# Effects of Interleukin-1 on Syntheses of Alkaline Phosphatase, Type X Collagen, and 1,25-Dihydroxyvitamin $D_3$ Receptor, and Matrix Calcification in Rabbit Chondrocyte Cultures

**Yukio Kato,* Kazuhisa Nakashima,* Masahiro Iwamoto,‡ Hiroshi Murakami,‖ Hiroko Hiranuma,‡ Tatsuya Koike,§ Fujio Suzuki,§ Hajime Fuchihata,‡ Yukio Ikehara,¶ Mitsuhide Noshiro,* and Akitoshi Jikko‡**

**Department of Biochemistry, School of Dentistry, Hiroshima University, Hiroshima 734, Japan; Departments of ‡Radiology and §Biochemistry, Faculty of Dentistry, Osaka University, Suita, Osaka 565, Japan; ‖Department of Pediatrics, Medical School, Osaka City University, Osaka 545, Japan; and ¶Department of Biochemistry, Fukuoka University, School of Medicine, Fukuoka 814-01, Japan*

## Abstract

**The effect of IL-1 on expression of the mineralization-related phenotype by chondrocytes was examined. In cultures of rabbit growth plate chondrocytes, IL-1 beta at 0.1 ng/ml caused 95% decreases in alkaline phosphatase activity, alkaline phosphatase mRNA levels, the incorporation of $^{45}Ca$ into insoluble material, and the calcium content during the hypertrophic stage. These effects of IL-1 beta were dose-dependent and were observed in 24–48 h. Furthermore, IL-1 beta suppressed increase in cell size and the syntheses of 1,25-dihydroxyvitamin $D_3$ receptor and type X collagen, other markers of hypertrophy, but had little effect on the synthesis of total protein including type II collagen. The inhibition of calcification was observed only when chondrocytes were exposed to IL-1 before the onset of calcification: IL-1 treatment from the mineralization stage had a marginal effect on $^{45}Ca$ incorporation into insoluble material. These results suggest that IL-1 inhibits chondrocyte hypertrophy and the onset of calcification in ossifying cartilage. (*J. Clin. Invest.* 1993. 92:2323–2330.) Key words: mineralization • cartilage • cytokine • ossification • fracture**

## Introduction

IL-1, produced by monocytes and other cell types, induces various immune responses and serves as a mediator of inflammation (1, 2). In addition, IL-1 has a variety of metabolic, endocrinologic and neurological actions (1, 2).

IL-1 has been shown to inhibit proliferation of overtly differentiated chondrocytes (3), although it stimulates proliferation of poorly differentiated chondrocytes (3) and fibroblasts (4). It also suppresses the syntheses of type II collagen (5) and proteoglycans (6) by cultured chondrocytes in certain conditions. In addition, it induces the releases of prostaglandin $E_2$, neutral metalloproteinases such as collagenase, and serine proteinases including plasminogen activator by synovial fibroblasts and chondrocytes (7–13), causing breakdown of the cartilage matrix (14). Furthermore, IL-1 stimulates formation of osteoclasts and bone resorption (15, 16). However, nothing is yet known about the effect of IL-1 on the expression of mineralization-related phenotypes by hypertrophying chondrocytes, because no good experimental model has been available.

Recently, we found that rabbit growth plate chondrocytes maintained as a pelleted mass in a centrifuge tube produce alkaline phosphatase (ALPase),[1] a marker of terminal differentiation (17), at as a high level as that in growth plates in vivo (18–21). Furthermore, the extracellular matrix synthesized by these cells is calcified during the hypertrophic stage in the absence of added beta-glycerophosphate or inorganic phosphate (18). This system of well-differentiated chondrocytes allowed us to examine the effects of IL-1 on chondrocyte hypertrophy and mineralization. Results showed that IL-1 beta at 0.01–0.1 ng/ml inhibited the increases in ALPase activity and mineralization in pelleted chondrocyte cultures. IL-1 also suppressed increase in cell size and the syntheses of ALPase mRNA, 1-alpha, 25-dihydroxyvitamin $D_3$ ($1,25(OH)_2$vitamin $D_3$) receptor and type X collagen in mass cultures on collagen gels. These results suggest that IL-1 inhibits the expressions of all terminal differentiation phenotypes by chondrocytes, and that it is a potent inhibitor of endochondral bone formation. These findings seem important for understanding the roles of IL-1 in fracture callus and arthritic cartilage.

## Methods

*Materials.* Recombinant preparations of human IL-1 alpha and beta were generous gifts from Dr. Y. Hirai (Otsuka Pharmaceutical Co., Tokushima, Japan). Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO), type II collagen was from Koken Co. (Osaka, Japan), and a bovine parathyroid hormone fragment [1–34] (PTH[1–34]) was from the Peptide Institute Inc. (Osaka, Japan). $1,25(OH)_2$vitamin $D_3$ was supplied by Dr. Y. Nishii (Chugai Pharmaceutical Co., Tokyo). 1,25-Dihydroxy[26,27-methyl-$^3H$]cholecalciferol (180 Ci/mmol) was obtained from Amersham International (Amersham, United Kingdom), and [$^{45}Ca$]$CaCl_2$ (37 mCi/mg) and L-[$^{35}S$]-methionine (800 Ci/mmol) was from New England Nuclear (Boston, MA).

*Cell culture.* Chondrocytes were isolated from growth plates of the ribs of 4-wk-old male New Zealand White rabbits, as described previously (22, 23). The cells ($8 \times 10^4$) were suspended in 1 ml of Eagle's MEM with 10% fetal bovine serum, 50 μg ascorbic acid, and 60 μg kanamycin (medium A), transferred to a 15-ml plastic centrifuge tube (model 25319; Corning Glass Inc., Corning, NY), and centrifuged at 500 *g* (1,500 rpm in a clinical centrifuge) for 5 min (18). The resulting

Address correspondence to Yukio Kato, Department of Biochemistry, School of Dentistry, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima City, 734, Japan.

