# EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON TRANSFORMING GROWTH FACTOR-β EXPRESSION AND BIOACTIVITY IN RAT OSTEOBLAST-ENRICHED CULTURES

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Nonsteroidal anti-inflammatory drugs (NSAIDs) have been reported to suppress bone remodeling in normal and repaired bones. Our previous results indicated that ketorolac and indomethacin suppressed proliferation, stimulated early differentiation, and induced apoptosis in cultured osteoblasts. Transforming growth factor-β (TGF-β) has been reported to enhance proliferation, suppress differentiation, and prevent apoptosis in osteoblasts. We proposed that one pathway of NSAID effects on osteoblast function might be through inhibition of the expression and/or bioactivity of TGF-β in osteoblasts. We tested the effects of ketorolac and indomethacin on the expression of TGF-$\beta_1$ mRNA and protein and the bioactivity of TGF-β in osteoblast-enriched cultures derived from fetal calvaria. The effects of prostaglandin $E_1$ ($PGE_1$) and $PGE_2$ on TGF-β expression and bioactivity were also examined in order to understand more about the role of prostaglandins in osteoblast function. Simultaneously, we estimated whether these NSAID effects on osteoblasts were prostaglandin-related. The results showed that 24-hour treatments with both PGEs and theoretic therapeutic concentrations of ketorolac and indomethacin had no significant effects on the levels of either transcription or translation of TGF-β or the post-translational function of TGF-β in osteoblasts. These results suggest that NSAIDs do not affect osteoblast function through changes in TGF-β action in osteoblasts.

**Key Words:** nonsteroidal anti-inflammatory drugs, prostaglandins, osteoblasts, transforming growth factor-β
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Nonsteroidal anti-inflammatory drugs (NSAIDs) are powerful drugs that relieve pain and suppress inflammation. However, NSAIDs have been reported to inhibit bone repair [1–6]. Ketorolac is a potent NSAID frequently used postoperatively. It acts by blocking the synthesis of prostaglandins at the cyclooxygenase pathway [7]. Our previous report indicated that ketorolac, in a rabbit model, inhibits repair of fractured bones and bone remodeling of intact bones [8]. We also found that ketorolac mainly suppresses the early stage of endochondral ossification during bone repair [9]. Our in vitro study revealed that NSAIDs inhibit proliferation and stimulate early differentiation through a prostaglandin-independent pathway in osteoblast-enriched cultures [10]. These findings

suggest that the effects of NSAIDs on osteoblast function might contribute to their suppressive effects on bone remodeling, especially at the early stage of endochondral ossification. However, the mechanisms by which NSAIDs affect osteoblast function remain unclear. This study was designed to investigate how NSAIDs affect osteoblast function.

Transforming growth factor-β, (TGF-β) is abundant in bone (200 μg/kg), and is synthesized by osteoblasts, osteocytes, and chondrocytes [11,12]. TGF-β inhibits bone resorption and stimulates bone formation, so it was thought to be an important coupling factor linking bone resorption and formation during remodeling [13–16]. It has also been reported to enhance proliferation and suppress differentiation during development in cultured osteoblasts [12]. Furthermore, TGF-β has an anti-apoptotic effect in osteoblasts [17]. NSAIDs inhibit proliferation and stimulate differentiation in osteoblast-enriched cultures derived from fetal rat calvaria [18], and have apoptotic effects on osteoblasts [19]. Thus, we proposed that TGF-β was a possible factor mediating the inhibitory, stimulatory, and/or inductive effects of NSAIDs on osteoblast proliferation, differentiation, and/or apoptosis, respectively. The role of TGF-$\beta_1$ in bone development and repair is better known than that of other types of TGF-β [20–22]. In this study, we investigated whether NSAIDs affect TGF-$\beta_1$ expression and bioactivity by examining the expression of TGF-$\beta_1$ mRNA and protein as well as TGF-β bioassay in osteoblast-enriched cultures after treatment with serial concentrations of ketorolac and indomethacin.

Prostaglandins, particularly prostaglandin $E_2$ ($PGE_2$), stimulate bone formation in vivo [23–30], but inhibit primary osteoblast proliferation in vitro [10,31, 32]. Prostaglandins are synthesized under autoregulation in osteoblasts [33]. Previous reports indicated that prostaglandin synthesis is blocked by NSAIDs in bone organ cultures [34] and in primary osteoblast cultures [10]. Some investigators propose that the suppressive effects of NSAIDs on bone repair result from the inhibition of prostaglandin synthesis in bone cells [35]. However, our previous report indicated that NSAIDs affect osteoblast function through a prostaglandin-independent pathway [10]. In this study, the effects of prostaglandins on TGF-β expression and bioactivity were measured to elucidate their role on osteoblast TGF-β expression and bioactivity. We also estimated whether the effects of NSAIDs on TGF-β expression were related to their inhibitory effects on prostaglandin synthesis.

