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Interleukin-1β Induces Interleukin-6 Production through the Production of Prostaglandin E_2 in Human Osteoblasts, MG-63 Cells

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This study was conducted to investigate the mechanism of interleukin-1β (IL-1β)-induced IL-6 production in human osteoblasts (MG-63 cells). Stimulation with IL-1β resulted in the production of IL-6 and prostaglandin E_2 (PGE_2). IL-6 production gradually increased and peaked 96 h after stimulation. IL-6 mRNA was detected between 4 and 72 h after IL-1β stimulation. The patterns of PGE_2 production and the expression of cyclooxygenase-2 (COX-2) mRNA were biphasic after stimulation. Actinomycin D, cycloheximide, indomethacin, and NS-398 (COX-2 inhibitor) suppressed the production of IL-6 and PGE_2. Anti-PGE_2 antibody markedly reduced the production of IL-6. In addition, stimulation with 17-phenyl-PGE_2, a PGE receptor-1 (EP-1 receptor) agonist, led to the expression of IL-6 mRNA after pretreatment with IL-1β. These findings indicate that IL-1β-induced IL-6 production in MG-63 cells involves the following sequence of steps: IL-1β-induced COX-2 activation, PGE_2 production, and EP-1 receptor signaling prior to IL-6 production.

Key words: EP-1 receptor, IL-1β, IL-6, PGE_2, osteoblasts.

Interleukin-1 (IL-1) was first defined as an initiator of the immune response to viral infection and an initiator of the inflammatory response to bacterial infection (*1-4*). In addition to the host defense mechanisms, IL-1, when produced in excess, plays a pathological role in the development of inflammatory diseases such as rheumatoid arthritis (RA) (*5, 6*). In RA, IL-1 has been demonstrated to stimulate the activities of lymphocytes (*7-9*) and to induce the production of collagenase by fibroblasts (*10*), synovial cells (*11*), and chondrocytes (*12*). Furthermore, recent studies indicate that IL-1 plays an important role in bone destruction in RA (*13-15*).

Bone destruction is caused by an imbalance between bone resorption and bone formation. There are some reports indicating that IL-1 stimulates the production of PGE_2 (*16*) and IL-6 (*17*) in mouse osteoblasts. In these reports, Sato *et al.* (*16*) demonstrated that PGE_2 produced by osteoblasts plays an important role in bone resorption in mice. In addition, Ono *et al.* (*18*) clarified the existence of PGE receptor-4 (EP-4) on mouse osteoclasts, and the EP-4 mediated mechanism of bone resorption. Besides PGE_2, IL-6 produced by osteoblasts also plays a role in bone resorption (*17*). Udagawa *et al.* (*19*) reported that osteoclast differentiation, which is the main phenomenon in bone resorption, depends on IL-6 production by osteoblasts and IL-6 receptor-mediated chain reactions. These suggest that IL-1-induced PGE_2 and IL-6 production by osteoblasts are important in bone destruction in RA. However, the precise mechanism of PGE_2 and IL-6 production by IL-1 in humans is still unclear. In the present study, we investigated possible mechanisms of IL-1β-induced IL-6 and PGE_2 production in human osteoblast MG-63 cells.

EXPERIMENTAL PROCEDURES

Materials—NS-398 and butaprost were provided by Taisho Pharmaceutical (Saitama) and Bayer AG (Wuppertal, Germany), respectively. Actinomycin D and indomethacin (Sigma Chemical MO, USA), cycloheximide (Nacalai Tesque, Kyoto), PGE_2 (Cascade Biochem, Berkshire, UK), 17-phenyl-PGE_2 (Biochem Research Laboratories, PA, USA), sulprostone (Cayman Chemical, MI, USA), IL-1β (Genzyme, MA, USA), IL-1 receptor antagonist (IL-1 Ra; Genzyme), and anti-PGE_2 antibody, (Cayman Chemical) were purchased from the indicated suppliers.