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1. *Abbreviations used in this paper:* ALPase, alkaline phosphatase; *p*NP, *para*-nitrophenyl phosphate; $1,25(OH)_2$vitamin $D_3$, 1-alpha,25-dihydroxyvitamin $D_3$.

cell pellet was incubated at 37°C under 5% $CO_2$ in air. Cultures were fed with fresh medium A (1 ml) 6 d after seeding, and thereafter, the medium was changed every other day. Ascorbic acid was added every other day during the whole culture period.

In other experiments, chondrocytes were seeded at high density ($1.5 \times 10^4$ cells per 6-mm microwell or $3 \times 10^5$ cells per 50-mm dish) into tissue culture dishes or type II collagen–coated dishes, and maintained in 0.2 or 10 ml of Eagle's medium, alpha-modification (Flow Laboratories, McLean, VA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 50 μg of ascorbic acid, 32 U of penicillin, and 40 μg of streptomycin per milliliter (medium B) or Dulbecco's medium, Iscove's modification (Flow Laboratories) supplemented with 10% fetal bovine serum, 50 μg/ml of ascorbic acid, and antibiotics (medium C). The cultures were fed with fresh medium B or medium C 3 d after seeding, and thereafter, the medium was changed every other day.

Fibroblasts were grown out from minced muscle tissue of 4-wk-old male New Zealand White rabbits on 10-cm plastic culture dishes in medium A. They were harvested with trypsin, seeded at $1.5 \times 10^4$ cells per 6-mm collagen-coated well, and maintained in medium B.

*Measurement of ALPase activity.* ALPase activity was measured by a modification of the method of Bessey et al. (24) with *para*-nitrophenyl phosphate (*p*NP) as substrate. Cell pellets or cell matrix layers from cultures were homogenized with a glass homogenizer in 0.9% NaCl/0.2% Triton X-100 at 0°C and centrifuged for 15 min at 12,000 *g*. The activity of the supernatant, which contained 95% of the total activity, was assayed in 0.5 M Tris-HCl buffer (pH 9.5) supplemented with 0.5 mM *p*NP and 0.5 mM $MgCl_2$. The mixture was incubated at 37°C for 10–30 min, and the reaction was stopped by addition of 0.25 vol of 1 M NaOH. Hydrolysis of *p*NP was monitored as change in $A_{410}$ in a spectrometer (Hitachi America Ltd., Brisbane, CA). *para*-Nitrophenol was used as a standard. 1 U of activity was defined as that catalyzing hydrolysis of 1 μmol of *p*NP/μg DNA · 30 $min^{-1}$. ALPase activity associated with matrix vesicles and released into the medium was also determined. For this, the medium was centrifuged at 800 *g* for 10 min to remove cells and cell debris, and the matrix vesicles were recovered from the supernatant by filtration as described by Habuchi et al. (25). Matrix vesicles recovered on filters (Millipore Corp., Bedford, MA) were solubilized with 2% Zwittergent (Calbiochem Corp., La Jolla, CA) in Tris-saline (0.15 M NaCl in 3.5 mM Tris-HCl, pH 7.4) (25), and portions of the solution were assayed for ALPase activity.

*Northern analysis.* Growth plate chondrocytes were seeded ($5 \times 10^5$ cells per 10-cm dish coated with type II collagen), and maintained in the presence of 30 ml of medium B. Total RNA was extracted by the guanidine HCl method (26). Samples of 15 μg of total RNA were denatured in the presence of 2.2 M formaldehyde and 50% formamide at 65°C for 10 min, subjected to electrophoresis on 1% agarose gel containing 0.66 M formaldehyde, and transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) (27). The RNA bound to the Nytran filter was prehybridized at 68°C with buffer containing 6 × SSC, 0.5% SDS, 5× Denhardt's solution, 0.1 mg/ml of sonicated salmon DNA and 10 mM EDTA for 1 h. The hybridization was carried out at 68°C for 15 h in the same buffer with a $^{32}P$-labeled ALPase cDNA probe (rat, universal type, 2.2 kb) (28), which was labeled with an oligolabeling kit (Pharmacia Japan, Tokyo).

*1,25(OH)$_2$vitamin $D_3$ binding.* Chondrocytes were seeded at high density ($3 \times 10^5$ cells per 50-mm dish) on type II collagen–coated dishes, and maintained in 10 ml of medium B. Cell layers were homogenized at 4°C in 0.7 ml of buffer A (10 mM Tris, pH 7.4, 2 mM EDTA, 5 mM dithiothreitol) containing 0.3 M KCl. The homogenate was centrifuged at 4°C at 226,000 *g* for 60 min. Portions of the cytosols (0.3–0.5 mg protein in 0.4 ml of buffer A) were incubated with 1-alpha, 25-dihydroxy[26,27-methyl-$^3H$]cholecalciferol (20,000 dpm/26 pg per 32 fmol) in the presence or absence of 200-fold excess of unlabeled 1,25(OH)$_2$vitamin $D_3$, as described previously (19).

*Collagen analysis.* Growth plate chondrocytes were seeded and maintained in the presence of medium C in 6-mm dishes. They were washed four times with PBS and twice with distilled water 19 d after seeding. The cell layers were homogenized with a Polytron for 3 min in 50 mM Tris/HCl buffer (pH 7.2) containing 1 M NaCl, 0.2% Triton X-100, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 25 mM EDTA, and 10 mM *N*-ethylmaleimide). The suspension was centrifuged at 3,000 *g* for 15 min at 4°C, and the pellet was incubated with pepsin at 0.4 mg/ml in 0.5 M acetic acid for 48 h at 4°C. The pepsin-resistant material was subjected to SDS-polyacrylamide gel electrophoresis in 4–20% acrylamide gradient gel under nonreducing conditions and was stained with Coomassie brilliant blue G.