## MATERIALS AND METHODS

### Osteoblast-enriched culture

Primary osteoblast-enriched cultures were prepared from parietal bone obtained from 21-day fetal Sprague-Dawley rats. The parietal bones were dissected free from sutures and periosteal layers as previously described [10,18]. Cells were released from bone chips by five 20-minute sequential collagenase digestions [36]. The last three digestions were pooled as an osteoblast-enriched cell suspension. Osteoblast-enriched cultures have characteristics of the osteoblast phenotype, as confirmed by high alkaline phosphatase expression and mineralization ability [10,18]. Cells were plated into 24-well plates ($5 \times 10^4$ cells/1.9 cm$^2$ well), 6-well plates ($1 \times 10^5$ cells/9.4 cm$^2$ well), or 75 cm$^2$ flasks in Dulbecco's modified Eagle medium (DMEM) containing 100 μg/mL L-glutamine, ascorbic acid, non-essential amino acids, gentamicin, 1% ITS$^+$ (aqueous solution containing insulin, transferrin, selenous acid, linoleic acid, and bovine serum albumin), and 10% fetal calf serum. Cultures were incubated in 5% $CO_2$ at 37°C and the medium was changed every 3 days.

### Treatments

Osteoblasts were grown to confluence, then rinsed and serum-deprived for 24 hours. After rinsing cultures three times with serum-free medium, cultures were incubated in conditioned media including ketorolac, indomethacin, $PGE_1$ or $PGE_2$ for 24 hours. $PGE_1$, $PGE_2$ and indomethacin were initially dissolved in 95% ethanol as stock agents. Conditioned media were prepared by diluting stock agents with serum-free medium to various concentrations of test agents. The final concentration of alcohol in conditioned media was 0.1% or less [37].

### Northern blot analysis

Total RNA was extracted from osteoblasts using the Stratagene micro-RNA isolation system (Stratagene Cloning Systems, La Jolla, CA, USA). RNA samples were quantified by spectrophotometry at 260 nm. The total RNA sample in the sample buffer (50% de-ionized formamide and 2.2 M formaldehyde) was denatured at 65°C for 5 minutes and resolved by electrophoresis

on 1.1% agarose gel. The RNA was then transferred onto a nitrocellulose membrane and cross-linked to the membrane using a UV cross-linker for 30 seconds. The membrane was hybridized with $^{32}$P-labeled cDNA probe. The TGF-$\beta_1$ cDNA probe was derived from purified plasmid DNA constructed using human TGF-$\beta_1$ cDNA (phTGF-$\beta$-2, ATCC). Human TGF-$\beta_1$ cDNA is highly cross-reactive with that of rats because the TGF-$\beta$ molecule is highly conserved between species [14]. The membrane was exposed with X-ray film (Kodak, Rochester, NY, USA) in an autoradiographic cassette at –70°C for 3 days. Membranes were re-probed with 1.7 kb EcoRV-ApaL1 DNA fragments cloned from the human $\beta$-actin cDNA as a positive control. Densitometry was quantified using Bio-Profil image analysis software (Vilber Lourmat, Marne, La Vallée, France).

## TGF-$\beta_1$ enzyme immunoassay

After 24 hours of incubation with test agent, the culture supernatants of both control and experimental cultures were collected for TGF-$\beta_1$ enzyme immunoassay with the human TGF-$\beta_1$ Quantikine kit (R&D Systems, Minneapolis, MN, USA). Latent TGF-$\beta$ was activated to its immunoreactive form prior to assay by acidification with 1 N HCl and neutralization with 1.2 N NaOH/0.5 M HEPES. Activated sample or standard TGF-$\beta$, 200 µL, was added to each well pre-coated with TGF-$\beta_1$ receptor type II. After 3 hours of incubation, the wells were washed and 200 µL of TGF-$\beta_1$ conjugate (polyclonal antibody against TGF-$\beta_1$ conjugated to horseradish peroxidase) was added and incubated for a further 1.5 hours. After aspiration and washing, 200 µL of enzyme substrate solution (tetramethyl-benzidine/hydrogen peroxidase) was added to each well and incubated for 20 minutes. The reaction was stopped with 50 µL of 2 N sulfuric acid and the wells were assessed at 450 nm using an enzyme immunoassay reader. All assays were performed in duplicate at room temperature. A standard curve was generated by plotting the log OD (optical density) as a function of log TGF-$\beta_1$ concentration. The TGF-$\beta_1$ concentration of a sample was calculated from the standard curve.

