 ng/ml); lane 4, IL-1 (1 ng/ml) plus bFGF (1 ng/ml).

ng/ml for 12, 24, 36, 48, and 72 h was analyzed for gelatinase and caseinase activity by the gelatin- and casein-substrate zymography techniques, respectively (Fig. 3, A and B). In addition to the production of a 92-kDa gelatinase (MMP-9), IL-1 at 1 ng/ml stimulated the production of a 57-kDa caseinase (MMP-3) in a time-dependent manner. This stimulation was marked because MMP-3 was not detected in the CM of a control culture (Fig. 3B). The production started to occur after 24 h and reached the maximum level after 48–72 h. Whereas the level of production of a 72-kDa gelatinase (MMP-2) was little changed by the treatment with IL-1.

Effect of an MMP Inhibitor on the IL-1-Stimulated Release of bFGF by RAC—To confirm that the release of bFGF by IL-1 was mediated by MMPs, we measured the bFGF concentration in the CM and cell layer after the addition of IL-1 in the presence of an MMP inhibitor. The MMP inhibitor inhibits the enzyme activity of MMPs, including MMP-1, -2, -9, and -3. When the MMP inhibitor, at 1 μM, was added together with IL-1 for 48 h, it partially suppressed the increase of bFGF in the CM by IL-1 and slightly increased the accumulation of bFGF in the cell layer by IL-1 (Fig. 4), suggesting that MMPs are at least partially involved in the release of bFGF by chondrocytes.

Effects of the Combination of IL-1 and bFGF on the Production of MMP-2 and -9 by RAC—bFGF has been shown to stimulate interstitial collagenase (MMP-1) production by endothelial cells (*27, 28*) and osteoblasts (*29*). To determine whether or not bFGF produced by IL-1-stimulated RAC has some effect on the production of MMPs by the cells, gelatin-substrate zymography was performed using the CM from chondrocytes treated with bFGF at 1

