Induction of Osteogenic Protein-1 Expression by Interleukin-1 β in Cultured Rabbit Articular Chondrocytes

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YOSHIDA, S., KUBOTA, Y., TOBA, T., HORIUCHI, S. and SHIMAMURA, T. *Induction of Osteogenic Protein-1 Expression by Interleukin-1 β in Cultured Rabbit Articular Chondrocytes*. Tohoku J. Exp. Med., 2002, 197 (2), 101-109 — To elucidate the effects of interleukin-1 β (IL-1 β) on osteogenic protein-1 (OP-1) gene expression in a polylayer culture of rabbit articular chondrocytes, we measured rabbit OP-1 mRNA using quantitative TaqMan reverse transcriptase-polymerase chain reaction (RT-PCR) techniques. Rabbit articular chondrocytes were isolated and cultured in minimum essential medium eagle α modification containing 10% fetal bovine serum for 7 days. IL-1 β was then added and cultures were continued for 48 or 96 hours. OP-1 gene expression was detected in cell cultures both with and without addition of IL-1 β . However, the level of expression was very low in the control group. OP-1 gene expression was significantly increased about 450- to 800-fold in IL-1 β -treated groups (0.1, 1, and 10 ng/ml) versus the control group. Evaluation of serial changes in OP-1 expression after addition of IL-1 β (10 ng/ml) revealed that OP-1 gene expression increased rapidly after addition of IL-1 β , reaching a peak at 48 hours, and then decreasing. Simultaneous assay of CD44 expression demonstrated a rapid increase, similar to that of OP-1 expression, following addition of IL-1 β : this was followed by a more gradual increase. Assay of hyaluronan synthase-2 (HAS-2) expression following addition of IL-1 β showed an increase after OP-1 expression had already reached a peak. Our results demonstrate that OP-1 expression is induced by IL-1 β and suggest that this expression, like that of HAS-2, may play a role as a protective mechanism against inflammatory cytokines. ——— osteogenic protein-1; interleukin-1 β ; articular chondrocytes

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Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGF- β) superfamily and are involved in the proliferation and differentiation of a variety of cells (Wozney et al. 1988; Özkaynak et al. 1990, 1991; Sampath et al. 1990). BMPs not only play important roles in bone development and growth, but they also exhibit a variety of other biological activities such as determination of mesodermal induction and body axis in the early embryo (Dudley et al. 1995; Winnier et al. 1995).

Osteogenic protein-1 (OP-1), also known as BMP-7, belongs to this BMP subgroup. The amino acid sequence of OP-1 is highly homologous (42%~88%) with other BMP subgroup factors. In addition, there is about 35% homology with TGF- β 1 (Sampath and Reuger 1994). OP-1 gene expression has been identified in bone, kidneys, heart, eyes and growth plates (Lyons et al. 1995). More recently, OP-1 expression has also been reported in articular cartilage (Chubinskaya et al. 2000). OP-1, therefore, exerts an effect on osteogenesis and articular cartilage metabolism. Under no addition of interleukin-1 β (IL-1 β), human recombinant OP-1 induces collagen and proteoglycan expression in cultured articular cartilage matrix (Chen et al. 1993; Flechtenmacher et al. 1996). In addition to these effects on articular cartilage matrix, human recombinant OP-1 also induces expression of hyaluronan (HA) and CD44, which are involved in the cartilage matrix structure (Nishida et al. 2000b).

Recently, OP-1 gene expression has been shown to be increased in articular chondrocytes of patients with osteoarthritis (Chubinskaya et al. 2000). This result suggested that chondrocytes attempt to withstand catabolic events by upregulation of OP-1 mRNA in pathological cartilage. Osteoarthritis and rheumatoid arthritis are disorders in which inflammatory cytokines, particularly IL-1, act as mediators of joint inflammation and destruction (Pujol and Loyau 1987; Arend and Dayer 1995). Based

on these background, we expected that OP-1 gene may be induced by inflammatory cytokine in the pathological disorders such as osteoarthritis. The present study was designed to investigate whether an inflammatory cytokine induced OP-1 gene expression. Quantitative TaqMan RT-PCR was used to evaluate the effects of IL-1 β on OP-1 gene expression in a polylayer culture of rabbit articular chondrocytes. We also investigated the effects of IL-1 β on CD44 and hyaluronan synthase-2 HAS-2 gene expression.