## TGF-$\beta$ bioassay

The inhibitory effect of TGF-$\beta$ on the proliferation of cloned mink lung epithelial cells (CCL-64, ATCC) reflects the bioactivity of TGF-$\beta$ [38,39]. After 24 hours of agent treatment, conditioned media were discarded and cultures were rinsed three times with serum-free medium. TGF-$\beta$ released from osteoblasts was collected by leaving 1 mL of serum-free medium in each well for another 24 hours. Serum-free medium containing TGF-$\beta$ was heated at 80°C for 15 minutes to convert latent TGF-$\beta$ to its active form. Media containing active TGF-$\beta$ was transferred to subconfluent, serum-deprived mink lung cell cultures for 24 hours, after which thymidine incorporation by the mink lung cell cultures was examined. To further confirm that the inhibition of proliferation in mink lung cell cultures was attributable to TGF-$\beta$ bioactivity, thymidine incorporation by mink lung cell cultures pre-cultivated in TGF-$\beta$ neutralized and non-neutralized osteoblast culture supernatants were compared. Pan-specific TGF-$\beta$ neutralizing antibody (20 µg/mL; R&D Systems) was added to neutralize TGF-$\beta$ in the active TGF-$\beta$–containing medium at 37°C for 1 hour prior to transferring to the mink lung cell cultures.

## Thymidine incorporation

Cloned mink lung cells were pulsed with 0.2 µCi/mL [$^3$H]thymidine 4 hours before harvest. At harvest, cells were washed with ice-cold PBS-thymidine (phosphate-buffered saline containing thymidine 1 µg/mL) for 5 minutes followed by 10% trichloroacetic acid (TCA) for 15 minutes, 5% TCA for 10 minutes, and 95% ethanol for 10 minutes. The cell layers were then solubilized in 0.05% sodium dodecyl sulfate/0.1 N NaOH. Aliquots of solubilized cells were mixed into liquid scintillant and counted in a beta counter.

## Statistical analysis

Data are expressed as the mean ± standard error of the mean of four wells from representative experiments. All experiments were repeated at least three times. Statistical significance of the effects of ketorolac, indomethacin and PGE$_2$ on TGF-$\beta_1$ synthesis and of NSAIDs on TGF-$\beta$ bioactivity was evaluated by the Kruskall-Wallis test and Dunn multiple comparisons, while that of the effects of TGF-$\beta$ on thymidine incorporation was assessed using the Mann-Whitney test.

## RESULTS

## TGF-$\beta$ mRNA expression

The relative densities of TGF-$\beta_1$/$\beta$-actin obtained from Northern blot analysis revealed no significant

differences among ketorolac- ($10^{-7}$–$10^{-4}$ M) and indomethacin-treated ($10^{-7}$–$10^{-5}$ M) and control osteoblast-enriched cultures (Figure 1). The results showed that 24-hour treatment with these broad ranges of concentrations of ketorolac and indomethacin had no significant effect on the expression of TGF-$\beta_1$ mRNA in osteoblasts. PGE$_2$ also had no significant effect on the expression of TGF-$\beta_1$ mRNA in osteoblast-enriched cultures over a concentration range of $10^{-9}$–$10^{-6}$ M (Figure 2).

## TGF-$\beta_1$ enzyme immunoassay

TGF-$\beta_1$ levels in culture supernatants were not significantly different among ketorolac- ($10^{-6}$ and $10^{-4}$ M) and indomethacin-treated ($10^{-6}$ and $10^{-4}$ M) and control osteoblast-enriched cultures (Figure 3). Nor did PGE$_2$ ($10^{-8}$ and $10^{-6}$ M) affect TGF-$\beta_1$ levels released from cultured osteoblasts (Figure 4).