Cell Culture—Cloned MG-63 human osteoblasts were obtained from American Type Culture Collection (ATCC; MI, USA). MG-63 cells were maintained in minimal essential medium (MEM; Gibco BRL, Berlin, Germany) containing 10% fetal calf serum (FCS; Nippon Bio-Supp. Center, Tokyo), 100 U/ml penicillin (Banyu Pharmaceutical, Tokyo), 100 μg/ml streptomycin (Gibco BRL), 2 mM glutamine (Kishida Chemicals, Osaka), and 1 mM non-essential amino acids (Gibco BRL) at 37°C in a humidified atmosphere of 95% air 5% CO_2. The cells (5×10^5 cells) were seeded into 175 cm^2 culture-flasks in 20 ml of culture medium. The medium was changed every 4 days and the cells were passaged after treatment with 0.05% trypsin-0.02% EDTA solution.

Determination of PGE_2 and IL-6—MG-63 cells (8×10^4 cells/ml) were plated in 48-well culture plates (Sumilon 48-well; Sumitomo Bakelite, Osaka) with 250 μl culture medium, and precultured for 24 h at 37°C in a humidified

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Abbreviations: IL, interleukin; PGE_2, prostaglandin E_2; IL-1 Ra, interleukin-1 receptor antagonist; COX, cyclooxygenase; EP receptor, prostaglandin E receptor; TNF, tumor necrosis factor.

atmosphere of 95% air 5% CO_2. MG-63 cells were stimulated with 2.0 ng/ml IL-1β in 125 μl culture medium. The culture medium was removed and subjected to EIA for PGE_2 (Cayman Chemical) and ELISA for IL-6 (Endogen, MA, USA).

RNA Isolation and RT-PCR Analysis—RNA was extracted using Isogen (Nippon Gene, Toyama) according to the manufacturer's instructions. Briefly, cells were added to 1.0 ml ISOGEN to which 0.2 ml of chloroform was then added, and the mixture was shaken vigorously for 30 s and incubated for a few minutes at room temperature. The suspension was centrifuged at 12,000 $\times g$ for 15 min at 4°C. The aqueous phase was then transferred to a fresh tube and 0.5 ml of isopropanol was added. After gently mixing, the samples were left for 10 min at room temperature, then vortexed vigorously, and centrifuged at 12,000 $\times g$ for 10 min at 4°C. The RNA precipitates were washed with 1 ml of 75% ethanol, suspended in 0.5 ml of RNAase-free H_2O, and frozen at −80°C until use. The amount of total RNA in each sample was measured spectrophotometrically at 260 nm (Gene Quant, Cambridge, UK), and the quality of the RNA was checked by electrophoresis. First strand cDNA was prepared using 1 μg RNA, superscript II reverse transcriptase, and random hexamers (Gibco BRL) according to the instructions of the manufacturer. In brief, a 12 μl reaction mixture containing 1 μg RNA and random hexamers was heated for 10 min at 70°C and immediately chilled on ice. The reaction was first preincubated for 5 min at 42°C in a mixture containing 2 μl 10 × buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2 μl 25 mM $MgCl_2$, 1 μl dNTP mix (10 mM), and 2 μl 0.1 M DTT. Superscript reverse transcriptase (200 U) was added and the mixture was incubated at 42°C for 50 min; the reaction was terminated by heating to 70°C for 15 min and chilling on ice. One microliter of first strand cDNA mixture was used for PCR amplification in 50 μl 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTP, 1 μM of each primer, and 2.0 U of Taq DNA polymerase (Takara, Shiga). Primer sequences were the following: For hCOX-1 (*20*), sense primer (5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′) and antisense primer (5′-GTGCATCAACACAGGCGCCTCTTC-3′); for COX-2 (*21*), sense primer (5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′) and antisense primer (5′-AGATCATCTCTGCCTGAGTATCTT-3′); for hIL-6 (*22*), sense primer (5′-ATGAACTCCTTCTCCACAAGCGC-3′) and antisense primer (5′-GAAGAGCCCTCAGGCTGGACTG-3′); for β-actin, sense primer (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′) and antisense primer (5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′). One cycle of PCR consisted of 94°C for 1.5 min (denaturing), 62°C for 1.5 min (annealing), and 72°C for 1.5 min (elongation). Twenty-five cycles for COX-1 and COX-2 and 35 cycles for IL-6 and β-actin were performed. The theoretical molecular lengths of the PCR products are 305, 303, 628, and 662 bp, respectively.

