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Effects of Nonsteroidal Anti-inflammatory Drugs and Prostaglandins on Osteoblastic Functions

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ABSTRACT. It has been reported that nonsteroidal anti-inflammatory drugs (NSAIDs) suppress bone repair and bone remodeling but only mildly inhibit bone mineralization at the earlier stage of the repair process. We proposed that the proliferation and/or the earlier stage of differentiation of osteoblasts may be affected by NSAIDs. This study was designed to investigate whether NSAIDs affect the proliferation and/or differentiation of osteoblasts and whether these effects are prostaglandin (PG) mediated. The effects of PGE_1 and PGE_2, indomethacin, and ketorolac on thymidine incorporation, cell count, intracellular alkaline phosphatase (ALP) activity, and Type I collagen content in osteoblast-enriched cultures derived from fetal calvaria were evaluated. The results showed that both PGs and NSAIDs inhibited DNA synthesis and cell mitosis in a time- and concentration-dependent manner. However, intracellular ALP activity and Type I collagen content were stimulated at an earlier stage of differentiation in osteoblasts. These results suggested that (i) the inhibitory effect of ketorolac on osteoblastic proliferation contributes to its suppressive effects on bone repair and remodeling *in vivo*; (ii) PGEs and NSAIDs may be involved in matrix maturation and biologic bone mineralization in the earlier stage of osteoblast differentiation; and (iii) the effects of ketorolac and indomethacin on cell proliferation and differentiation may not be through the inhibition of the synthesis of PGE_1 or PGE_2. BIOCHEM PHARMACOL **58**;6:983–990, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. osteoblast; rat calvarium; NSAID; prostaglandin; proliferation; differentiation

NSAIDs¶ are powerful agents to relieve the pain and inflammation of orthopedic patients. However, inhibitory effects of NSAIDs on bone repair have been reported [1–7]. Our previous report indicated that ketorolac, which is a potent NSAID blocking the synthesis of PGs at the cyclooxygenase pathway [8], inhibits bone repair and bone remodeling in a rabbit model [9]. Ketorolac diminishes the torsional stiffness, maximum torque, and energy absorption of demineralized bone matrix grafted ulnae in torsional testing [9]. We also found in a histologic study that ketorolac suppresses bone formation after 6 weeks of treatment, but affects mineralization only minimally within 4 weeks of medication [10]. Therefore, we believe that the suppressive effect of ketorolac on bone formation and biomechanical properties may be through its effect on the proliferation and/or differentiation of osteoblasts.

PGs (PGE_2 in particular) stimulate bone formation *in vivo* [11–19]. PGE_1 has similar effects on bone formation in mice [20]. It also has been reported that NSAIDs inhibit the synthesis of PGs in bone organ culture [21]. Therefore, it was suggested that the suppressive effects of NSAIDs on bone repair are due to the inhibitory effect of PG synthesis in bone cells [22]. A recent report indicated that PGE_2 is synthesized in osteoblasts and that its synthesis is autoregulated [23]. However, whether the inhibitory effect of ketorolac on bone repair is mediated by inhibiting the synthesis of PGs in bone cells remains unclear.

Type I collagen biosynthesized by osteoblasts is the major component of the organic bone matrix. The potential of the osteoblast to produce Type I collagen may reflect its activity of extracellular matrix biosynthesis during osteoblast differentiation. ALP is produced abundantly by osteoblasts and plays an important role in extracellular mineralization [24]. Therefore, in this study we tested the intracellular ALP activity and Type I collagen level as markers of osteoblast differentiation. Previous reports indicated that PGE_2 stimulates ALP activity in cloned osteoblastic MC3T3-E1 cells [25], and inhibits mineralization in cultures of rat bone marrow-stromal cells [26]. But the effect of

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¶ *Abbreviations*: NSAIDs, nonsteroidal anti-inflammatory drugs; ALP, alkaline phosphatase; PGs, prostaglandins; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; DMEM, Dulbecco's modified Eagle's medium; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence; and TCA, trichloroacetic acid; and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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PGE_2 on intracellular ALP activity in primary osteoblasts is poorly understood.