*Determination of total protein synthesis.* Chondrocytes in 6-mm collagen-coated dishes were exposed to IL-1 beta at 0 or 100 pg/ml 6 d after seeding in 0.1 ml of a 9:1 (vol/vol) mixture of methionine-free MEM and Dulbecco's modified Eagle's medium in the presence of [$^{35}S$]methionine (10 μCi/ml) for 24 h. Total protein in cell layer fraction was subjected to SDS-polyacrylamide gel electrophoresis in 4–20% acrylamide gradient gel. After electrophoresis, the gels were processed for fluorography, and exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY).

*Determinations of $^{45}Ca$ uptake and calcium.* Pelleted cultures were exposed to 1 ml of MEM supplemented with $^{45}CaCl_2$ (2 μCi/culture) 3 h before the end of incubation. Cultures on collagen-coated dishes were exposed to 0.1 ml of Eagle's medium, alpha-modification with $^{45}CaCl_2$ (1 μCi/culture) 3 h before the end of incubation. Cell pellets or cell matrix layers were homogenized in 0.9% NaCl/0.2% Triton X-100 at 0°C, and then centrifuged for 15 min at 12,000 *g*. After separation of the supernatant, the precipitate was washed with 0.1 M $CaCl_2$ in 0.05 M Tris-HCl, pH 7.4, at 20°C for 30 min to remove exchangeable $^{45}Ca$, and was then solubilized by incubation in 0.5 M HCl for 3 h at 20°C (29). Aliquots were used to determine $^{45}Ca$ radioactivity.

For determination of the calcium content, cell pellets or cell matrix layers were washed five times with PBS (calcium- and magnesium-free) and then heated at 800°C for 10 h. The ash was dissolved in 6 M HCl solution containing 1% lanthanum chloride, and its calcium content was determined with an atomic absorption spectrometer (model AA-640; Shimadzu, Kyoto, Japan). Staining of cell matrix layers with alizarin red was performed as described (30).

*Determinations of DNA and uronic acid.* Cell pellets or cell matrix layers were washed with PBS, and homogenized at 0°C in 1 ml of 0.9% NaCl/0.2% Triton X-100. The homogenate (0.1–0.3 ml) was incubated at 37°C for 16 h with 1 mg/ml of Pronase E (Protease type XIV; Sigma Chemical Co.) in 3 ml of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.9% NaCl, 1 mM $CaCl_2$, and 0.2% Triton X-100. The resulting digest was used for determinations of DNA (31) and uronic acid (32).

## Results

*Effects of IL-1 on induction of ALPase and calcification in pelleted chondrocyte cultures.* Rabbit growth plate chondrocytes were seeded as a pelleted mass in a centrifuge tube, and maintained in medium A. In these cultures, the DNA content started to increase on day 3, and reached a plateau on day 10 (18–21). ALPase activity started to increase after cessation of cell division on day 10, and reached a maximum on day 21 (Fig. 1 *A*) (18–21). The addition of IL-1 beta on day 10 or 16 suppressed the increase in ALPase activity (Fig. 1 *A*). This effect of IL-1 beta on ALPase was reversible: after a shift from medium containing IL-1 to IL-1–free medium, the ALPase activity promptly increased to a level similar to that in cultures not exposed to IL-1 (Fig. 1 *A*). The inhibitory effect of IL-1 beta was detectable at 10 pg/ml and maximal at 100 pg/ml (Fig. 1 *B*).

The incorporation of $^{45}Ca$ into insoluble material started to increase on day 12 and reached a maximum on day 16–18 (Fig. 2 *A*). The addition of IL-1 beta on day 10 or 16 suppressed this increase in the incorporation of $^{45}Ca$ into insoluble material.

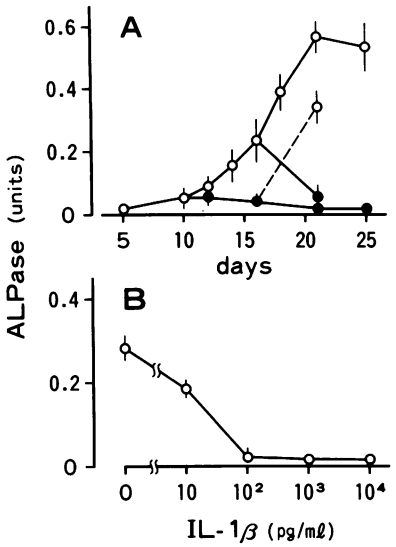


*Figure 1.* Effects of IL-1 beta on ALPase activity in chondrocyte cultures. Rabbit growth plate chondrocytes were seeded and maintained in centrifuge tubes, as described in Methods. (*A*) Human IL-1 beta was not added (○) or was added at 100 pg/ml (●) 10 or 16 d after cell seeding. IL-1 beta was removed from some cultures on day 16 (*dotted line*). (*B*) Increasing concentrations of IL-1 beta were added 10 d after cell seeding, and ALPase activity was determined on day 16. Values are averages±SD for four cultures.

The inhibition of calcification could be detected 2 d after addition of IL-1. The inhibition of $^{45}Ca$ incorporation by IL-1 beta was maximal at 100 pg/ml (Fig. 2 *B*). Table I shows that IL-1 beta suppressed the increase in the calcium content of pelleted cultures during the hypertrophic stage.

*Effects of IL-1 on ALPase activity and ALPase mRNA levels in chondrocyte cultures on collagen-coated dishes.* Rabbit growth plate chondrocytes were seeded at high density into collagen-coated dishes, and maintained in medium B. These chondrocyte cultures on collagen-coated dishes were used in all subsequent studies because their ALPase activity and calcification were 3 to 10 times those in pelleted cultures (33).