Neutralization with Anti-PGE_2 Antibody—To examine

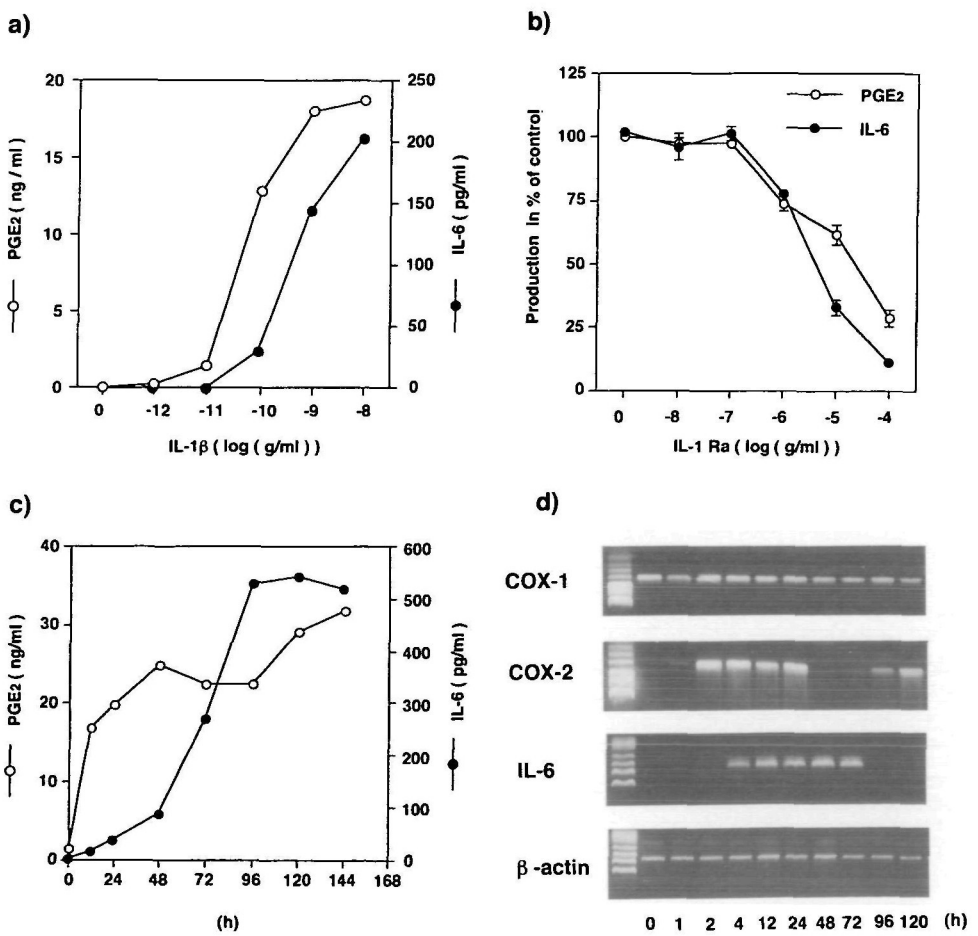

Fig. 1. **(a) Effect of IL-1β on PGE_2 and IL-6 production by MG-63 cells.** MG-63 cells were treated with IL-1β for 96 h. Values are the mean ± SE of three experiments. **(b) Effect of IL-1-Ra on IL-1β-induced PGE_2 and IL-6 production by MG-63 cells.** PGE_2 and IL-6 were measured in conditioned medium at 48 and 96 h after stimulation with 0.5 ng/ml IL-1β, respectively. Values are the mean ± SE of four determinations. **(c) Effect of the duration of treatment with IL-1β on the production of PGE_2 and IL-6.** Values are the mean ± SE of three experiments. **(d) Effect of the duration of treatment with IL-1β on the expression of COX-1, COX-2, and IL-6 mRNA.** MG-63 cells (2×10^5 cells) were stimulated with 0.5 ng/ml IL-1β for 12 h. Total RNA was extracted according to the manufacturer's instructions (Isogen).