The present study was designed to evaluate the effects of NSAIDs on the proliferation and differentiation of osteoblasts and to investigate whether these effects are mediated by the inhibition of PGs. The mitosis and DNA replication of osteoblasts were tested at cell confluence, and intracellular ALP activity and Type I collagen content were examined at various times after cells were cultured with ketorolac, indomethacin, PGE_1, or PGE_2.

MATERIALS AND METHODS

Materials

DMEM, L-glutamine, non-essential amino acids, ascorbic acid, fetal bovine serum, and trypsin-EDTA were purchased from Gibco-BRL. Gentamicin, PGE_1, PGE_2, indomethacin, and an alkaline phosphatase assay kit were obtained from the Sigma Chemical Co. ITS^+ (a combination of insulin, transferrin, selenium, linoleic acid, and bovine serum albumin) was purchased from Collaborative BRL, and collagenase from the Worthington Corp. [^{3}H]Thymidine, PVDF membrane, and ECL reagent were obtained from New England Nuclear. A PGE_2 enzyme immunoassay (EIA) system was purchased from Amersham, and ketorolac from Syntex. Culture plates were obtained from Corning.

Calvarial Cell Culture

Primary osteoblast-enriched cultures were prepared from parietal bones obtained from fetal Sprague–Dawley rats of 21 days gestation. Fetal rats were removed from the uteri of mother rats using a sterile technique. Parietal bones were dissected free from the sutures. The periosteal layers from both sides of the bones were removed carefully. The bones were cut into chips and washed with sterilized Hanks' solution. Cells were released from the bone chips by five 20-min sequential collagenase digestions [27, 28]. The last three digestions were pooled to provide an osteoblast-enriched cell suspension. The characteristics of osteoblastic phenotype were confirmed by high alkaline phosphatase expression, and mineralization ability by von Kossa stain [29, 30]. Cells were plated into 24-well plates (5×10^4 cells/1.9 cm^2 well) and 6-well plates (1×10^5 cells/9.4 cm^2 well) in DMEM containing 100 μg/mL of L-glutamine, ascorbic acid, non-essential amino acids, gentamicin, 1% ITS^+, and 10% fetal bovine serum. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°. Medium was changed every 3 days. Conditioned media were serum-free media supplemented with various concentrations of test agents. Stock test agents of PGE_1, PGE_2, and indomethacin were initially dissolved in 95% ethanol. Then all the test agents were diluted with serum-free medium, so that the final concentration of ethanol was 0.1% or less in the conditioned media [31].

Determination of Cell Proliferation

Cells were grown in plates to confluence (6-well for cell count, 24-well for thymidine incorporation), rinsed, and serum-deprived for 24 hr. Cultures were then rinsed three times with serum-free medium and incubated with conditioned media (defined above) for 6 or 24 hr.

THYMIDINE INCORPORATION. In cultures treated for 24 hr, cells were pulsed with 0.2 μCi/mL of [^{3}H]thymidine 4 hr before harvest. In cultures treated for 6 hr, conditioned media were removed, cells were rinsed three times with serum-free medium, and then incubated in serum-free medium with 0.2 μCi/mL of [^{3}H]thymidine (specific activity 25 Ci/mmol) for the remainder of the 24 hr. At harvest, cells were washed with ice-cold PBS-thymidine (phosphate-buffered saline containing 1 μg/mL of thymidine) for 5 min followed by 10% TCA for 15 min, 5% TCA for 10 min, and 95% ethanol for 10 min. Then the cell layers were solubilized in 0.05% SDS/0.1 N NaOH. Aliquots of solubilized cells were mixed into liquid scintillant and counted in a beta counter.

CELL COUNT. Cells were released from culture plates by 0.25% trypsin-EDTA and stained with 1% trypan blue. Cell number was determined by using a hemocytometer.

