Effects of interleukin-1 β on insulin-like growth factor-I autocrine/paracrine axis in cultured rat articular chondrocytes

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Abstract

Objective—To clarify the interaction of tissue destruction and repair of articular cartilage during inflammation, the effects of interleukin-1 β (IL-1 β) on the expression of insulin-like growth factor I (IGF-I), its receptor, and its binding proteins were examined.

Methods—Articular chondrocytes from five week rats were cultured in serum free medium treated with IL-1 β (1-100 U/ml) for 24 hours. The concentration of IGF-1 in the conditioned medium was measured by RIA, and IGF-BP were analysed by immunoligand blotting method. IGF-I receptors were also examined by [¹²⁵I]IGF-I binding study.

Results—IL-1 β induced the secretion of IGF-I and IGF-binding protein in chondrocytes; this was not inhibited by indomethacin (5 μ g/ml). IL-1 β also increased the number of IGF-I receptors but had no effect on receptor affinity. IL-1 β inhibited chondrocyte proliferation, while exogenous IGF-I and growth hormone stimulated chondrocyte cell growth. IL-1 β did not change IGF-I mRNA levels.

Conclusion—IL-1 β up-regulated the IGF-I autocrine/paracrine axis in cultured articular chondrocytes. These observations provide insight into the critical role played by IL-1 β in tissue destruction and repair, and into the direct interaction between cytokines and growth factors associated with inflammatory arthropathy.

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A stable equilibrium exists between the synthesis and degradation of extracellular matrix for the maintenance of normal cartilage function. Imbalances in this tissue, resulting either from the inhibition of matrix synthesis or from accelerated degradation, causes cartilage degeneration, ultimately leading to the destruction of cartilage tissue, that is seen in osteoarthritis or rheumatoid arthritis.¹

Interleukin-1 β (IL-1 β) inhibits chondrocytes from producing proteoglycan and stimulates the degradation of cartilage matrix.²⁻⁵ Chondrocytes are reported to produce IL-1 β .⁶ Furthermore, the concen-

tration of IL-1 β released from synovial cells, monocytes or macrophages in the synovial fluid of patients with rheumatoid arthritis is reported to be elevated.^{7,8} This cytokine is thought to regulate the local metabolism of chondrocytes in an autocrine/paracrine fashion. Its precise function, however, is still unknown. There is a report that IL-1 β appears during the early phase of wound healing and is important for triggering repair.⁹ In articular cartilage, tissue repair is seen in the early phase of osteoarthritis.¹⁰ After injection of IL-1 β into the rabbit knee joint, glycosaminoglycan synthesis was initially depressed, but at one week the rate of synthesis had almost doubled compared with the normal side.¹¹ In addition, IL-1 receptor antagonist protein has recently been reported to be effective as a form of gene therapy in experimental arthritis.¹²

Insulin-like growth factor I (IGF-I) plays an important role in increasing matrix synthesis^{13,14} and the expression of IGF-I mRNA in articular chondrocytes is increased in osteoarthritis.¹⁵ This evidence indicates the possibility of tissue repair in articular chondrocytes due to the increase of growth factors, such as IGF-I, in response to cytokines such as IL-1 β . This study was therefore carried out to clarify the effects of IL-1 β on cultured articular chondrocytes and to determine whether or not this regulates IGF-I, IGF-binding proteins (BP) production, and the relevant levels in these cells. The role played by IGF-I in the autocrine/paracrine axis in inflammatory arthropathy was also examined.

Materials and methods

MATERIALS

Recombinant human IL-1 β (specific activity 1×10^7 U/mg) was a gift from Otsuka Pharmaceutical (Tokushima, Japan); the IGF-I assay kit developed by the Nichols Institute was supplied by Eiken Chemical (Tokyo, Japan). The prostaglandin E2 (PGE₂) RIA kit and [¹²⁵I]-Iodotyrosyl IGF-I were purchased from Amersham International (Tokyo, Japan). Eagle's Minimum Essential Medium was obtained from Nissui Pharmaceutical (Tokyo, Japan) and fetal bovine serum from Gibco (Grand Island, NY). Collagenase type 2A was obtained from Sigma Chemical (St Louis, MO). The other materials used were commercial products of the highest grade available.