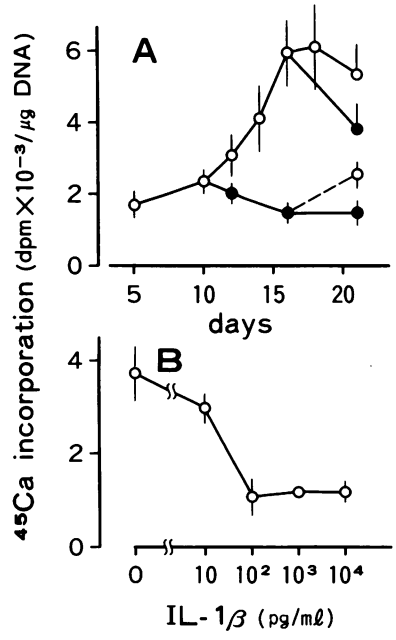


*Figure 2.* Effects of IL-1 beta on $^{45}Ca$ incorporation into insoluble material in chondrocyte cultures. $^{45}CaCl_2$ was added at 2 μCi/ml to the culture medium 3 h before the end of incubation. Rabbit growth plate chondrocytes were seeded and maintained in centrifuge tubes, as described in Methods. (*A*) IL-1 beta was not added (○) or was added (●) at 100 pg/ml 10 d after cell seeding. IL-1 beta was removed from some cultures on day 16 (*dotted line*). (*B*) IL-1 beta, at the indicated concentrations, was added from day 10, and $^{45}Ca$ uptake was measured on day 16. Values are averages±SD for four cultures.

*Table I. Effects of IL-1 Beta on the Calcium Content of Chondrocytes in Pelleted Cultures*

| IL-1 beta | DNA | Calcium |
|---|---|---|
| *ng/ml* | *μg/culture* | |
| 0 | 7.1±1.2 | 6.4±2.5 |
| 0.1 | 7.0±0.6 | 1.8±1.2 |
| 1 | 6.7±0.7 | 1.4±0.7 |

Rabbit growth plate chondrocytes were seeded and maintained in centrifuge tubes, as described in Methods. Human IL-1 beta was added 10 d after cell seeding. The contents of DNA and calcium were determined on day 30. The contents of DNA and calcium on day 10 were 5.7 and 0.6 μg/culture, respectively. Values are averages±SD for four cultures.

In cultures on collagen-coated dishes, IL-1 beta suppressed the increase in ALPase activity in the cell layer dose dependently (Fig. 3 *A*): treatments with IL-1 beta at 10, 100, and 1,000 pg/ml caused 30, 40, and 60% decreases, respectively, in ALPase activity in 24 h. This reduction in ALPase activity in the cell layer did not result from its release into the medium, because IL-1 did not increase the ALPase activity in the medium (Fig. 3 *B*). IL-1 alpha at 10–1,000 pg/ml also suppressed ALPase activity dose dependently (Fig. 3).

Northern analysis showed that growth plate chondrocytes, but not permanent chondrocytes (not shown), express liver/bone/kidney-type ALPase mRNA (Fig. 4). The size (2.7 kb) of rabbit chondrocyte ALPase mRNA was the same as that of rat liver ALPase mRNA (28). IL-1 beta at 100 pg/ml caused 50, 70, and 95% decreases in the ALPase mRNA content of growth plate chondrocytes after 24, 48, and 96 h, respectively (Fig. 4). In similar conditions, IL-1 beta had little effect on the level of fibroblast growth factor receptor mRNA (*flg* transcript) (data not shown).

*Effects of IL-1 on the syntheses of 1,25(OH)$_2$vitamin D$_3$ receptor and type X collagen.* In chondrocyte cultures, binding of 1,25(OH)$_2$vitamin D$_3$ increased during the hypertrophic stage (Fig. 5 *A*) (19, 33). The affinity (kD, 0.49 nM) of the 1,25(OH)$_2$vitamin D$_3$ receptor and the number of binding

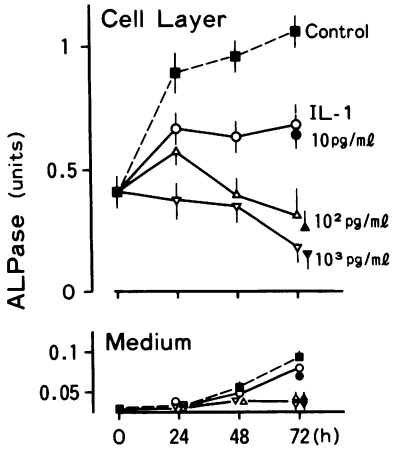


*Figure 3.* Effects of increasing concentrations of IL-1 alpha and beta on ALPase activity in rabbit growth plate chondrocyte cultures on collagen-coated dishes. Cells were seeded and maintained as described in Methods. IL-1 alpha, at 10 (●), 100 (▲) or 1,000 pg/ml (▼), or IL-1 beta, at 10 (○), 100 (△), or 1,000 pg/ml (▽), was added from day 6. ALPase activities in the cell layer and medium were measured 24, 48, and 72 h after addition of IL-1. Values are averages±SD for four cultures.