PGE_2 Assay

Cells grown in 24-well plates were treated similarly to those in the thymidine incorporation experiments. After 6 or 24 hr of agent treatments, the conditioned media were collected for assay using an Amersham PGE_2 enzyme immunoassay system. Each assay well contained 50 μL of sample or standard PGE_2 and 50 μL of PGE_2 antiserum. After a 3-hr incubation in a cold room at 2–8°, 50 μL of PGE_2 conjugate (PGE_2 conjugated with horseradish peroxidase) was added. Wells were incubated in the cold room for another hour. Enzyme substrate (3,3′,5,5′-tetramethyl benzidine/hydrogen peroxide in 20% dimethyl formamide) was added and incubated for 30 min at room temperature. After 100 μL of 1 M sulfuric acid was added, wells were read at 450 nm with an EIA reader. All the assays were performed in duplicate. A standard curve was generated by plotting the percent B/B_o (the corrected optical density ratio of standard to zero standard) as a function of the log PGE_2 concentration. The PGE_2 concentration of samples was calculated from the standard curve.

ALP Activity Assay

Cells in 6-well plates reached confluence 7 days after plating. Cells were harvested following the incubation of control or conditioned media for 48 hr at 10, 15, or 20 days after cell plating. Cultures were washed three times with Ca^{2+}-Mg^{2+}-$NaHCO_3$-free PBS. Cells were scraped off into 600 μL of 0.1% Triton X-100. After storage at −20°,

samples were thawed, and cells were disrupted by sonication for 30 sec. ALP was assayed in 100 μL of cell lysate according to the method of Bessey *et al.* [32] by using a Sigma diagnostic assay kit. Total protein was assayed according to the method of Bradford [33] using a Bio-Rad protein assay kit. The specific activity of ALP was calculated as units/mg protein.

Western Blot Analysis

Type I collagen was measured by western blot analysis [34]. Cells in 6-well plates were harvested at 10 or 15 days after plating. The processes of drug treatment and cell lysis were as described above in the ALP assay. Cell lysates with identical protein amounts were mixed with dissociation buffer [0.18 M Tris–HCl, pH 6.8, 3% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue]. The samples were boiled for 5 min and resolved with 7.5% SDS–PAGE. Proteins were transferred onto PVDF membranes using a semi-dry electroblotter (Owl Scientific Inc.). Membranes were blocked overnight with 10% nonfat dry milk in Tris-buffered saline-non-fat tween (TBS-t) and incubated with rabbit polyclonal antibody (Biodesigns) against rat Type I collagen at a dilution of 1:1000 for 1 hr. Membranes were washed with TBS-t twice for 15 min and three times for 5 min and then incubated with secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase (Transduction Laboratories) at a dilution of 1:2000 for 1 hr. Membranes were washed again as described above. Autoradiography was performed using an ECL reagent. The densities of the bands were quantified by densitometry.

Statistic Analysis

Data are expressed as the means ± SEM of 3–4 wells from representative experiments. All experiments were performed at least three times. Statistical significance was evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. $P < 0.05$ was considered significant.

RESULTS

Cell Proliferation

The effects of NSAIDs on proliferation of primary rat osteoblasts were inhibitory. Ketorolac caused a 35.5% decrease ($P < 0.01$) in thymidine incorporation at a concentration of 1000 μM after a 6-hr treatment, while 7.3 to 99% decreases were found over a concentration range of 0.1 to1000 μM (0.1 μM, $P < 0.05$; 1–1000 μM, $P < 0.01$) after 24 hr of treatment (Fig. 1). Indomethacin caused 16.6 to 58.8 and 18 to 82.6% decreases in thymidine incorporation over a concentration range of 0.1 to 100 μM (0.1 μM, $P < 0.05$; 1–100 μM, $P < 0.01$) after 6- and 24-hr treatments, respectively (Fig. 2). The effects of PGs on DNA synthesis in osteoblasts were also inhibitory. PGE_1