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CHONDROCYTE CULTURE

Chondrocytes were isolated from the articular cartilage of five week old male Sprague-Dawley rats, in accordance with methods detailed elsewhere.¹⁶ Each experiment involved 10 rats. In brief, cartilage removed from both the shoulders and the femurs was dissected into small pieces. The pieces were treated with trypsin for 30 minutes, followed by a two hour treatment with 0.2% collagenase. The cells released after digestion were seeded at 1×10^5 cells/ml in Eagle's MEM medium containing 10% fetal bovine serum plus glutamine and were grown to confluence in an atmosphere of 5% CO₂/95% air at 37°C. When primary cultures reached confluency, the monolayers were washed with PBS and enzymatically detached by a mixture of 0.1% trypsin and 0.02% EDTA, and subcultured for the definitive experiments. Cells were seeded into 100 mm diameter dishes for RNA preparation and IGFBP detection, into 24-well plates for other assays, at a density of 1×10^4 cells/cm² in Eagle's MEM with 10% FCS.

MEASUREMENT OF IGF-I PRODUCTION

Medium IGF-I concentration was measured with a RIA, using the commercially available human IGF-I assay kit developed by the Nichols Institute with a rabbit polyclonal anti-human IGF-I antiserum. The sensitivity of IGF-I was 0.05 ng/nl. This IGF-I kit measures free IGF-I, and there was no cross reactivity with IGF-II and insulin. The samples were neither extracted nor treated before the kit was used to determine the IGF-I levels.

MEASUREMENT OF PGE₂ PRODUCTION

PGE₂ concentration in the medium was determined with an RIA kit. Before analysing extracted samples using this assay system it is necessary to convert extracted prostaglandin E₂ into its methyl oximate derivative using the methyl oximation reagent provided.

DETECTION OF IGF-BINDING PROTEINS

To analyse IGFBP, chondrocytes were cultured to confluence in tissue culture plates (100 mm diam) in Eagle's MEM containing 10% FCS, and then incubated for a further 24 hours in 5 ml serum-free medium. Test substances (IL-1 β , Indomethacin) were added in the medium and incubations were continued for 24 hours. Conditioned medium (5 ml) were collected and concentrated 20-fold by lyophilisation, which was then reconstituted in Laemmli sample buffer in preparation for electrophoretic analysis.¹⁷ Prepared samples and standards were fractionated under non-reducing conditions on 12% SDS-polyacrylamide gels for four hours at 100 V, then transferred electrophoretically to nitrocellulose membrane. After electroblotting, IGFBP were detected by a ligand blotting method described by Hossenlopp *et al.*¹⁸ Briefly, the membrane was soaked first for 30 minutes in saline (0.15 M NaCl-0.01 M Tris HCl, Ph 7.4) containing

3% Nonidet P-40, then for two hours in saline containing 1% BSA, and finally for 10 minutes in saline containing 0.1% Tween-20. It was then exposed to 400 000 cpm/ml [¹²⁵I]IGF-I in saline and incubated overnight. The membrane was washed in saline and exposed to Kodak film for four days at -70°C.

[¹²⁵I]IGF-I BINDING STUDY

Cells were plated at a density of 2×10^4 cells/well in 24-multiwells in Eagle's MEM containing 10% FCS. When the cultures reached confluency, the medium was replaced with 1 ml serum-free medium and maintained for 24 hours, and then incubated with or without IL-1 β for a further 24 hours; 1.2×10^5 cells/well were used for these binding studies. After the medium was aspirated, monolayers were washed with phosphate buffered saline and then incubated with [¹²⁵I]-labelled IGF-I (50 000 cpm) plus various concentrations of unlabelled IGF-I. After incubations were carried out at 4°C for 12 hours, cell layers were rinsed twice with cold PBS and solubilised to determine radioactivity in a gamma counter.