## TGF-$\beta$ bioassay

After osteoblast culture supernatants were neutralized with TGF-$\beta$ antibody, thymidine incorporation by mink
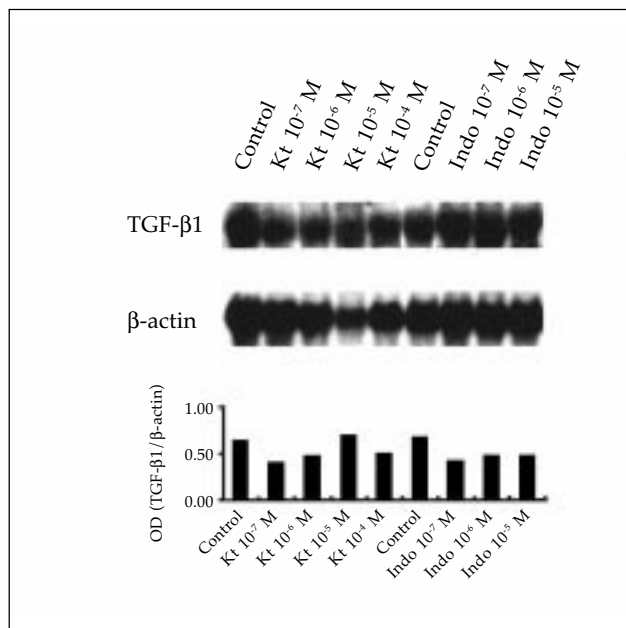
**Figure 1.** *Effects of ketorolac and indomethacin on transforming growth factor-$\beta_1$ (TGF-$\beta_1$) mRNA expression in osteoblast-enriched cultures. Representative Northern blot images of TGF-$\beta_1$ and $\beta$-actin from total RNA of osteoblasts. Cells were incubated with serum-free medium (control) or conditioned media containing various concentrations of ketorolac (Kt) or indomethacin (Indo) for 24 hours. The densities of the bands were quantified by densitometry. OD (TGF-$\beta_1$/$\beta$-actin) = ratio of optical densities of TGF-$\beta_1$ and $\beta$-actin.*
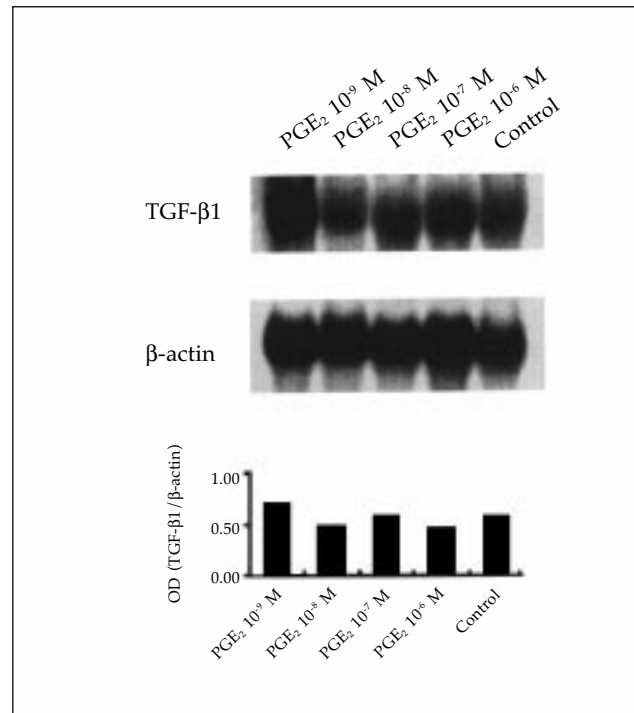
**Figure 2.** *Effects of prostaglandin E$_2$ (PGE$_2$) on transforming growth factor-$\beta_1$ (TGF-$\beta_1$) mRNA expression in osteoblast-enriched cultures. Representative Northern blot images of TGF-$\beta_1$ and $\beta$-actin from total RNA of osteoblasts. Cells were incubated with serum-free medium (control) or conditioned media containing various concentrations of PGE$_2$ for 24 hours. The densities of the bands were quantified by densitometry. OD (TGF-$\beta_1$/$\beta$-actin) = ratio of optical densities of TGF-$\beta_1$ and $\beta$-actin.*
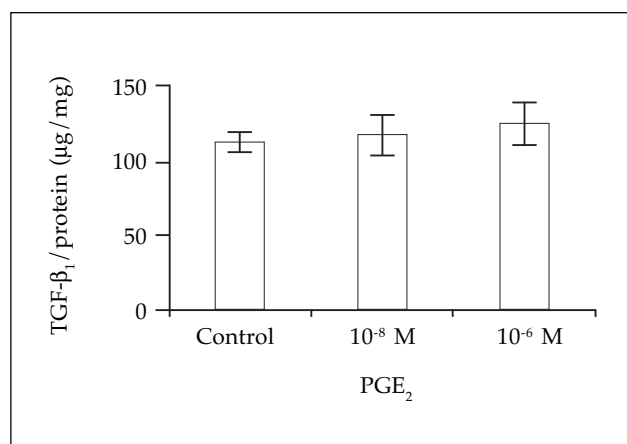