the effect of PGE_2 depletion in the medium without the influence of the phagocytic activity of MG-63 cells, the technique shown in Fig. 3a was employed. Costar transwells (Costar, MA, USA) without microporous membranes were coated with anti-PGE_2 antibody in 0.1 M carbonate/bicarbonate coating buffer, pH 9.6, at 4°C overnight. The transwells were then blocked with 5% bovine serum albumin-phosphate balanced saline (BSA-PBS) at 37°C for 2 h. In addition, 500 μl of MG-63 cells (8.0×10^4 cells/ml) were precultured in 24-well culture plates (Falcon, Beckon Dickinson, NJ, USA) for 24 h at 37°C. Finally, 500 μl of 1.0 ng/ml IL-1β was added, the transwells already coated with anti-PGE_2 antibody were inserted, and incubation at 37°C for 96 h was performed.

RESULTS

The Induction of IL-6 and PGE_2 by IL-1β Stimulation—IL-1β (10^{-12}–10^{-8} g/ml) induced the production of IL-6 and PGE_2 in a dose-dependent manner (Fig. 1a). The concentrations of IL-1β to causing 50% of to the maximum PGE_2 and IL-6 production were 5×10^{-11} and 5×10^{-10} g/ml, respectively. Tumor necrosis factor-α (TNF-α) (10^{-12}–10^{-8} g/ml) did not induce IL-6 and PGE_2 production under similar conditions (data not shown). Next, to examine whether the induction of IL-6 and PGE_2 occurs *via* the IL-1 receptor, MG-63 cells were stimulated with IL-1β in the presence of an IL-1 Ra. The production of IL-6 and PGE_2 was suppressed by IL-1 Ra in a concentration-related manner (Fig. 1b).

Time Course for the Production of IL-6 and PGE_2, and mRNA Expression of IL-6, COX-1, and COX-2—When MG-63 cells were stimulated with 0.5 ng/ml IL-1β, IL-6 was generated and reached a maximum within 96 h (Fig. 1c). No expression of IL-6 mRNA was detected in unstimulated cultures, whereas the expression of IL-6 mRNA was detected within 4–72 h after stimulation (Fig. 1d). The production of PGE_2 was biphasic with peaks at 48 and 144 h after stimulation with IL-1β (Fig. 1c). COX-1 mRNA was expressed constitutively. The expression of COX-2 mRNA was also biphasic, detected in two periods between 2 and 24 h (first period) and after 96 h (second period) after stimulation (Fig. 1d).

Effects of Protein Synthesis Inhibitors and COX Inhibitors on PGE_2 and IL-6 Production—IL-1β-induced IL-6 production (96 h) was suppressed almost completely by 1 μg/ml actinomycin D or 10 μg/ml cycloheximide. PGE_2 production after 48 h was also greatly inhibited by treatment with actinomycin D or cycloheximide (Fig. 2a).

Indomethacin (10^{-6} M), which inactivates COX-1 and COX-2, resulted in the almost complete suppression of both IL-6 and PGE_2 production. NS-398 (10^{-6} M), a specific inhibitor of COX-2, suppressed IL-6 production almost completely and PGE_2 production by 75% (Fig. 2b).