MATERIALS AND METHODS

Materials

Minimum essential medium eagle alpha modification (α -MEM), trypsin type 250, collagenase type IA, kanamycin monosulfate, ascorbic acid (L-ascorbic acid 6-palmitate) and human recombinant IL-1 β were purchased by Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased by Gibco BRL (Eggenstein, Germany).

Isolation of chondrocytes

Articular chondrocyte samples were obtained from the shoulder, hip, and knee joints of mature Japanese white rabbits, weighting about 3 kg according to the method of Maeda et al. (2001). Articular cartilage was crushed with a scalpel, then treated with 10 ml of 0.1% trypsin at 37°C for 30 minutes to isolate chondrocytes from the extracellular matrix. Chondrocytes were centrifuged at 1500 $\times g$ for 5 minutes, and then washed with phosphate buffered saline without calcium and magnesium to remove the trypsin. Chondrocytes were incubated for 4 hours in 20 ml α -MEM containing 0.15% collagenase, passed through a 100 μ m (pore size) nylon sieve, and then washed with α -MEM.

Chondrocyte polylayer cultures

Isolated cells were suspended in α -MEM containing 10% FBS, 50 μ g/ml of ascorbic acid

and 60 μ g/ml of kanamycin. The chondrocytes (1.0×10^5 cells/ml) were transferred into 2 ml polypropylene microtubes (Assist Co., Ltd., Tokyo) and centrifuged at $1500 \times g$ for 5 minutes. Pelletized cells were incubated for 4 days at 37°C in 5% CO₂ and 95% air. The medium was exchanged, and pelletized cells were incubated for another 3 days. The medium was replaced with serum-free medium containing IL-1 β , followed by incubation for an additional 48 or 96 hours.

RNA extraction and cDNA synthesis

Total RNA was extracted using FastRNA™ Kit-Green (Bio 101, Carlsbad, CA, USA). Extracted RNA was treated with DNase I (RNase free, Takara, Kyoto) to avoid DNA contamination. The cDNA was synthesized from 0.1 μ g of total RNA using a random nucleotide primer and reverse transcriptase derived from the avian myeloblastosis virus (RNA-PCR Kit Ver. 2.1, Takara) under the following conditions: 30°C for 10 minutes, 42°C for 60 minutes and 95°C for 5 minutes (Noonan and Roninson 1988).

Determination of nucleotide sequence of rabbit OP-1 gene PCR product

The nucleotide sequence of the rabbit OP-1 gene was not previously determined. We there-

fore prepared the first OP-1 primers and determined the nucleotide sequence of the rabbit OP-1 gene PCR product using RT-PCR. The first OP-1 PCR primers were prepared in previously reported highly conserved regions among humans, dogs, and mice (Özkaynak et al. 1990, 1991; Ishibashi et al. 1993). We confirmed that PCR amplified products were of the expected sizes, then performed sequencing according to the dye terminator and dye primer methods using a ABI PRISM™ 377 DNA Sequencing System (PE Applied Biosystems, Tokyo). ABI PRISM™ Cycle Sequencing Kits (PE Applied Biosystems) were used according to the protocols supplied by the manufacturer.

Primers and probes

The PCR primers and probe for assay of OP-1 expression were prepared based on the determined rabbit OP-1 nucleotide sequence, using Primer Express™ software (PE Applied Biosystems) (Table 1).