lung cell cultures was significantly elevated regardless of whether cultures were pre-cultivated in supernatants from control and prostaglandin- or NSAID-treated osteoblast cultures ($p < 0.01$) (Figures 5 and 6). Thymidine incorporation by mink lung cell cultures pre-cultivated in osteoblast culture supernatants was significantly reduced compared with that by cells that were not pre-cultivated in osteoblast culture supernatants (blank control) ($p < 0.01$) (Figure 7). Treated and control osteoblast culture supernatants had similar proliferation inhibitory effects to TGF-$\beta_1$ (1 ng/mL) in mink lung cell cultures (Figure 7). These results confirm that all the osteoblast cultures in these experiments released bioactive TGF-$\beta$ which inhibited thymidine incorporation in mink lung cell cultures. In comparison with the bioactivities of TGF-$\beta$ released from control and PGE$_1$- ($10^{-8}$ M), PGE$_2$- ($10^{-8}$ M), ketorolac- ($10^{-5}$ M), and indomethacin-treated ($10^{-5}$ M) osteoblast cultures, there were no significant differences among groups (Figure 7).
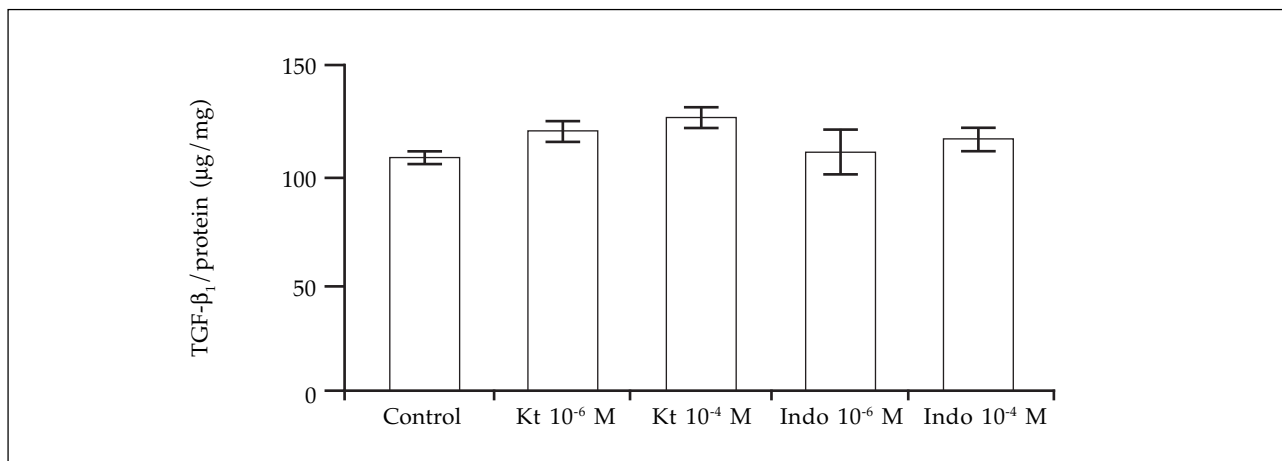
**Figure 3.** *Effects of ketorolac and indomethacin on transforming growth factor-β₁ (TGF-β₁) synthesis in osteoblast-enriched cultures. Osteoblasts were incubated with serum-free medium (control) or conditioned media containing various concentrations of ketorolac (Kt) or indomethacin (Indo) for 24 hours. TGF-β₁ secreted in culture supernatant was measured using TGF-β₁ enzyme immunoassay. Each bar represents the mean ± standard error of the mean of four replicate cultures. The assay was performed in duplicate for each culture. Data were evaluated by the Kruskall-Wallis test.*
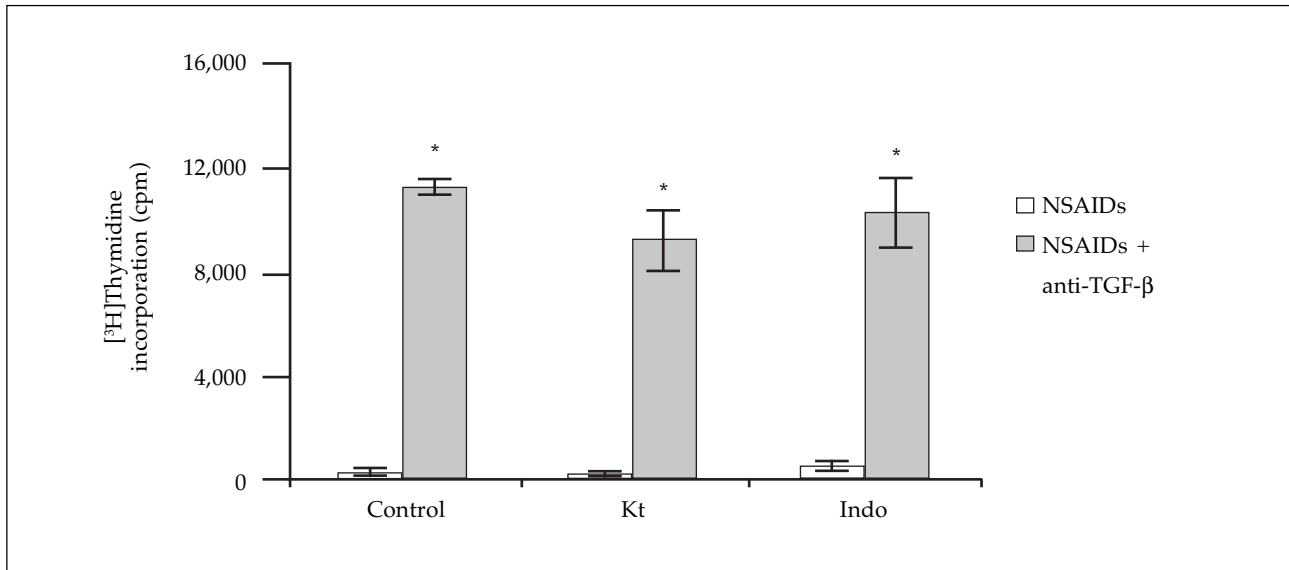
## DISCUSSION