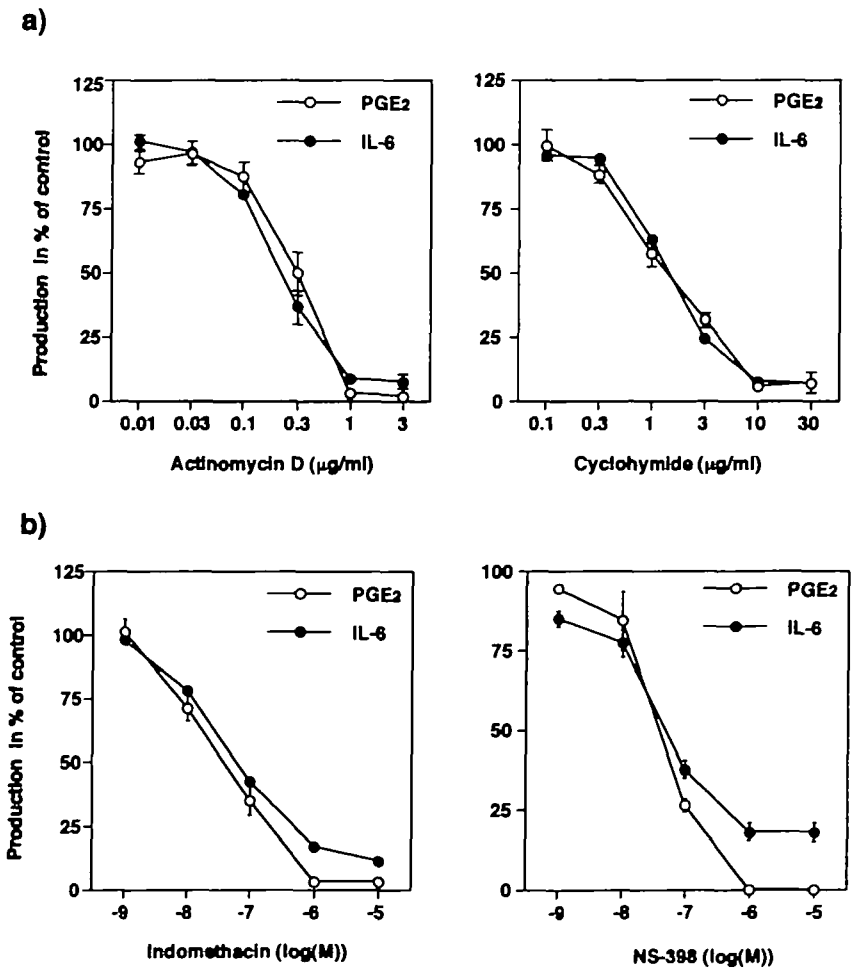

Fig. 2. (a) Effect of actinomycin D and cycloheximide on the production of PGE_2 and IL-6. (b) Effect of indomethacin and NS-398 on the production of PGE_2 and IL-6. MG-63 cells were stimulated with 0.5 ng/ml IL-1β for 48 h (PGE_2) and 96 h (IL-6).