Semiquantification of rabbit OP-1, CD44 and HAS-2 gene expression

RT-PCR was performed to confirm OP-1 expression. OP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCRs were conducted using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, NJ, USA). A

TABLE 1. *The primers and probes used in PCR and cDNA product size*

Gene	Primer and probe sequence (5' to 3')	Product size
OP-1	F: AGAGGGTGCGGCTGTCAA	114
	R: CTACATCCCGGGAGCGCTTT	
	Probe: TCCCTGCCCAAGTGCTCCTGCAG	
CD44	F: CAGCTGACCTCTGCAAGGC	78
	R: TGAACCCATACCTGCAGGTCTC	
	Probe: CCTGGTCCAGGGCCTTCTGCATCT	
HAS-2	F: TCAAGCATGGTATCTGAATCACAA	118
	R: AAAGTGTCTTGCATCATGCAGAAA	
	Probe: CCTGTACATAATCCACGCTTCGTCCCA	
GAPDH	F: CAACGTGTCGGTCGTGGA	72
	R: GTATGACGACATCAAGAAGGTGGT	
	Probe: TGACCTGCCGCTGGAGAAAGCTGCTAA	

F, forward; R, reverse.

TaKaRa PCR Thermal Cycler MP (Takara) was used with a pre-PCR heat step of 95°C for 5 minutes and heat steps of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute over a total of 30 cycles. PCR amplified products were analyzed by 8% polyacrylamide gel electrophoresis (PAGE) with ethidium bromide staining. For assays of OP-1, CD44 and HAS-2 gene expression, quantitative TaqMan RT-PCR was performed using an ABI PRISM™ 7700 Sequence Detector (PE Applied Biosystems). This TaqMan PCR method uses 0.01 µg of cDNA, 1×TaqMan Universal PCR Master Mix (PE Applied Biosystems), 0.2 µM of primer, and 0.1 µM of probe. Reaction products for mRNA extracted from Japanese white rabbits were used as the standard curve. Amplification conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 95°C for 15 seconds and 60°C for 1 minute over a total of 50 cycles. Generated data were analyzed using Sequence Detector 1.6 software. Each mRNA level was corrected for GAPDH mRNA level and semiquantified.

Statistical analysis

Data were expressed as the mean ± s.d. A multiple comparison test Bonferroni/Dunn (Dunn's procedure as a comparison procedure) was used, and *p*-values of less than 0.05 were considered significant.

RESULTS

Determination of nucleotide sequence of rabbit OP-1 gene PCR product

The nucleotide sequence for the rabbit OP-1 gene PCR product was highly homologous with previously reported human, dog, and mouse OP-1 gene sequences (Fig. 1). The homology was 92.0% between rabbits and humans, 90.9% between rabbits and dogs, and 88.6% between rabbits and mice.

Effect of each concentration of IL-1β on OP-1 gene expression after culture for 48 hours

To investigate the effects of IL-1β on OP-1 gene expression, we replaced the medium with serum-free medium, added 0 (control), 0.1, 1 or 10 ng/ml of IL-1β to each culture tube, continued cultures for an additional 48 hours, and then assayed gene expression. OP-1 gene expression in the cultured rabbit articular chondrocytes was initially confirmed using RT-PCR. Bands for OP-1 gene were confirmed for each IL-1β treated and control group (Fig. 2A). However, the level of OP-1 expression in the control group was extremely low. OP-1 expression was significantly increased in each IL-1β-treated group vs. the control group (about 450-fold higher in the 0.1 ng/ml group, 600-fold higher in the 1 ng/ml group, and 800-fold higher in the 10 ng/ml group) (Fig. 2B).

Serial changes in OP-1, CD44 and HAS-2 gene expression after adding IL-1β

To evaluate serial changes in OP-1, CD44 and HAS-2 gene expression after adding IL-1β, we replaced the medium with serum-free medium, added 10 ng/ml of IL-1β to each culture tube, and assayed levels of expression at multiple culture time points (0, 8, 16, 24, 48, 72 and 96 hours). OP-1 expression after the addition of IL-1β vs. the control group demonstrated a rapid increase, with about a 50-fold rise at 8 hours. OP-1 expression continued to increase, reaching a peak at 48 hours (about 800-fold increase), and then decreasing (Fig. 3A).