RNA EXTRACTION AND NORTHERN BLOT ANALYSIS

Chondrocyte RNA was extracted with acid-guanidium thiocyanate and phenol chloroform.¹⁹ Thirty micrograms of total RNA was denatured and separated by electrophoresis on 1% agarose formaldehyde gel as described previously.²⁰ After electrophoresis, the RNA was blotted on to a nitrocellulose membrane. It was then prehybridised and hybridised with a ³²P-labelled cDNA probe. Hybridisation signals were detected by exposure to x ray film at -70°C. Mouse IGF-I cDNA was provided by G I Bell.²¹ To confirm the specificity of articular chondrocytes, we used rat $\alpha 1$ (II) collagen cDNA and rat aggrecan cDNA, provided by Y Yamada.²²⁻²⁴

Values were expressed as the mean (SEM). Data were compared using Student's 2-tailed *t*-test, and *p* values less than 0.05 were considered significant.

Results

Cell specificity in rat articular chondrocytes

As we used second passaged rat chondrocytes, we tried to ascertain whether the specific characteristics of articular chondrocytes were maintained. Type II collagen and aggrecan were selected as chondrocyte-specific markers. Type II collagen mRNA species (5.5 Kb) and aggrecan mRNA expression (9 Kb) were detected (fig 1). These results confirmed that the chondrocytes used in these studies had the characteristics of articular chondrocytes in that they produced type II collagen and aggrecan.

EFFECTS OF IL-1 β ON IGF-I LEVELS IN THE CULTURE MEDIUM

Confluent cultured rat articular chondrocytes were incubated with IL-1 β (1-200 U/ml) in

Figure 1 Expression of $\alpha 1(II)$ collagen and aggrecan mRNA. The second passaged cells were subjected to Northern gel analysis, as described in Materials and methods. $\alpha 1(II)$ collagen mRNA was detected in size 5.5 kb, aggrecan mRNA was detected in about 9 kb.

serum-free medium for 24 hours, and the conditioned media were assayed for IGF-I. IL-1 β stimulated the release of IGF-I at 50 U/ml and higher concentrations. The maximal stimulation of IGF-I release was relatively constant after stimulation with IL-1 β at 50 to 200 U/ml (fig 2). Further stimulation by IL-1 β was not observed after 48–72 hours of treatment (data not shown).

The effects of indomethacin and cyclohexamide on IL-1 β -induced IGF-I release were also examined. The chondrocytes were stimulated with IL-1 β (2–200 U/ml) in serum-free medium for 24 hours and the conditioned media were assayed for PGE₂. With increasing concentrations of IL-1 β , the PGE₂ levels were notably elevated. When indomethacin (5 μ g/ml) was added, in the presence of IL- β ,

Figure 2 IGF-I levels in culture medium after 24 hour treatment with or without IL-1 β . IL-1 β (50–200 U/ml) significantly increased the IGF-I concentration of the medium. [$n = 4$, mean (SE), * $p < 0.001$].

Figure 3 Prostaglandin E₂ levels in culture medium after 24 hour treatment. IL-1 β significantly stimulated PGE₂ levels in the chondrocytes medium, however, indomethacin (5 μ g/ml) completely blocked the synthesis of PGE₂. [$n = 4$, mean (SE), * $p < 0.001$].

the synthesis of PGE₂ was completely blocked (fig 3).

However, indomethacin did not inhibit the release of IGF-I induced by IL-1 β , although cyclohexamide, a protein synthesis inhibitor, significantly reduced the IGF-I secretion induced by IL-1 β (fig 4).

DEGRADATION STUDY OF IGF-I IN CULTURED MEDIA

Using two different methods, we examined whether IGF-I in the medium was degraded by IL-1 β .

(1) [¹²⁵I]IGF-I (100 000 cpm/well) was added to the serum-free medium of confluent chondrocytes with or without added IL-1 β and the degradation of this radiolabelled ligand was determined by measuring the radioactivity. Incubation medium, collected at various times, was mixed with 10% trichloroacetic acid. After 30 minutes of incubation at 4°C, the radioactivity of the precipitate was determined in a

Figure 4 Effects of indomethacin and cyclohexamide on IL-1 β -induced IGF-I secretion. Indomethacin (5 μ g/ml) did not inhibit this secretion, while cyclohexamide (1 μ g/ml) significantly inhibited it. [$n = 4$, mean (SE), * $p < 0.001$].