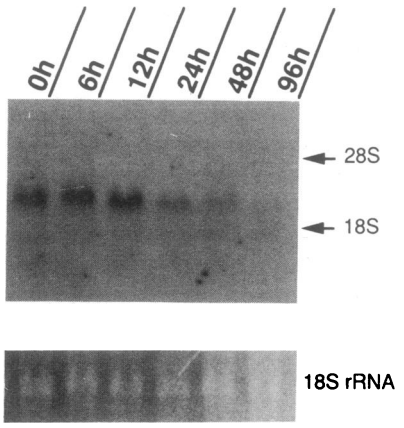


*Figure 4.* Effects of IL-1 beta on ALPase mRNA levels. Chondrocytes were seeded, and maintained as described in Methods. IL-1 at 100 pg/ml was not added or was added 6–96 h before the end of incubation. (*A*) Northern hybridization of total RNA from day 20 cultures with a liver/bone/kidney-type ALPase cDNA probe. (*B*) Ethidium bromide staining of the gels.

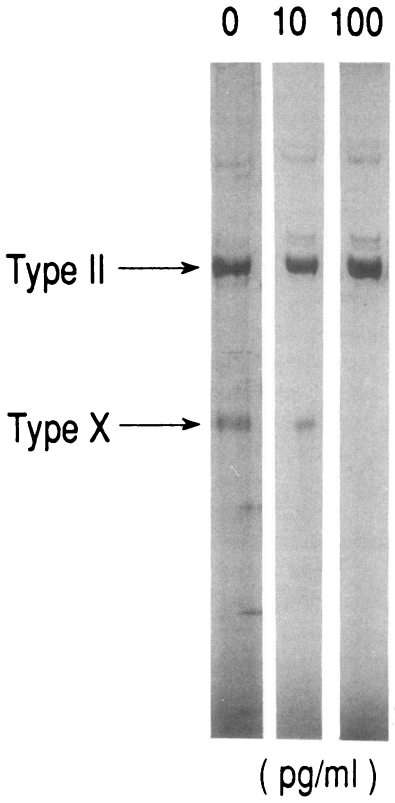


*Figure 6.* Effects of IL-1 beta on type II and X collagen contents of chondrocyte cultures. Chondrocytes were seeded and maintained in the presence of medium C in 6-mm dishes. IL-1 beta was not added or was added at 10 or 100 pg/ml from day 6. Collagen contents in day 19 cultures were determined as described in Methods.

sites per cell (11,000) in hypertrophic chondrocytes in culture were similar to those (0.22–0.55 nM and 12,000, respectively) in the growth plate in vivo (19, 33). Treatment with IL-1 beta at 1–100 pg/ml decreased the binding of 1,25(OH)$_2$vitamin $D_3$ dose dependently (Fig. 5 *B*).

Treatment with IL-1 beta at 10–100 pg/ml decreased the content of type X collagen, but had little effect on the type II collagen level (Fig. 6). This cytokine at 100 pg/ml had little effect on [$^{35}$S]methionine incorporation into cellular proteins, as judged by SDS-polyacrylamide gel electrophoresis, although it increased the syntheses and releases of procollagenase and prostromelysin into the medium (data not shown), as expected from previous studies (11).

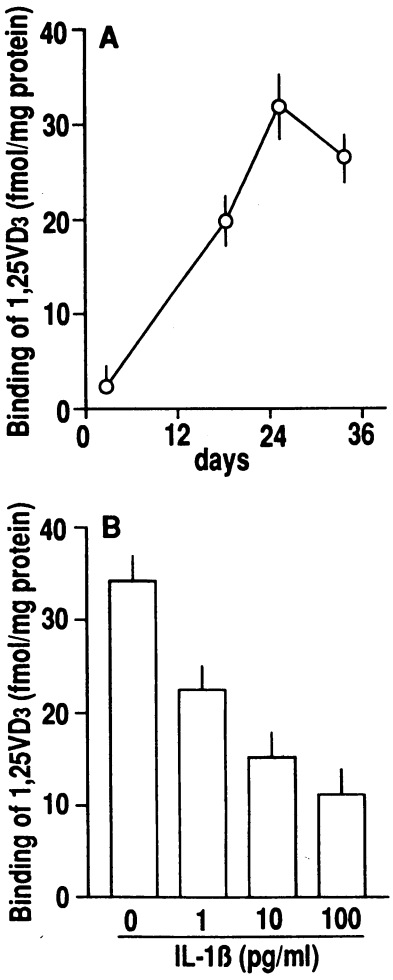


*Figure 5.* Effects of IL-1 beta on increase in binding of 1,25(OH)$_2$vitamin $D_3$ to chondrocytes during the hypertrophic stage. Rabbit growth plate chondrocytes were seeded and maintained on collagen-coated dishes, as described in Methods. (*A*) On the indicated days, the cells were homogenized, and the cytosols were incubated with $^3$H-labeled 1,25(OH)$_2$vitamin $D_3$ in the presence or absence of 200-fold excess of nonradioactive 1,25(OH)$_2$vitamin $D_3$ at 20°C for 1.5 h. Specific binding was calculated by subtracting nonspecific binding (< 20%) from the total binding. (*B*) IL-1 beta was not added or was added at 1, 10, or 100 pg/ml from day 8. The binding of 1,25(OH)$_2$vitamin $D_3$ was determined on day 25. Values are averages±SD for four cultures.

*Effects of IL-1 on cell size and mineralization in PTH-treated or PTH-free cultures.* The biochemical data presented in Figs. 1–6 suggest that IL-1 inhibits terminal differentiation of chondrocytes into the hypertrophic stage. In fact, phase microscopic analyses showed that chondrocytes on collagen-coated dishes became hypertrophic in the absence of IL-1, but not in its presence (Fig. 7). This effect of IL-1 on chondrocyte size was detectable in 24 h (data not shown) and became prominent in 48 h (Fig. 7).

In mass cultures on collagen-coated dishes, treatments with IL-1 alpha and beta for 6 d suppressed the incorporation of $^{45}$Ca into insoluble material dose dependently, the concentrations of IL-1 beta and alpha for half maximal inhibition being < 10 pg/ml (Fig. 8). Accordingly, the calcium content of mass cultures maintained with IL-1 beta at 100 pg/ml was only 6% of that of IL-1–free cultures (Table II).