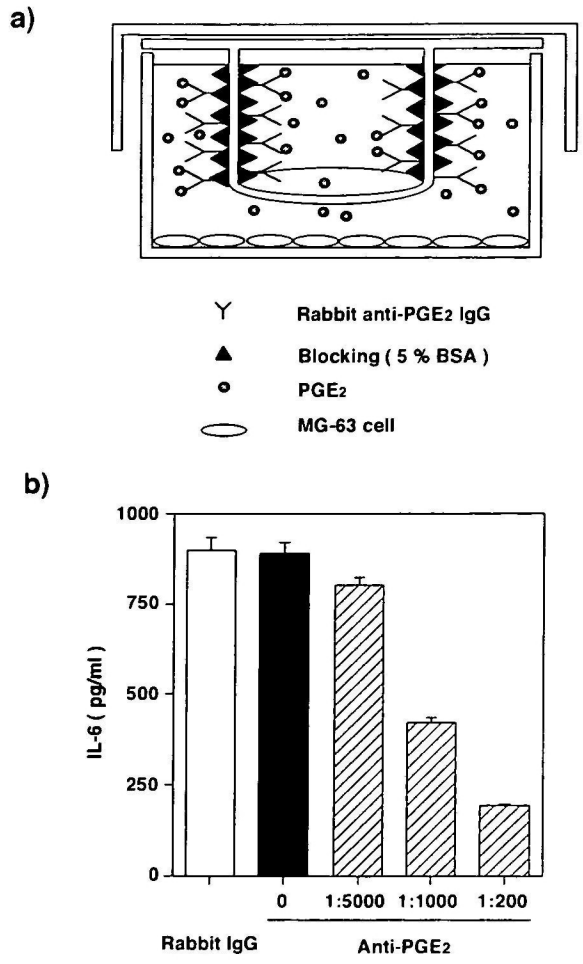

Fig. 3. **(a) Model for the neutralization of released PGE_2 by MG-63 cells. (b) Effect of anti-PGE_2 antibody on the production of IL-6.** The methods are summarized in "EXPERIMENTAL PROCEDURES."

Effect of Anti-PGE_2 Antibody on IL-6 Production—To investigate the relationship between the inductions of IL-6 and PGE_2 by IL-1β, the effect of anti-PGE_2 antibody on IL-6 production was examined. In preliminary experiments, MG-63 cells showed a phagocytic activity when treated with zymosan, and then IL-6 production was enhanced by zymosan in a concentration-dependent manner (data not shown). Therefore, the experiment shown in Fig. 3a (the methods are described in "EXPERIMENTAL PROCEDURES") was designed and carried out. Treatment with normal rabbit IgG did not affect IL-6 production. Treatment with anti-PGE_2 antibody for 96 h caused a dose-dependent decrease in IL-1β-induced IL-6 production by MG-63 cells (Fig. 3b).

Effects of PGE_2 and Its Agonists on the Expression of IL-6 mRNA—Neither PGE_2 nor EP receptor agonists induced IL-6 production in MG-63 cells without pretreatment with IL-1β (data not shown). Therefore, we examined the effect of EP receptor agonists on IL-6 production after pretreatment with IL-1β. NS-396 obviously suppressed the expression of IL-6 mRNA induced by IL-1β. In cells treated with NS-398, 17-phenyl-PGE_2, a PGE_2 and EP-1 receptor agonist, induced IL-6 mRNA expression, while

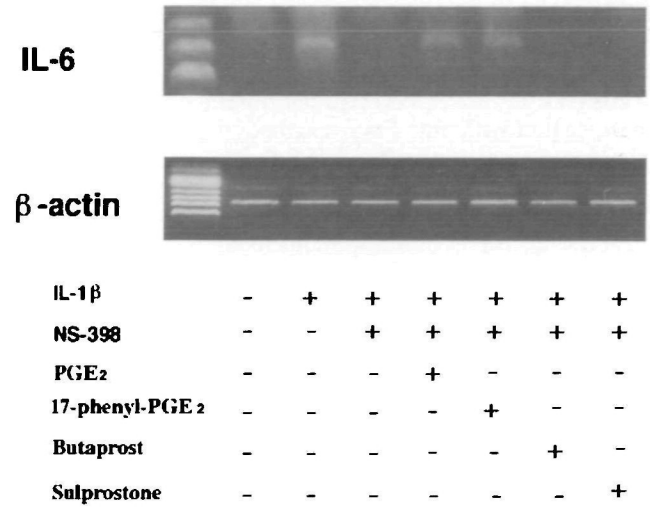

Fig. 4. **Effects of PGE_2 and its agonists on the expression of IL-6 mRNA.** MG-63 cells (2×10^5 cells) were first stimulated with 0.5 ng/ml IL-1β and treated with NS-398 (10^{-5} M) for 24 h. Following treatment, the cells were washed and incubated with PGE_2, 17-phenyl-PGE_2, butaprost, or sulprostone at 10^{-5} M for another 24 h. Total RNA was extracted according to the manufacturer's instructions (Isogen).

butaprost, an EP-2 receptor agonist, and sulprostone, an EP-3 receptor agonist, did not (Fig. 4).