The major pathway of the anti-inflammatory and analgesic effects of NSAIDs is to inhibit the synthesis of prostaglandins that mediate inflammation and pain. However, several recent reports have indicated that some NSAIDs affect various cellular functions through a prostaglandin-independent pathway. Some NSAIDs alter the glucosaminoglycan synthesis of chondrocytes by affecting cytokines or growth factors [40,41]. S-isomers of NSAIDs up-regulate the expressions of interleukin-10 (IL-10) and tumor necrosis factor, and down-regulate IL-6 in murine peritoneal macrophages [42]. Autocrine secretion of TGF-β in osteoblasts modulates the function of osteoblasts and osteoclasts. Thus, TGF-β acts as an important coupling factor during the normal bone remodeling cycle, and as a significant growth factor stimulating bone repair [14,43]. Previous reports have indicated that TGF-β stimulates proliferation, prevents apoptosis, and suppresses differentiation of osteoblasts in vitro [12,17]. Our previous results showed that NSAIDs suppress bone repair in vivo as well as inhibit proliferation, stimulate differentiation, and induce apoptosis in cultured osteoblasts [8–10]. Accordingly, we hypothesized that these NSAID effects occurred through inhibition of TGF-β production and/or action in osteoblasts. In this study, we investigated the effects of indomethacin and ketorolac on the expressions of mRNA and protein as well as the bioactivity of TGF-β. We tested a broad range of concentrations of ketorolac ($10^{-7}$–$10^{-4}$ M) and indomethacin ($10^{-7}$–$10^{-5}$ M). However, these agents had a non-significant effect on the expression of TGF-β₁ mRNA in osteoblast-enriched cultures. We also evaluated the amount of TGF-β₁ released from osteoblast-enriched cultures after NSAID treatment



**Figure 4.** *Effects of prostaglandin E₂ (PGE₂) on transforming growth factor-β₁ (TGF-β₁) synthesis in osteoblast-enriched cultures. Osteoblasts were incubated with serum-free medium (control) or conditioned media containing various concentrations of PGE₂ for 24 hours. TGF-β₁ secreted in culture supernatant was measured using TGF-β₁ enzyme immunoassay. Each bar represents the mean ± standard error of the mean of four replicate cultures. The assay was performed in duplicate for each culture. Data were evaluated by the Kruskall-Wallis test.*