In another series of studies, treatment of chondrocyte cultures with IL-1 beta at 5 pg/ml from day 8 caused 60–70% decreases in the uronic acid content and ALPase activity on day 35. These inhibitions of proteoglycan accumulation and ALPase activity were reversible, when the cytokine was removed from the cultures from day 19. On the other hand, treatment with IL-1 beta at 1 or 5 pg/ml during the early stage of hypertrophy (days 8–18) suppressed subsequent mineralization irreversibly, as judged by alizarin red staining (Fig. 9).

Next, chondrocytes were exposed to IL-1 beta at different times after seeding. The addition of IL-1 beta from day 16 decreased $^{45}$Ca incorporation into insoluble material, whereas its addition from day 23 or 63 had a marginal effect (Table III).

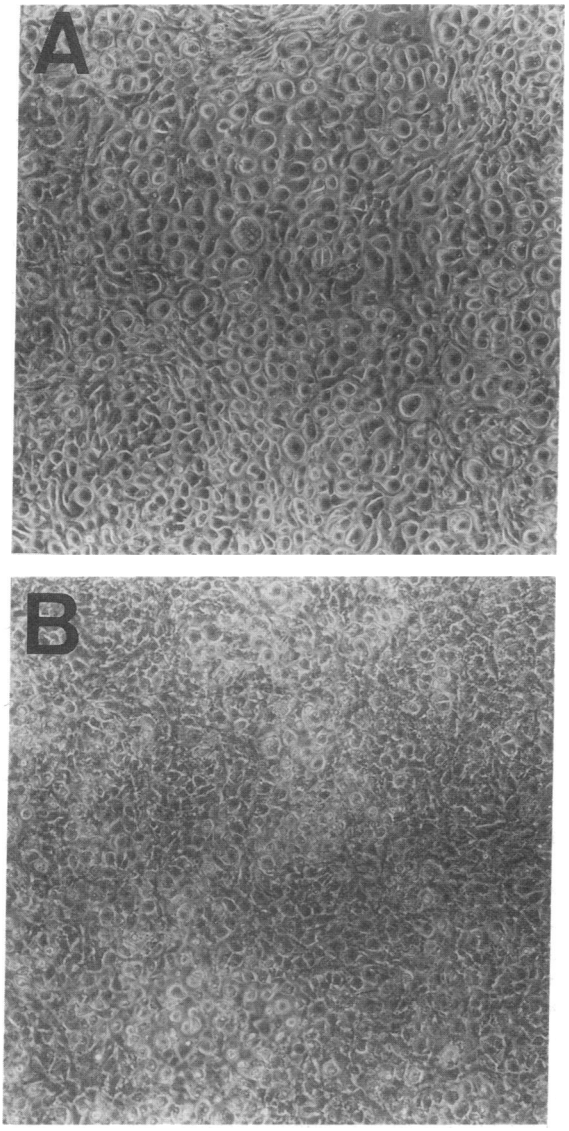


*Figure 7.* Morphology of chondrocytes grown with (*B*) or without (*A*) IL-1 beta. Cells were seeded and maintained as described in Fig. 3. IL-1 beta at 0.1 ng/ml was added from day 6. Pictures were taken on day 8.

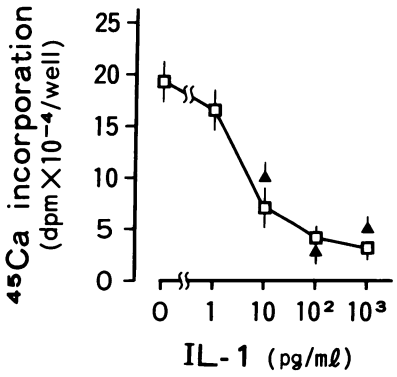


*Figure 8.* Effects of increasing concentrations of IL-1 alpha and beta on $^{45}Ca$ incorporation into insoluble material in chondrocyte cultures on collagen-coated dishes. Cells were seeded and maintained as described in Methods. IL-1 alpha (▲), or beta (□), at the indicated concentrations, was added from day 16, and $^{45}Ca$ incorporation into insoluble material was measured on day 22. Values are averages±SD for four cultures.

*Table II. Effects of IL-1 Beta on the Calcium Content in Growth Plate Chondrocytes on Type II Collagen-coated Dishes*

| Cell | Treatment | DNA | Calcium |
|---|---|---|---|
| | | *μg/culture* | *μg/culture* |
| Chondrocytes (d10) | — | 1.5 | < 1 |
| Chondrocytes (d35) | — | 2.1±0.5 | 27.3±6.5 |
| | IL-1 beta | 1.7±0.3 | 1.7±0.3 |
| Fibroblasts (d35) | — | 1.1 | < 1 |

Rabbit growth plate chondrocytes and fibroblasts were separately seeded at high density and maintained on type II collagen-coated dishes, as described in Methods. IL-1 beta (100 pg/ml) was added from day 10. The contents of DNA and calcium were determined on day 35. Values are averages for duplicate cultures or those±SD for four cultures.

In these conditions, the addition of the cytokine from day 16 or 23 decreased the uronic acid (proteoglycan) content to 5–30% of the control levels (Table III). Thus inhibition of calcification by IL-1 was dissociated from cytokine stimulation of proteoglycan degradation.

Previous studies have shown that PTH inhibits terminal differentiation of chondrocytes (21). Consistent with this finding, treatment with PTH for 6 d delayed subsequent increases in ALPase activity (Fig. 10 *A*) and $^{45}Ca$ uptake (Fig. 10 *B*). The addition of IL-1 beta to cultures previously exposed to PTH from day 23 suppressed $^{45}Ca$ uptake (Table III), perhaps because of the delay in calcification.