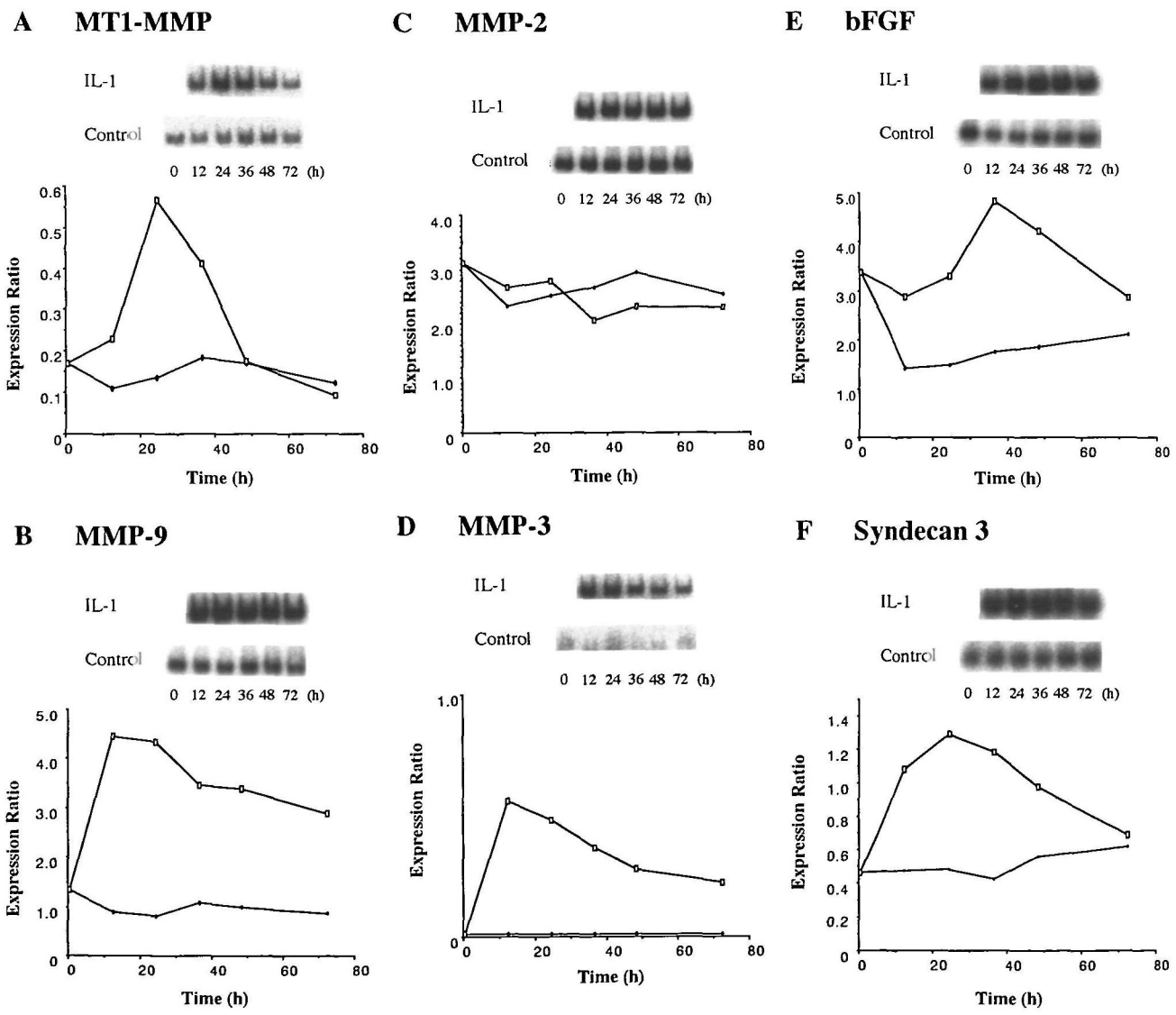

Fig. 6. **Time-dependent effect of IL-1 on the expression of mRNAs of MMPs, bFGF, and syndecan 3 in RAC.** When RAC cultured in a 6-cm diameter dish reached confluence, the cells were washed with DMEM containing 0.5% FBS and then incubated in an aliquot of fresh medium in the presence (□) or absence (●) of IL-1 at 1 ng/ml. Total RNA was isolated at the indicated times. RT-PCR was performed, and the PCR products were subjected to electrophoresis and exposed to imaging plates as described under "MATERIALS AND METHODS." The graphs show the relative expression levels compared with the level of 18S rRNA (photographs not shown). (A) MT1-MMP, (B) MMP-9, (C) MMP-2, (D) MMP-3, (E) bFGF, (F) syndecan 3.

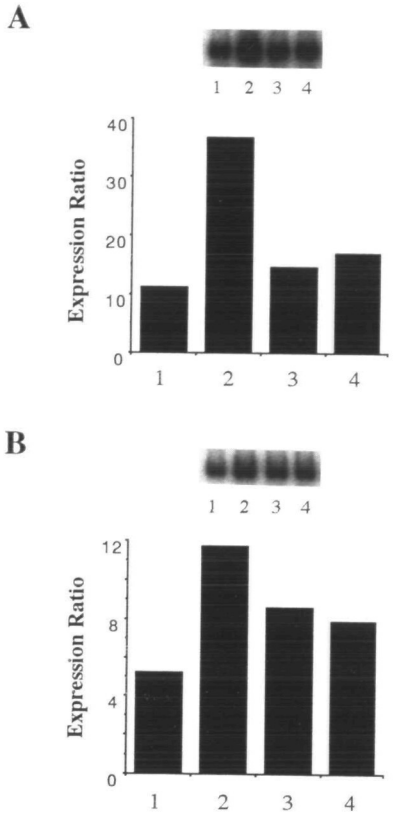

Fig. 7. **Effect of NMA on the expression of mRNAs of MMP-9 and bFGF in RAC.** When RAC cultured in a 6-cm diameter dish reached confluence, the cells were washed with DMEM containing 0.5% FBS and then incubated in an aliquot of fresh medium containing IL-1 in the presence or absence of NMA for 36 h. Other experimental procedures were the same as those described in Fig. 6. (A) MMP-9, (B) bFGF. Lane 1, control; lane 2, IL-1 (1 ng/ml); lane 3, NMA (3 mM); lane 4, IL-1 (1 ng/ml) plus NMA (3 mM).

ng/ml in the presence or absence of IL-1 at 1 ng/ml for 48 h (Fig. 5). bFGF alone stimulated the production of MMP-9 by RAC. The maximal stimulated level was almost the same as that with IL-1 (Fig. 5). When bFGF was added together with IL-1, the production of MMP-9 was much more stimulated. On the other hand, the production of MMP-2 was little affected by these treatments. These findings suggest that bFGF-stimulated MMP-9 production and IL-1-stimulated MMP-9 production occur through independent pathways.