**Figure 5.** *Effects of transforming growth factor-β (TGF-β) released from nonsteroidal anti-inflammatory drug (NSAID)-treated osteoblasts on thymidine incorporation by mink lung epithelial cell cultures. Osteoblasts were incubated with serum-free medium (control) or conditioned media containing $10^{-5}$ M of ketorolac (Kt) or indomethacin (Indo) for 24 hours. Media containing TGF-β were collected and activated. From each well, 0.5 mL of active TGF-β–containing medium was transferred into mink lung epithelial cell cultures (□ NSAIDs), while the other 0.5 mL was pre-treated with pan-specific TGF-β neutralizing antibody prior to transferring (▨ NSAIDs + anti-TGF-β). Each bar represents the mean ± standard error of the mean of four replicate cultures. Data were evaluated by the Mann-Whitney test. \*Thymidine uptake of cultures neutralized with anti-TGF-β antibody was significantly elevated compared to non-neutralized cultures,* $p < 0.01$.
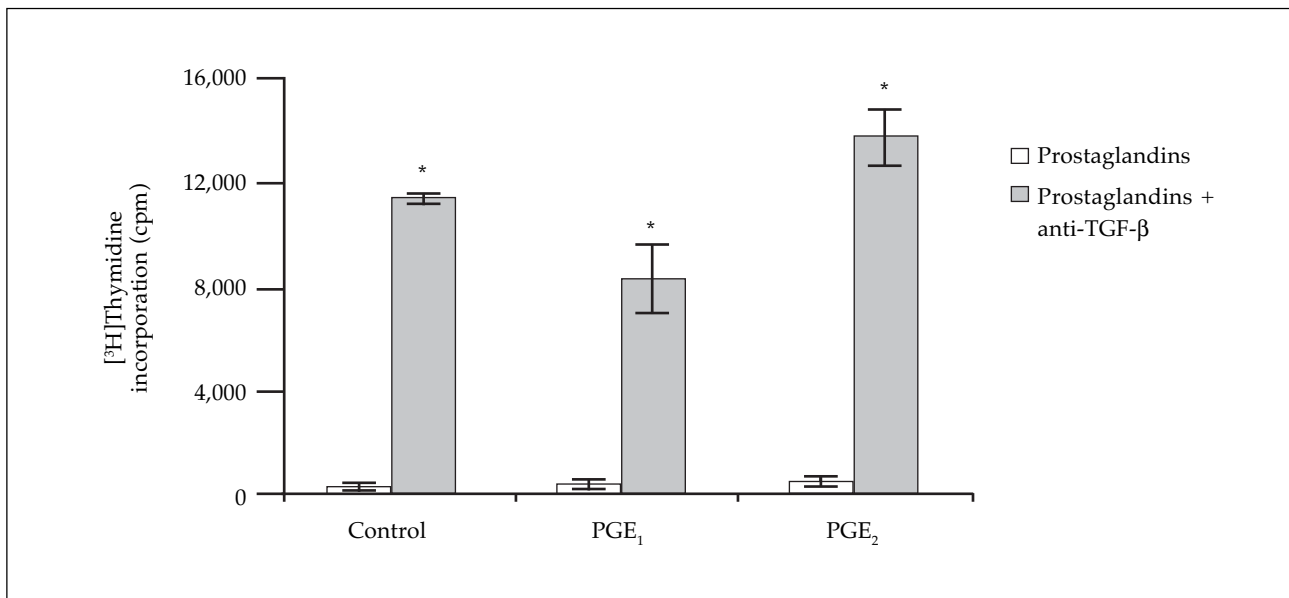