Figure 5 Displacement of [125 I]IGF-I from rat articular chondrocyte monolayers by increasing concentrations of unlabelled IGF-I (0.01–200 ng/ml). (n = 3.)

Figure 6 Scatchard analysis of IGF-I specific binding in rat articular chondrocyte with or without added IL-1 β . The Kd values, which means binding affinity, reflected by the slopes are similar, but the Bmax, which means overall binding capacity, derived from abscissa intercept is greater for articular chondrocytes treated with IL-1 β than that for cells not so treated.

Figure 7 Western blot analysis of IGF binding proteins in culture medium. Samples were fractionated on 12% SDS-polyacrylamide gels and then transferred on to nitrocellulose membrane. After electroblotting, the membrane was soaked for 30 minutes in saline (0.15 M NaCl-0.01 M Tris HCl, pH 7.4) containing 3% Nonidet P-40, then for two hours in saline containing 1% BSA, and finally for 10 minutes in saline containing 0.1% Tween-20. It was then exposed to 400 000 cpm/ml [125 I]IGF-I in saline and incubated overnight. The membrane was then washed in saline and exposed to Kodak film for four days at -70°C .

Figure 8 Northern gel analysis of IGF-I mRNA expression in cultured articular chondrocytes. Lane 1; control, lane 2; IL-1 β treatment. Lane 3 shows IGF-I mRNA extracted from MC3T3-E1. Cultured chondrocytes (1×10^6) were subjected to total RNA extraction. Each lane contained 30 μg of total RNA. After hybridisation with [32 P]-mIGF-I cDNA, the blot paper was autoradiographed for 48 hours, as described in the Method section. The same paper was washed and then IGF-I mRNA was detected in size 9 kb.

gamma counter (Aloka). The results were expressed as percentages of the radioactivity in the control medium without IL-1 β . There was no significant difference in the time courses (data not shown).

(2) Unlabelled IGF-I (50 ng/ml) was added to the serum-free medium of confluent chondrocytes with or without IL-1 β . IGF-I content in the medium was determined by RIA at various times. No degradation of the IGF-I medium occurred in the presence of IL-1 β during 40 hour incubation (data not shown).

[125 I]IGF-I BINDING ON CHONDROCYTES

Figure 5 shows the binding of [125 I]IGF-I to chondrocytes incubated with various concentrations of unlabelled IGF-I. Half maximal binding was obtained at a concentration of 1 ng/ml and that of 1.5 ng/ml–2 ng/ml in the IL-1 β treated cells. Scatchard analysis showed that the dissociation constant (Kd) of IGF-I binding was similar for control and IL-1 β -treated cells. In contrast, the number of IGF-I receptors per cell was significantly increased, in a dose-dependent manner, after the addition of IL-1 β (fig 6).

EFFECTS OF IL-1 β ON DNA SYNTHESIS

Confluent chondrocytes were cultured in serum-free medium with or without IL-1 β for 24 hours and [3 H]-thymidine incorporation was determined. There was no increase in the uptake of [3 H]-thymidine. However, IGF-I and growth hormone stimulated chondrocyte DNA synthesis (data not shown).

IGF-BINDING PROTEINS IN THE MEDIUM

We examined IGF-binding proteins in the serum-free medium of confluent chondrocytes incubated with or without IL-1 β (100 U/ml) for 24 hours. Control medium without IL-1 β contained a major band at 30 kD and a faint band at 41 kD. The intensity of the 41 kD band was increased in the medium when it was treated with IL-1 β . Almost the same results were obtained in the medium of chondrocytes incubated with IL-1 β and indomethacin (5 μ g/ml) (fig 7).

EFFECTS OF IL-1 β ON THE EXPRESSION OF IGF-I mRNA

The level of IGF-I mRNA was not altered in IL-1 β -treated cells (fig 8).