DISCUSSION

The present findings indicate that IL-1β-induced IL-6 production is initiated by the production of PGE_2 and activation of EP1 receptor pathways in human osteoblasts, MG-63 cells. Previous studies on IL-1-induced IL-6 production by osteoblasts have mainly used the murine osteoblast system, especially MC3T3 cells (*16, 17*). There have been very few opportunities to examine the IL-1-induced IL-6 production in human osteoblasts, however that was the goal of this investigation. The present data indicate that the chain reaction inducing IL-6 after stimulation with IL-1 in human osteoblasts is generally similar to that in the mouse system, but with minor differences.

In the experiments, the kinetics of IL-1β-induced IL-6 production in MG-63 cells were examined. IL-1β was found to induced the production of PGE_2 and IL-6 in a dose-related fashion. PGE_2 production was induced by lower concentrations of IL-1β than those needed to induce the production of IL-6. In this experiment, the dose response was measured 96 h after IL-1β stimulation. As indicated in Fig. 1c, the time courses for the production of PGE_2 and IL-6 are different, suggesting the possible existence of chain reactions in IL-1β induced IL-6 production.

In the present study, IL-1β was found to induce PGE_2 production in a biphasic fashion. The IL-1-induced biphasic production of PGE_2 has been reported for MC3T3 mouse osteoblasts (*23*). In MC3T3 cells, IL-1 clearly induces COX-2 mRNA expression in an autocline manner (*23*). In MG-63 cells, a biphasic production of PGE_2 corresponding closely to the expression of COX-2 mRNA was detected after stimulation with IL-1β. This suggests that similar mechanisms underly human and murine osteoblast activation in terms of PGE_2 production.

Regarding the role of PGE_2 in IL-1-induced IL-6 production, there have been some reports indicating that IL-6 production occurs after the production of PGE_2 in other systems (*24-31*). Murakami *et al.* (*23*), Shacter *et al.* (*24*), Lu *et al.* (*25*), and Anderson *et al.* (*27*) demonstrated IL-1β-induced IL-6 production *via* PGE_2 production in *in vivo* systems by pharmacological studies using NSAIDs. In *in vitro* systems, the participation of PGE_2 has been demonstrated by the potentiation of IL-6 production by pretreatment with PGE_2 in human synovial fibroblasts (*28*), human gingoval fibroblasts (*29*), and human astrocytoma cells (*30, 31*). Additionally, Kozawa *et al.* (*32*) reported that exogenous PGE_2 directly induces the production of IL-6 by murine osteoblasts. The difference in the mode of action of PGE_2 in murine osteoblasts and human osteoblasts was demonstrated in this study. Here, PGE_2-induced IL-6 production appeared only after the MG-63 cells were stimulated with IL-1β. This shows the necessity of IL-1β priming to induce the effect of PGE_2 in MG-63 cells *via* the production of IL-6. In addition, this effect of PGE_2 is mediated by EP1 receptor. At present, four different kinds of EP receptors have been reported: EP1, EP2, EP3, and EP4. The pathophysiological role of each EP receptor has been investigated extensively using specific agonists to each EP (*33*). As it is difficult to obtain EP4-specific agonists, we examined EP1, EP2, and EP3 agonists. The present findings indicate the MG-63 activation process of EP1 receptors in human osteoblasts. Kozawa *et al.* (*32*) reported that PGE_2 stimulates IL-6 synthesis in MC3T3 cells through both Ca^{2+} mobilization from the extracellular space *via* EP1 receptors and c-AMP production *via* EP2 receptors. Our findings confirm the participation of the EP1 pathway in human osteoblasts. Further investigation into the mechanism of the PGE_2-induced production of IL-6 in MG-63 cells using EP4 agonists is necessary.

In conclusion, the present findings indicate that IL-1β stimulates the production of IL-6 in human osteoblasts, MG-63 cells. Simultaneously, IL-6 production is induced by the production of PGE_2 and the activation of EP1 receptor pathways.

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