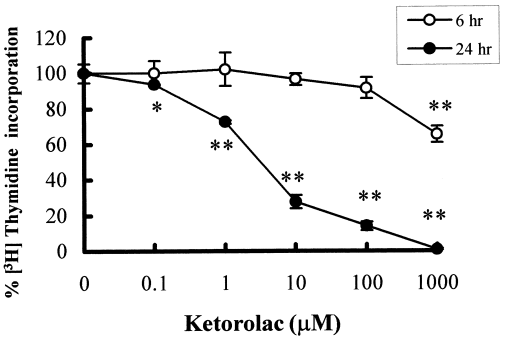

FIG. 1. Time–and concentration–response effects of ketorolac on thymidine uptake. Each point represents the mean ± SEM of four replicate cultures. (One hundred percent incorporation = 73,800 ± 3,370 or 62,930 ± 660 cpm for data from 6- or 24-hr treated cultures, respectively.) Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Key: (6 hr) 6-hr treatment with ketorolac; (24 hr) 24-hr treatment with ketorolac; (*) $P < 0.05$, in comparison with the control culture; and (**) $P < 0.01$, in comparison with the control culture.

caused 31–53% (100–1000 nM) and 27–60% (10–1000 nM) decreases ($P < 0.01$) in thymidine incorporation after 6- and 24-hr treatments, respectively (Fig. 3). PGE_2 caused 24.3 to 65.2% decreases in thymidine incorporation over a concentration range of 10–1000 nM (10 nM, $P < 0.05$; 100-1000 nM, $P < 0.01$) after 6 hr of treatment, while 33 to 89.5% decreases were found in 24 hr-treated cultures over a concentration range of 0.1 to 1000 nM (0.1 nM, $P < 0.05$; 1–1000 nM, $P < 0.01$) (Fig. 4). Cell numbers in cultures were decreased significantly upon treatment with NSAIDs; 14.3 to 50.7% decreases were caused by ketorolac (0.1–1000 μM) ($P < 0.01$) and 27.7 to 84.6% decreases by PGE_2 (0.1–1000 nM) ($P < 0.01$) after 24 hr of treatment (Fig. 5). The inhibitory effects of ketorolac, indomethacin,

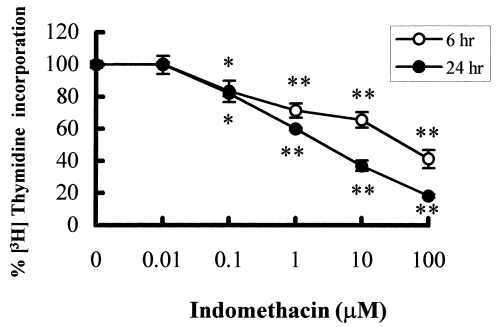

FIG. 2. Time–and concentration–response effects of indomethacin on thymidine uptake. Each point represents the mean ± SEM of four replicate cultures. (One hundred percent incorporation = 72,780 ± 1000 or 54,190 ± 570 cpm for data from 6- or 24-hr treated cultures, respectively.) Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Key: (6 hr) 6-hr treatment with indomethacin; (24 hr) 24-hr treatment with indomethacin; (*) $P < 0.05$, in comparison with the control culture; and (**) $P < 0.01$, in comparison with the control culture.

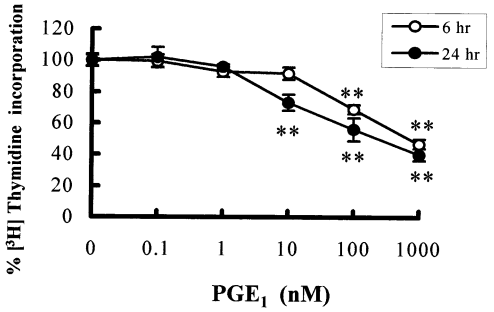

FIG. 3. Time–and concentration–response effects of PGE_1 on thymidine uptake. Each point represents the mean ± SEM of four replicated cultures. (One hundred percent incorporation = 69,300 ± 4,900 or 51,930 ± 1,090 cpm for data from 6- or 24-hr treated cultures, respectively.) Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Key: (6 hr) 6-hr treatment with PGE; (24 hr) 24-hr treatment with PGE; and (**) $P < 0.01$, in comparison with the control culture.

PGE_1, and PGE_2 on osteoblastic proliferation were time- and concentration-dependent.