Discussion

In this study, we have demonstrated for the first time that IL-1 β stimulates articular chondrocytes to release IGF-I and IGFBP into culture medium. The specificity and properties of articular chondrocytes after treatment were confirmed by the presence of type II collagen and aggrecan mRNAs. IGF-I release was observed at 50 U/ml of IL-1 β , the response being dose-dependent. The response was not time-dependent, and medium IGF-I concentrations were almost the same after incubation with IL-1 β for 24 and 48 hours (data not shown). The intracellular IGF-I levels were too low to compare the differences between control and IL-1 β treatment. As there was a possibility that the degradation of IGF-I in the medium was modulated by proteinases induced by IL-1 β , we examined IGF-I degradation after adding labelled or unlabelled IGF-I, and confirmed that the changes of IGF-I degradation did not occur in this system. This shows that IL-1 β temporarily stimulated chondrocytes to release IGF-I without stimulating the synthesis of IGF-I, as shown by the finding that IL-1 β did not stimulate the expression of IGF-I mRNA. As IL-1 β acts to increase the secretion of IGF-I without affecting its mRNA level, several possibilities are proposed. However, the precise mechanism, translational control of the IGF-I secretory control, remains to be clarified.

IL-1 is reported to stimulate mouse calvaria to produce IGF-I by a prostaglandin-dependent mechanism,²⁵ and PGE₂ has been reported to stimulate IGF-I production in rat osteoblast enriched cultures.²⁶ In our study, however, indomethacin (5 μ g/ml) did not block the release of IGF-I from chondro-

cytes stimulated by IL-1 β , which is considered to be a PGE₂-independent mechanism. In contrast, the findings that cyclohexamide inhibited IGF-I production demonstrated that new protein synthesis was required for IL-1 β to exert its action on the release of IGF-I.

IGFBP play an important role in regulating IGF availability.²⁷ IGFBP in human serum consist of five molecular forms, 41.5, 38.5, 34, 30 and 24 kD; 41.5 and 38.5 kD BP are regulated by growth hormone, corresponding to IGFBP-3.²⁸ It is reported that IGFBP-3 was not detected in rabbit chondrocytes medium, but that the 30 kD form was predominant.²⁹ In this study, we showed that rat chondrocytes produced two major bands of IGFBP, that is, 30 and 41 kD. In the control medium the 30 kD band was distinct, while the 41 kD band increased after the addition of IL-1 β . This 41 kD band corresponds to IGFBP-3 in human serum. However, the exact role of the 41 kD BP is still unknown.

The concentration of IGF-I induced by IL-1 β in the medium was too low (less than 0.5 ng/ml) to affect the proliferation of chondrocytes by an autocrine mechanism. That these levels of IGF-I do not overcome the inhibitory effect of IL-1 β on DNA synthesis is shown by findings that the incorporation of 3 H-thymidine or 35 S-sulphate in rabbit chondrocytes was stimulated by IGF-I at concentrations of over 10 ng/ml.³⁰ Furthermore, a 100 ng/ml concentration of IGF-I was necessary for the inhibition of IL-1 induced matrix degradation of proteoglycans in an explant culture of pig articular cartilage,³¹ findings not inconsistent with our results.

In experimental arthritis, the non-responsiveness of chondrocytes to IGF-I has been reported by Joosten.³² In our study, however, there was no damage to chondrocyte IGF-I receptors, rather, the number of IGF-I receptors was increased by IL-1 β . As it has been reported that prostaglandin F₂ α stimulated the number of IGF-I receptors of MC3T3-E1,³³ it is possible that the increase of IGF-I receptors in articular chondrocytes may be mediated by prostaglandins, independently of the IGF-I secretion pathway.

Cartilage tissues are known to be destroyed by PGE₂ and metalloproteinases released from chondrocytes stimulated by IL-1 β . Conversely, IL-1 β may trigger tissue repair by releasing IGF-I. The complexity of IGF-I, BP, and receptor interaction, and the up-regulation of these growth factors by IL-1 β , might contribute to the stable balance that exists between the synthesis and the degradation of extracellular matrix. The IL-1 β -induced IGF-I autocrine/paracrine axis, however, is not sufficient to repair tissue destruction.

In conclusion, various cytokines and growth factors may be important in promoting the repair of articular cartilage degradation. IL-1 β may affect the post-receptor pathways of IGF-I signal transduction to inhibit tissue repair in the articular cartilage.

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