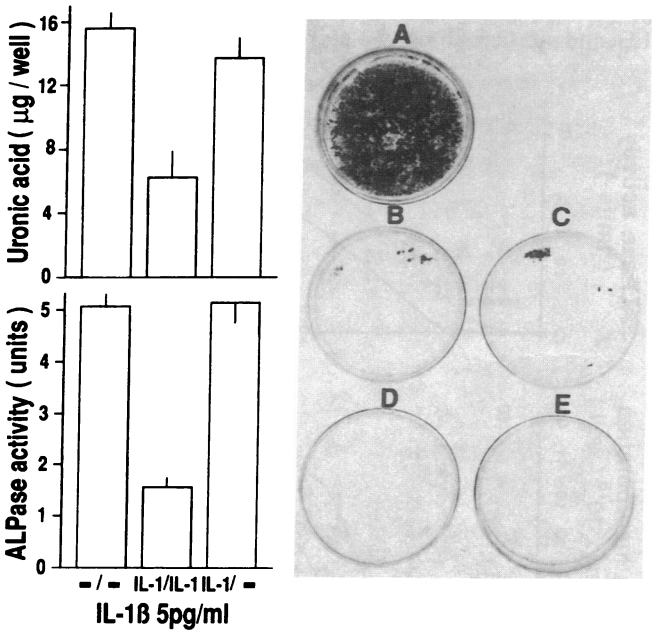


*Figure 9.* Effects of limited exposure to IL-1 on the uronic acid content and ALPase activity in chondrocyte cultures. Cells were seeded and maintained as described in Methods. IL-1 beta, at 0 (*A*), 1 (*B* and *C*) or 5 pg/ml (*D* and *E*), was added from day 8 for 10 (*C* and *E*) or 27 (*B* and *D*) d. The uronic acid level and ALPase activity were determined on day 35. Values are averages±SD for four cultures. The cell matrix layers on day 35 were stained with alizarin red.

*Table III. Effects of IL-1 Beta on $^{45}Ca$ Incorporation into Insoluble Material and the Uronic Acid Content in Growth Plate Chondrocytes on Type II Collagen-coated Dishes*

| Days in culture | Addition of IL-1 (period) | Treatment with PTH (d3–d9) | $^{45}Ca$ uptake | Uronic acid |
|---|---|---|---|---|
| | | | *dpm × $10^{-4}$/culture* | *μg/culture* |
| 10 | — | − | 0.5±0.2 | a |
| 18 | — | − | 5.4±1.6 | a |
| | d12–d18 | − | 1.1±0.6 | a |
| 22 | — | − | 11.7±2.8 | 26.1±4.1 |
| | d16–d22 | − | 2.3±0.5 | 2.7±2.3 |
| | — | + | 1.7±0.2 | 39.5±6.5 |
| | d16–d22 | + | 1.3±0.1 | 2.3±0.5 |
| 29 | — | − | 46.6±2.9 | 27.1±1.3 |
| | d23–d29 | − | 38.9±1.8 | 9.6±3.8 |
| | — | + | 37.3±1.3 | a |
| | d23–d29 | + | 13.2±6.2 | a |
| 69 | — | − | 103.7±4.5 | a |
| | d63–d69 | − | 99.6±1.3 | a |
| | — | + | 81.9±8.3 | a |
| | d63–d69 | + | 58.7±10.1 | a |

a, not determined; d, day (e.g., d3 = day 3). Rabbit growth plate chondrocytes were seeded at high density and maintained on type II collagen-coated dishes, as described in Methods. IL-1 beta at 100 pg/ml was added for 6 d at the indicated times. Values are averages±SD for four cultures.

*Effects of IL-1 on ALPase activity and calcification in the presence of indomethacin.* IL-1 is a strong stimulator of prostaglandin $E_2$ production in synovial cells and chondrocytes (7–13), and prostaglandins mediate some actions of IL-1 (34, 35).

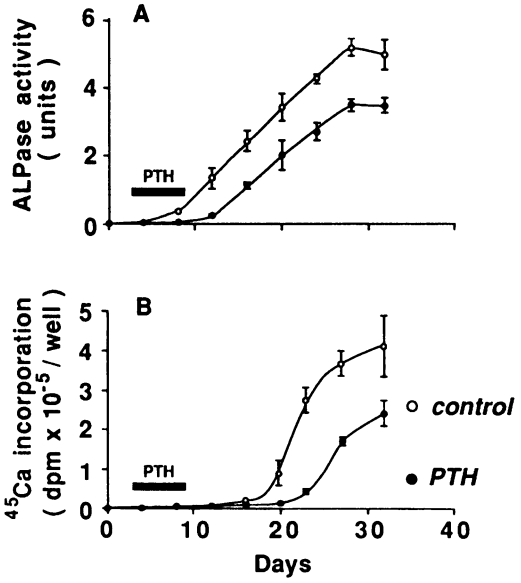


*Figure 10.* Effects of limited exposure to PTH on the induction of ALPase (*A*) and calcification (*B*) in chondrocyte cultures. Cells were seeded and maintained as described in Methods. The cultures became confluent on day 3. PTH[1-34], at $2 \times 10^{-8}$ M, was added from day 3 for 6 d. Values are averages±SD for four cultures.