PGE_2 Assay

The PGE_2 level in the culture medium was measured to confirm the inhibitory effect of NSAIDs on PG synthesis. PGE_2 levels were decreased by 12.1 to 97.5% and 24.1 to 93.1% compared with control cultures upon treatments with ketorolac and indomethacin, respectively, over a concentration range of 1–100 μM in cultures treated for 6 hr (Table 1). Furthermore, PGE_2 levels in cultures treated for 24 hr were decreased by 92.2 and 94.6% upon treatment with 10 μM ketorolac and indomethacin, respectively (data not shown).

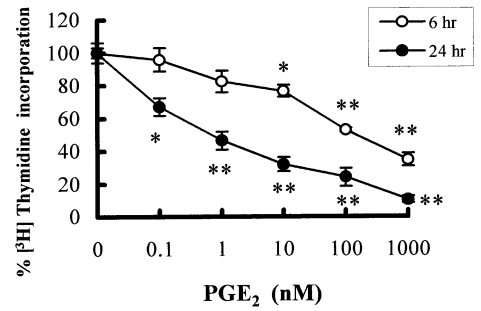

FIG. 4. Time–and concentration–response effects of PGE_2 on thymidine uptake. Each point represents the mean ± SEM of four replicated cultures. (One hundred percent incorporation = 79,960 ± 4,840 or 51,280 ± 1,530 cpm for data from 6- or 24-hr treated cultures, respectively.) Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Key: (6 hr) 6-hr treatment with PGE_2; (24 hr) 24-hr treatment with PGE_2; (*) $P < 0.05$, in comparison with the control culture; and (**) $P < 0.01$, in comparison with the control culture.

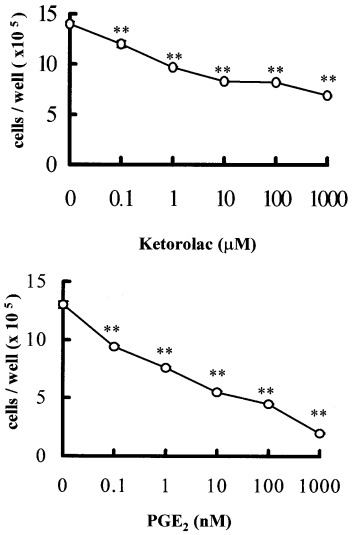

FIG. 5. Concentration–response effects of a 24-hr treatment with ketorolac (upper panel) and PGE_2 (lower panel) on cell number accumulation. Each point represents the mean ± SEM of triplicate cultures. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Key: (**) $P < 0.01$, in comparison with the control culture.

ALP Activity

Treatment with ketorolac (0.1–100 μM) resulted in a concentration-dependent increase ($P < 0.01$) in intracellular ALP activity of osteoblasts at 10 days after plating. The maximal effective concentration of ketorolac was 100 μM, which increased ALP activity by 1.7-fold in comparison with control cultures, whereas treatment with 1000 μM ketorolac showed a non-significant effect on ALP activity (Fig. 6). PGE_2 also had a stimulatory effect on ALP activity. Concentrations of 0.1 to 1000 nM increased ALP activity by 0.5- to 1.2-fold (0.1–100 nM, $P < 0.01$; 1000 nM, $P < 0.05$); the maximal effective concentration was 1 nM (Fig. 7). However, at 15 or 20 days after cell plating, ALP activities of osteoblasts were not affected significantly by either ketorolac or PGE_2 (Figs. 6 and 7).

TABLE 1. Effects of NSAIDs on PGE_2 synthesis in osteoblast-enriched cultures

Treatment	Concn (μM)	PGE_2 (nM)
Control		0.58 ± 0.04
Kt	0.1	0.54 ± 0.03
Kt	1	0.45 ± 0.02*
Kt	10	0.10 ± 0.02†
Kt	100	0.02 ± 0.003†
Ind	0.1	0.60 ± 0.04
Ind	1	0.44 ± 0.02*
Ind	10	0.11 ± 0.02†
Ind	100	0.04 ± 0.004†

PGE_2 levels in control and conditioned media were assayed after 6 hr of treatment with NSAIDs. Data are means ± SEM of triplicate cultures, evaluated by one-way ANOVA and Scheffe's method. Abbreviations: Kt, keterolac; and Ind, indomethacin.