Effect of IL-1 on the Expression Levels of mRNAs of MMPs, MT1-MMP, bFGF, and Syndecan 3 in RAC—To determine whether or not the production of MMPs and bFGF stimulated by IL-1 is due to the induction of gene expression, we performed RT-PCR using each primer with [α-^{32}P]dCTP. The mRNA levels of MT1-MMP and MMP-9 increased as early as 24-36 h and 12-24 h after the addition of IL-1, respectively, and then decreased gradually (Fig. 6, A and B). The mRNA level of MMP-3 also increased as early as 12 h after the addition of IL-1, but there was almost no transcript of MMP-3 in a control culture (Fig. 6D). Conversely, the mRNA level of MMP-2 did not change remarkably (Fig. 6C). The mRNA level of bFGF increased as early as 12 h after the addition of IL-1, the maximum level being reached after 36 h (Fig. 6E). The decrease in bFGF mRNA after 12 h in a control culture was reproducible and may be due to the effect of the decreased serum concentration. The mRNA level of syndecan 3, a bFGF binding proteoglycan, increased as early as 12-24 h after the addition of IL-1, and then decreased gradually (Fig. 6F).

Effect of NMA on the Expression of mRNAs of MMP-9 and bFGF in RAC—To determine whether or not NO mediates IL-1-stimulated production of MMP and bFGF at the transcriptional level or post-transcriptional level, the effects of NMA, an inhibitor of NO production, on the mRNA levels of MMP-9 and bFGF were investigated. As shown in Fig. 7, NMA inhibited the expression of MMP-9 mRNA and bFGF mRNA. These findings suggest that NO is involved in the expression of the mRNAs of MMP-9 and bFGF.

DISCUSSION

Recently, we showed that the accumulation of bFGF released into the CM after treatment with IL-1 began to increase after 36 h, and reached a maximum level at 72 h (Ref. *20*, Fig. 2A). In the present study, we also demonstrated that bFGF in the cell layer accumulated after the addition of IL-1 (Fig. 2B). The accumulation was apparent as early as 24 h after the addition of IL-1 and reached a maximum after 48 h. Moreover, the mRNA level of bFGF increased as early as 12 h after the addition of IL-1 (Fig. 6E). These findings indicate that IL-1 stimulates not only the release of bFGF, but also its production and its gene expression in cultured articular chondrocytes. Although Rivera *et al.* (*30*) found that IL-1β increases the bFGF mRNA levels in rat brain on *in situ* hybridization, the present study demonstrated for the first time that IL-1β stimulates the production of bFGF in articular chondrocytes at both the protein and mRNA levels.

It has been reported that MMP-9 digests native collagens IV, V, and XI, elastin, proteoglycan and gelatin (*31-34*). Although MMP-9 exhibits proteoglycan-degrading activity at pH 7.5, MMP-3 is a proteinase considered to be a major proteoglycan-degrading enzyme in cartilage. In the present study, we demonstrated that not only MMP-9 but also MMP-3 production preceded bFGF release from the chondrocyte cell layer (Figs. 2 and 3), further supporting that the MMPs induced by IL-1 degrade the RAC matrix composed of collagens and proteoglycans, resulting in the release of bFGF from the chondrocyte matrix.

There have been several reports that IL-1β induces cartilage degradation (*1, 2*), and that IL-1-stimulated chondrocytes synthesize proteoglycan-degrading enzymes, such as MMP-9 and MMP-3 (*6, 7*). When the MMP inhibitor, which suppresses the activity of MMPs, including MMP-1, -2, -3, and -9, was added to an RAC culture together with IL-1, the increased level of bFGF release from RAC caused by IL-1 was suppressed, and the accumulation of bFGF in the cell layer was slightly potentiated (Fig. 4). These findings suggest that the IL-1-stimulated release of bFGF from chondrocytes is not the result of overproduction of bFGF, although IL-1 stimulates the production of bFGF at the transcriptional level in these cells.

IL-1 stimulated the expression of mRNA of MMP-3 and -9 in RAC (Fig. 6), indicating that the production of these

MMPs stimulated by IL-1 is regulated at the transcriptional level. On the other hand, the mRNA level of MMP-2 did not change remarkably. Lefebvre *et al.* (*7*) found that IL-1 and TNF-α had no effect on the production of the 72-kDa gelatinase (proMMP-2) by chondrocytes on gelatin zymography. However, Sato *et al.* (*35*) reported that proMMP-2 can be activated by MT1-MMP on the cell membrane of invasive tumor cells. The expression of MT1-MMP in lung and gastric carcinomas is well correlated with the activation of proMMP-2 (*36, 37*), and recently, Ohuchi *et al.* (*8*) reported that MT1-MMP digests fibrillar collagen types I, II, and III, which are only cleaved by interstitial collagenases, MMP-1, -8, and -13. Moreover, inflammatory cytokines such as IL-1 and TNF-α stimulate the expression of mRNA of MT1-MMP in cultured articular chondrocytes of OA, and articular chondrocytes of OA, in which MT1-MMP appears, co-expressed MMP-2 in an immunohistological study (*38*). In the present study, the mRNA level of MT1-MMP, which can activate proMMP-2, was increased by the stimulation by IL-1. Therefore, although IL-1 did not stimulate the expression of MMP-2 at either the mRNA or protein level in an RAC culture, MT1-MMP may directly or indirectly *via* MMP-2 play an important role in IL-1-stimulated cartilage degradation.