CD44 expression, like that of OP-1, also rapidly increased after addition of IL-1β vs. the control group. An increase of about 7-fold was observed at 8 hours. CD44 expression continued to gradually increase up to 96 hours (about a 7.5-fold increase) (Fig. 3B). HAS-2 expression did not significantly differ between the IL-1β group and control group up to 48 hours. However, a significant increase was

Rabbit 5'	ATGGTCATGAGCTTCGTCAACCTCGTGGAACACGACAAGGAATTCCTCCACCCGCGCTAC	
Human	-----T-----A-----	
Dog	-----G--T---A--G-----A--T---	
Mouse	-----A-----T---A-----T--A---	
Rabbit	CACCACCGCGAGTTCCGGTTCGATCTTTCCAAGATCCCCGAGGGAGAAGCAGTGACGGCC	
Human	----T--A-----T-----A--A--G----T--C----A	
Dog	-----G-----A-----C-----A--A--G----T-----T--A	
Mouse	----T--G-----G-----C---CGG----C--A	
Rabbit	GCCGAGTTCCGGATCTACAAGGA CTACATCCGGGAGCGCTTT GACAACGAGACGTTCCGC	
Human	----A-----A-----C-----T-----G	
Dog	----A-----A-----C-----A-----G	
Mouse	----A--A-----T-----G--A-----C---AG	
Rabbit	ATCCGCGTGTACCAGGTGCTGCAGGAGCACTTGGGCAGGGAGTCGGACCTCTTCCTG CTT	
Human	---A---T--T-----C-----A---T-----C	
Dog	---A---T-----A---G-----G	
Mouse	---ACA--C--T-----C-----CA-----T---G	
Rabbit	GACAGCCGCACCCTCT GGGCCTCGGAGGAGGGGTGGCTGGTCTTCGACATCACGGCCACC	
Human	-----T-----C-----G--T-----A-----	
Dog	-----C-----G-----A-----	
Mouse	-----A-----T--T-----C--T---G--T--T---A-----	
Rabbit	AGCAACCACTGGGTGGTCAACCCTCGGCACAACCTGGGCCTGCAGCTCTCGGTGGAGACT	
Human	-----T--G-----G	
Dog	-----A--A-----GC-----C	
Mouse	-----T-A-----T-----C	
Rabbit	CTGGATGGGCAGAGCATCAACCCCA-3'	Homology
Human	-----	92.0%
Dog	T---C-----	90.9%
Mouse	-----	88.6%

Fig. 1. Determination of nucleotide sequence of rabbit OP-1 gene PCR product. Homology was 92.0% between rabbits and humans, 90.9% between rabbits and dogs, and 88.6% between rabbits and mice. The frames indicate the positions of rabbit OP-1 PCR primers used in assays.

observed at 72 hours following the addition of IL-1 β (i.e., after OP-1 expression had already peaked). HAS-2 expression increased about 2-fold by 96 hours (Fig. 3C).

DISCUSSION

Inflammatory cytokines play an important

role as mediators of local inflammation and joint destruction in the pathogenesis of osteoarthritis and rheumatoid arthritis. IL-1 β plays a key role in this process. IL-1 β is predominantly produced by synovial cells and causes cartilage matrix destruction by stimulation of matrix metalloproteinases and active oxygen

Fig. 2. OP-1 and GAPDH expression in rabbit articular chondrocytes by RT-PCR (A) and effect of IL-1 β on OP-1 expression in chondrocytes (B). A: PCR bands for OP-1 expression (114 bp) are shown in the upper panel, and PCR bands for GAPDH expression (72 bp) are shown in the lower panel. The DNA maker was ϕ X174 DNA-Hae III digest. B: Effects of each concentration of IL-1 β on OP-1 expression are shown. Chondrocytes were cultured for 48 hours after adding IL-1 β . OP-1 expression is corrected for GAPDH expression. Values are mean $\pm$ s.d. ($n=15$). The asterisks indicate significant difference at $**p<0.01$.