*$P < 0.05$, in comparison with the control culture.

†$P < 0.01$, in comparison with the control culture.

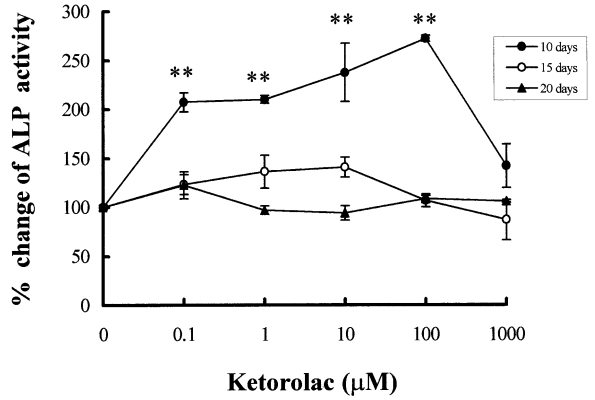

FIG. 6. Effects of ketorolac on ALP activity at various days in culture. Cells were incubated for 10, 15, or 20 days after plating. Each point represents the mean ± SEM of triplicate cultures. (One hundred percent activity = 1.26 ± 0.09, 3.46 ± 0.70, or 14.3 ± 0.80 SigU/mg protein for data from cultures at 10, 15, or 20 days after plating, respectively. A Sigma unit is defined as that amount of enzyme activity that will liberate 1 mol of *p*-nitrophenol/hr under the test conditions of the method of Bessey *et al.* [32].) The ALP activity assay was performed in duplicate in each culture. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Key: (**) $P < 0.01$, in comparison with the control culture.

Type I Collagen

Ketorolac (1–1000 μM) increased the intracellular levels of Type I collagen by 1.1- to 4.4-fold in osteoblast-enriched cultures 10 days after cells were plated; the maximal effective concentration was 10 μM. Fifteen days after plating, 1.5- to 3.3-fold increases were noted over the concentration range of 0.1 to 1000 μM (Fig. 8). The effect of indomethacin (0.01–100 μM) on the synthesis of Type

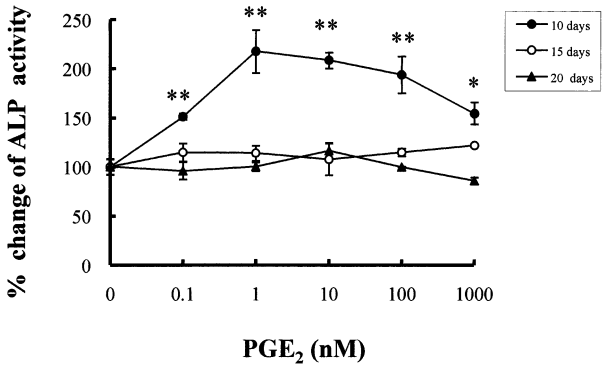

FIG. 7. Effects of PGE_2 on ALP activity at various days in culture. Cells were incubated for 10, 15, or 20 days after plating. Each point represents the mean ± SEM of triplicate cultures. (One hundred percent activity = 1.06 ± 0.08, 3.06 ± 0.60, or 14.1 ± 0.80 SigU/mg protein for data from cultures at 10, 15, or 20 days after plating, respectively.) The ALP activity assay was performed in duplicate in each culture. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Key: (*) $P < 0.05$, in comparison with the control culture; and (**) $P < 0.01$, in comparison with the control culture.