*Table IV. Effect of Cyclooxygenase Inhibition on IL-1 Beta Stimulated-chondrocyte Cultures*

| Indomethacin | ALPase | | $^{45}Ca$ incorporation | |
|---|---|---|---|---|
| | Control | IL-1 | Control | IL-1 |
| *M* | | | *dpm × $10^{-3}$/culture* | |
| 0 | 2.43±0.31 | 0.32±0.07 | 61.0±8.6 | 20.1±1.1 |
| $10^{-6}$ | 2.62±0.23 | 0.56±0.15 | 68.0±13.2 | 22.4±3.2 |
| $10^{-5}$ | 1.68±0.28 | 0.58±0.18 | 43.7±9.3 | 19.6±1.4 |

Rabbit growth plate chondrocytes were seeded at high density and maintained on type II collagen-coated dishes, as described in Methods. IL-1 beta (100 pg/ml), indomethacin or both were added from day 6, and ALPase activity was measured on day 12. Alternatively, IL-1-beta (100 pg/ml), indomethacin, or both were added from day 16, and $^{45}Ca$ uptake was measured on day 20. Indomethacin alone had no effect on the cell morphology. Values are averages±SD for four cultures.

The cyclooxygenase inhibitor indomethacin, at $10^{-6}$ M, has been shown to abolish IL-1 beta–induced production of prostaglandin $E_2$ in human articular chondrocytes (13). However, indomethacin, at $10^{-6}$ or $10^{-5}$ M, did not reverse the inhibitions by IL-1 beta of ALPase and calcification in rabbit chondrocyte cultures (Table IV).

## Discussion

In fracture callus, arthritic cartilage, and growth plate cartilage, some populations of chondrocytes undergo a series of changes including proliferation, matrix formation, hypertrophy (terminal differentiation), and mineralization, before replacement of the cartilage by new bone. In the present study, the effects of IL-1 on terminal differentiation of chondrocytes and mineralization were examined in pelleted cultures and mass cultures on collagen-coated dishes. In these systems, mineralization is coupled with terminal differentiation of the chondrocytes (18–21). Scarcely any mineralization was observed in cultures of fibroblasts or normal articular chondrocytes, perhaps because these cells undergo terminal differentiation at low rates in vivo (33, 36, 37). Studies by electron microscopy and Fourier transform infrared spectroscopy have shown that apatite crystals in the chondrocyte cultures are similar to those in growth plates in vivo, but differ markedly from carbonated bone apatites (33).

IL-1 alpha and beta suppressed the induction of ALPase and calcification in pelleted cultures and mass cultures. The inhibitions of ALPase and calcification were observable 24 and 48 h, respectively, after addition of IL-1. This suggests that IL-1 has a direct effect on the expression of the mineralization-related phenotype of chondrocytes. Northern analysis showed that IL-1 decreased the steady-state level of liver/bone/kidney-type ALPase mRNA to an extent that roughly corresponded to the decrease in ALPase activity. These observations suggest that IL-1 regulates ALPase activity at a pretranslational level.

IL-1 inhibited the increase in the chondrocyte size and the syntheses of 1,25(OH)$_2$vitamin $D_3$ receptor and type X collagen, other markers of terminal differentiation. Thus, this cytokine inhibited the expressions of all differentiation phenotypes by hypertrophic chondrocytes. The specificity of the inhibition

of terminal differentiation is indicated by lack of the effect of IL-1 on the syntheses of type II collagen and total protein.

Early addition of IL-1 beta suppressed $^{45}Ca$ incorporation into insoluble material during the initial mineralizing process, but its later addition had little effect. Later addition of PTH also had little effect on $^{45}Ca$ incorporation into insoluble material (data not shown). Thus, the mineralization may become independent of the hormone and cytokines in a later phase. In any case, our results suggest that IL-1 inhibits the onset of calcification by suppressing chondrocyte hypertrophy, although it may also affect calcification by causing breakdown of certain extracellular macromolecules.

Previous studies have shown that infusion of IL-1 beta into normal mice (38) or production of IL-1 beta by certain tumors (39) induces hypercalcemia by stimulating bone resorption. The present results suggest that this cytokine also affects endochondral bone formation by inhibiting chondrocyte hypertrophy and mineralization. At sites of bone fracture, IL-1 stimulates resorption of nonfunctional bone fragments. IL-1 may impair fracture healing by inhibiting chondrocyte hypertrophy and mineralization, when it is formed in excess as a result of prolonged inflammation.

In arthritic cartilage, IL-1 causes reduction in matrix synthesis and enhances matrix degradation (40). In a later stage, some chondrocyte clusters show increased syntheses of DNA and matrix: other chondrocytes become hypertrophic and induce formation of osteophytes to compromise dysfunction of the joint. Our results, taken together with previous observations (3, 5, 6), suggest that IL-1 suppresses these reparative responses of arthritic chondrocytes.

Whether IL-1 plays a regulatory role in normal cartilage is not known. However, normal chondrocytes, as well as arthritic chondrocytes, produce IL-1 in culture (41–43). Furthermore, IL-1 gene expression is found in the hypertrophic zone of the growth plate in situ, whereas it is undetectable in elastic cartilage (44). In preliminary studies, we found that the concentrations of IL-1 beta required for stimulation of syntheses of procollagenase and prostromelysin in cultures of hypertrophic chondrocytes are at least 30-fold lower than those in cultures of articular chondrocytes (Nakamura, S., Y. Okada and Y. Kato, unpublished observation). These observations suggest that IL-1 plays special roles in growth plate cartilage.

Previous studies have shown that IL-1 inhibits the syntheses of DNA (3), proteoglycan (5), and collagen (6) by chondrocytes in certain culture conditions. However, IL-1 increased DNA synthesis or proteoglycan synthesis in other culture systems (45, 46). On the other hand, IL-1 consistently suppressed ALPase activity and mineralization in various culture systems with different batches of rabbit and porcine chondrocytes (data not shown).

We conclude from the present study that IL-1 alpha and beta at low concentrations (1–10 pg/ml) inhibit chondrocyte hypertrophy and mineralization. Our results seem important for understanding the actions of IL-1 in fracture callus, arthritic cartilage, and growth plate cartilage.

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