There have been several reports that syndecan 3, a heparan sulfate proteoglycan, binds to bFGF (*39, 40*) and the growth factor being accumulated on the cell surface (*41, 42*). The level of syndecan 3 mRNA started to increase as early as 12 h, reached a maximum level after 24 h, and then gradually decreased (Fig. 6F). This increase slightly preceded the increase in the expression of bFGF mRNA. Although we did not determine the change in the amount of the gene product of syndecan 3, the gene expression of syndecan 3 may cause transient accumulation of newly synthesized bFGF on the chondrocyte cell surface after 12-24 h and some of the accumulated bFGF may stimulate transient stimulation of DNA synthesis in the chondrocytes themselves (*20*). After that, MMP production increases, and the MMPs may degrade proteoglycans including syndecan 3, resulting in the release of bFGF bound to syndecan 3.

Articular chondrocytes synthesize NO when treated with inflammatory cytokines, such as IL-1, TNF-α, and lipopolysaccharide, *in vitro* (*20, 43*). Murrell *et al.* (*44*) reported that IL-1β and TNF-α induced NOS activity in bovine and human cartilage. In the present study, we showed that this increase in NO production was due to iNOS induction. Murrell *et al.* (*44*) also reported that IL-1 stimulated not only NO production but also stromelysin and collagenase activity, and that these activities are inhibited by competitive inhibitors of NOS. We reported that the NO inhibitor, NMA, inhibited IL-1-stimulated MMP-9 production by RAC in culture (*20*). In the present study, we further demonstrated that NMA inhibited the expression of mRNA of MMP-9 (Fig. 7A). These findings indicate that NO produced by IL-1 stimulates MMP-9 production at the transcriptional level. Moreover, NMA inhibited the IL-1-stimulated increase in bFGF mRNA (Fig. 7B), indicating that NO produced by IL-1 also stimulates bFGF production at the transcriptional level. These are very important findings because they prove that the IL-1-increased MMP and bFGF levels in the CM are not due to cell death or degeneration.

There have been several reports that bFGF stimulates the expression of MMP-1 (interstitial collagenase) in various cells (*27-29, 45*), and angiogenic preparations that contain bFGF stimulate collagenase in human endothelial cells (*46*). Moreover, it has been reported that articular cartilage-degradation in the rabbit is induced through the synergism of bFGF and IL-1β (*47-49*). With regard to the synergism of IL-1 and bFGF, Chandrasekhar and Harvey (*48*) have suggested that the potentiation of IL-1 catabolic effects by bFGF is related to its ability to induce additional IL-1 receptors on the chondrocyte cell surface without changing their affinity. In the present study, we also demonstrated that when bFGF at 1 ng/ml alone was added to an RAC culture, the level of production of MMP-9 was nearly the same as that on the addition of IL-1 at 1 ng/ml (Fig. 5), and that the simultaneous addition of IL-1 and bFGF caused greater stimulation of the production of MMP-9 than the addition of IL-1 or bFGF alone (Fig. 5). These findings suggest that IL-1 causes both MMP and bFGF production to increase, and that the produced bFGF contributes to the long-lasting production of MMP-9.

In conclusion, the present study demonstrated the following new findings. (i) IL-1 augmented the production of bFGF at both the protein and mRNA levels in RAC in culture. (ii) Not only MMP-9 but also MMP-3 and MT1-MMP are involved in IL-1-stimulated bFGF release from the matrix of the cells. (iii) IL-1 stimulated the production of these MMPs at the transcriptional level. (iv) NO production by IL-1 is due to iNOS, and is involved in the IL-1-stimulated gene expression of MMPs and bFGF. (v) IL-1-stimulated bFGF production may cause further degradation of the matrix *via* MMP production. (vi) The IL-1-stimulated bFGF release is not simply due to overproduction of bFGF but also to degradation of the matrix including syndecan 3. It is through these molecular mechanisms that the degradation of articular cartilage followed by angiogenesis in the synovium toward cartilage is thought to occur in arthritic joints.

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