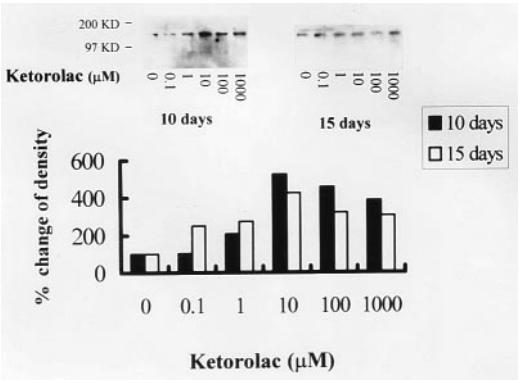

FIG. 8. Effects of ketorolac on the synthesis of Type I collagen at various days in culture. Cells were incubated for 10 or 15 days after plating. Representative ECL films from three independent assays of western blot analysis show binding of antiserum to Type I collagen. The densities of the bands were quantified by densitometry.

I collagen was also stimulatory; 0.5- to 4.8-fold and 1.0- to 1.5-fold increases were noted 10 and 15 days after cell plating, respectively (Fig. 9). The stimulatory effects of both ketorolac and indomethacin on the synthesis of Type I collagen in osteoblasts were more pronounced at 10 days than at 15 days after cells were plated. PGE_2 (0.1–1000 nM) increased Type I collagen content of osteoblasts by 1.2- to 4.2-fold 10 days after cell plating; the maximal effective concentration was 10 nM. However, no obvious change in the levels of intracellular Type I collagen was found at any concentration of PGE_2 15 days after plating (Fig. 10).

DISCUSSION

PGE_2 is one of the potent PGs that stimulate bone formation *in vivo* and DNA synthesis in calvaria organ culture [19, 35, 36]. In cloned osteoblastic cell lines, both stimulatory and inhibitory effects of PGE_2 on proliferation or differentiation were reported [19]; PGE_2 inhibited the

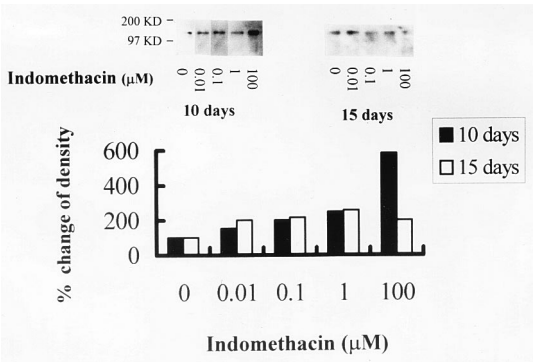

FIG. 9. Effects of indomethacin on the synthesis of Type I collagen at various days in culture. Cells were incubated for 10 or 15 days after plating. Representative ECL films from three independent assays of western blot analysis show binding of antiserum to Type I collagen. The densities of the bands were quantified by densitometry.

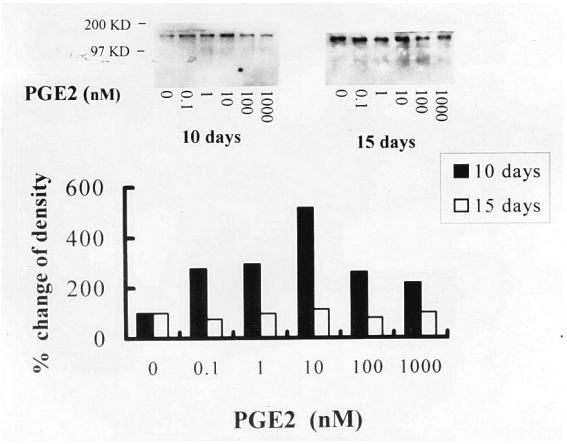

FIG. 10. Effects of PGE_2 on the synthesis of Type I collagen at various days in culture. Cells were incubated for 10 or 15 days after plating. Representative ECL films from three independent assays of western blot analysis show binding of antiserum to Type I collagen. The densities of the bands were quantified by densitometry.