(Dayer and Demczuk 1981; Arend and Dayer 1995). IL-1 β also inhibits proteoglycan and collagen synthesis in the cartilage matrix (Tyler and Benton 1988; Arner and Pratta 1989). We

have previously reported that IL-1 β decreases $\alpha 1$ (II), $\alpha 2$ (VI), $\alpha 1$ (IX) and $\alpha 2$ (XI) collagen gene expression in polyayer cultures of rabbit articular chondrocytes (Tajima et al. 1995;

Fig. 3. Serial changes in OP-1, CD44 and HAS-2 expression after adding IL-1 β . Chondrocytes were cultured for 0, 8, 16, 24, 48, 72, and 96 hours after adding IL-1 β . A: OP-1 expression. B: CD44 expression. C: HAS-2 expression. ●, IL-1 β 10 ng/ml; ○, control group. Values are mean $\pm$ s.d. ($n=9$). The asterisks indicate significant difference at ** $p < 0.01$, and * $p < 0.05$.

Goto et al. 1999).

In addition, IL-1 α has been shown to increase CD44 expression in articular chondrocytes (Chow et al. 1995; Nishida et al.

2000a). These previous reports suggest that IL-1 stimulates the expression of CD44, the chondrocyte receptor of HA, due to up-regulate of HA internalization. Thus, it is considered that the catabolic state of chondrocytes may enhance HA turnover. Furthermore, we have also reported that IL-1 β increases CD44 and RHAMM gene expression (Toba et al. 1997; Onodera et al. 1998; Tajima et al. 1999).

OP-1 is modified to the mature form after translation (Gray and Mason 1990; Özkaynak et al. 1990). In this study, we attempted to clarify the transcriptional regulation of OP-1 gene expression in a polylayer culture of rabbit articular chondrocytes and examined whether IL-1 β was involved in regulation of OP-1 gene expression. We found extremely low levels of OP-1 gene expression in the control cells. However, the level of expression rapidly increased in cells with the addition of IL-1 β . Moreover, OP-1 gene expression significantly increased 450- to 800-fold in an IL-1 β concentration-dependent manner. In a study by Huck et al. (1997), human recombinant OP-1 prevented decreases in proteoglycan synthesis caused by low concentrations of IL-1 β . Thus, the induction of OP-1 gene expression by IL-1 β may help new synthesis of matrix to maintain the microenvironment of the cartilage.

We evaluated serial changes in OP-1 gene expression following addition of IL-1 β . At 8 hours after adding IL-1 β , the level of OP-1 gene expression already increased about 50-fold vs. the control group, while $\alpha 1$ (II) collagen and aggrecan expression did not change significantly (data not shown). Significant decreases in $\alpha 1$ (II) collagen and aggrecan expression were observed from 16 hours (data not shown). Furthermore, OP-1 gene expression after addition of IL-1 β rapidly increased in comparison to expression of CD44 and HAS-2. These results suggest that increased OP-1 gene expression may represent direct regulation mediated via IL-1 receptors rather than secondary regulation due to destruction of cartilage matrix.

In this study, up-regulation of HAS-2 gene expression was observed after OP-1 gene expression. This result suggests that OP-1 may assist HAS-2 gene expression in IL-1 β -treated chondrocytes. However, the previous study reported that IL-1 α stimulated the expression of HAS-2 gene after 48 hours culture in human articular chondrocytes (Nishida et al. 2000a). Compared to our study, there are differences; IL-1 α or IL-1 β , alginate beads culture or polylayer pellet culture, human articular chondrocytes or rabbit articular chondrocytes. Further studies will be required to show the specific action of endogenous OP-1.

In conclusion, our study in a polylayer culture of rabbit articular chondrocytes is the first to confirm that IL-1 β remarkably increases levels of OP-1 gene expression, although it promotes destruction of cartilage matrix. This finding suggests that increased OP-1 expression serves as a protective mechanism against articular cartilage matrix destruction caused by the inflammatory cytokine IL-1 β .

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