expression of procollagen mRNA in PY1a cells [37], and had biphasic effects on DNA synthesis, while stimulating ALP activity in MC3T3-E1 cells [25, 38]. However, the effects of PGEs on the proliferation of primary osteoblasts have rarely been reported. Two previous reports indicated that PGE_2 inhibits primary osteoblastic proliferation at 10^{-8} and 10^{-7} M [39] and inhibits basal DNA synthesis at 10^{-6} M [40]. Our data showed a time- and concentration-dependent inhibitory effect of PGE_2 on osteoblastic DNA replication and cell mitosis over a concentration range of 0.1 to 1000 nM. PGE_1 (10–1000 nM) also was found to have a time- and concentration-dependent inhibitory effect on DNA synthesis. In addition, the results of the present study showed that 6 or 24 hr of treatment with ketorolac (1000 or 0.1 to 1000 μM) and indomethacin (1–100 μM) inhibited osteoblastic proliferation in a time- and concentration-dependent manner. These results support our previous hypothesis that the inhibitory effects of ketorolac on bone repair may involve osteoblast proliferation [10]. Moreover, in our experimental system, it was demonstrated that PG synthesis was inhibited by NSAIDs, whereas both PGs (PGE_1 and PGE_2) and NSAIDs (ketorolac and indomethacin) had inhibitory effects on osteoblast proliferation, suggesting that the inhibitory effects of ketorolac and indomethacin may not be mediated by PGE_1 or PGE_2. It is possible that NSAIDs inhibit osteoblastic proliferation through pathways other than PG synthesis. A recent report indicated that sulindac, a NSAID, caused cell cycle arrest by way of inhibiting cyclins and cyclin-associated kinase activity in rat enterocytes [41]. Other reports also indicated that NSAIDs caused cell cycle arrest and induced apoptosis by a PG-independent pathway in colon cancer cell lines [42, 43]. Accordingly, further study to investigate the effects of NSAIDs on cell cycle kinetics and the associated proteins such as cyclins and cyclin-dependent kinases in osteoblasts is needed.

Type I collagen, which constitutes about 90% of the organic bone matrix, is synthesized by osteoblasts during the later proliferation period and earlier matrix maturation period in fetal rat calvarial-derived osteoblast culture [44, 45]. ALP is an important enzyme during the maturation of bone extracellular matrix, which contributes to the biologic mineralization by hydrolyzing phosphate ester, stimulating calcium phosphate precipitation, and hydrolyzing inorganic pyrophosphate (an inhibitor of hydroxyapatite formation) [24]. Our data showed that PGE_2 stimulated ALP activity and Type I collagen synthesis in rat osteoblasts in culture at 10 days but not at 15 and 20 days after plating, implying that PGE_2 may be involved in the earlier stage of bone matrix maturation and the subsequent bone mineralization. This stimulatory effect of PGE_2 on ALP activity may contribute to the increased bone formation demonstrated in previous *in vivo* studies [35], although the effect on osteoblastic proliferation was inhibitory. Our data showed that NSAIDs had a significant stimulatory effect on type I collagen synthesis and intracellular ALP activity at 10 days after cells were plated. The results suggested that the stimulatory effect on ALP activity and Type I collagen synthesis may not be through the inhibition of PGE_2 synthesis. It is likely that some other factors had mediated the action of ketorolac on ALP activity. Cytokines play important roles in ALP expression during bone remodeling, e.g. transforming growth factor β has an inhibitory effect on osteoblastic ALP activity [46, 47], whereas platelet-derived growth factor has a stimulatory effect on osteoblastic ALP activity [48]. Further study on cytokines that affect ALP activity and collagen synthesis during bone differentiation is needed.

Our previous *in vivo* study had demonstrated that ketorolac inhibits bone repair significantly but has only a limited suppressive effect on bone mineralization in the earlier stage of repair [10]. Together with the present results, it implies that NSAIDs can only affect osteoblast proliferation and the earlier stage of differentiation during the osteoblast developmental process. The stimulatory effect of ketorolac on ALP activity of osteoblasts could not explain its inhibitory effect on bone mineralization during the earlier stage of the repair process *in vivo*. However, in addition to alkaline phosphatase, there are other factors that are involved in biologic mineralization such as acidic phospholipid, phosphatidylinositol, and osteocalcin [49–51]. The effect of ketorolac on bone mineralization would result from its sum effect on all the factors involved in mineralization. The inhibitory effect of NSAIDs on proliferation may be one of the most important mechanisms contributing to their inhibitory effects on bone repair in fractures and bone remodeling.

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