**Figure 6.** *Effects of transforming growth factor-β (TGF-β) released from prostaglandin E (PGE)-treated osteoblasts on thymidine incorporation in mink lung epithelial cell cultures. Osteoblasts were incubated with serum-free medium (control) or conditioned media containing $10^{-8}$ M of $PGE_1$ or $PGE_2$ for 24 hours. Media containing TGF-β were collected and activated. From each well, 0.5 mL of active TGF-β–containing medium was directly transferred into mink lung epithelial cell cultures (□ prostaglandins), while the other 0.5 mL was pre-treated with pan-specific TGF-β neutralizing antibody prior to transferring (▨ prostaglandins + anti-TGF-β). Each bar represents the mean ± standard error of the mean of four replicate cultures. Data were evaluated by the Mann-Whitney test. \*Thymidine uptake of cultures neutralized with anti-TGF-β antibody was significantly elevated compared to non-neutralized cultures,* $p < 0.01$.
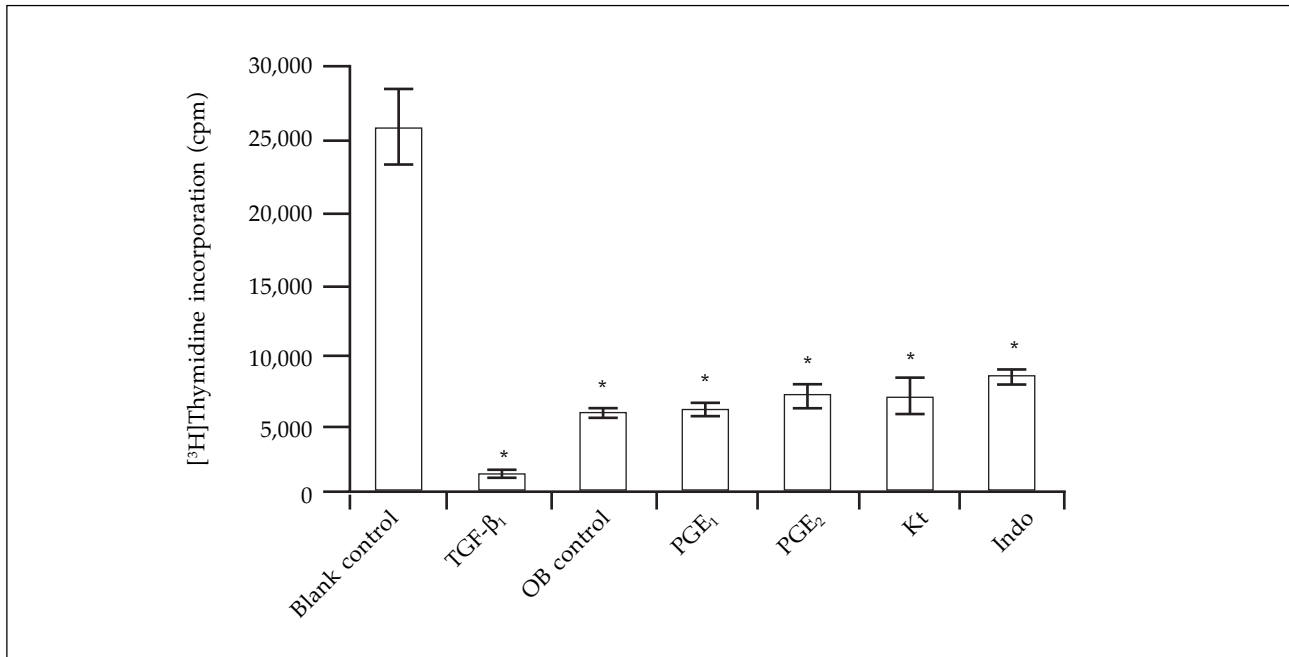
**Figure 7.** *Effects of nonsteroidal anti-inflammatory drugs (NSAIDs) and prostaglandin Es (PGEs) on the bioactivities of osteoblast transforming growth factor-β (TGF-β). Osteoblasts were incubated with serum-free medium (OB control) or conditioned media containing $10^{-5}$ M of ketorolac (Kt) or indomethacin (Indo) or $10^{-8}$ M of $PGE_1$ or $PGE_2$ for 24 hours. Media containing TGF-β were collected, activated, and then transferred into mink lung epithelial cell cultures. Thymidine incorporations of mink lung cell cultures incubated with serum-free medium (blank control), $TGF-\beta_1$, or osteoblast culture supernatants (OB control, $PGE_1$, $PGE_2$, Kt, or Indo) were measured. Each bar represents the mean ± standard error of the mean of four replicated cultures. Data were evaluated by the Kruskall-Wallis test and Dunn multiple comparisons. \*Thymidine uptake of all cultures was significantly less than in blank control cultures, $p < 0.01$.*

over the range of therapeutic concentrations ($10^{-6}$ and $10^{-4}$ M). Ketorolac and indomethacin did not significantly change the total fraction (active plus latent forms) of $TGF-\beta_1$ secreted from osteoblasts, suggesting that 24-hour treatment with therapeutic concentrations of ketorolac and indomethacin may not affect osteoblast $TGF-\beta_1$ expression at either the transcriptional or translational level. In order to exclude methodologic error, the TGF-β bioassay used in this study was carefully confirmed to reflect the bioactivity of TGF-β released in osteoblast-enriched cultures. The theoretic therapeutic concentration ($10^{-5}$ M) of both NSAIDs had a non-significant effect on TGF-β bioactivities in osteoblast-enriched cultures. This suggests that ketorolac and indomethacin may not affect the post-translational function of TGF-β produced by osteoblasts.

The biologic effects of TGF-β on modulation of bone and cartilage metabolism are significant but complicated. Some investigators indicated that TGF-β might act on bone or cartilage directly [44–47], while others demonstrated that TGF-β acts by stimulating prostaglandin synthesis [16,48–50]. Ota et al showed that $PGE_2$ stimulated the production of hepatocyte growth factor in human colonic fibroblast cultures [51]. Accordingly, the interactions between prostaglandins and growth factors might modulate their biologic function in various tissues. In the present study, we investigated the effects of PGEs on TGF-β expression and bioactivity in order to understand the role of prostaglandins other than their direct actions on osteoblasts. The results indicate that a broad concentration range of $PGE_2$ ($10^{-9}$–$10^{-6}$ M) had no significant effect on TGF-β mRNA expression. Treatment with $PGE_2$ ($10^{-8}$ M and $10^{-6}$ M) did not significantly change the amount of $TGF-\beta_1$ secreted by osteoblast-enriched cultures. $PGE_1$ and $PGE_2$ ($10^{-8}$ M) showed no significant effects on the bioactivity of TGF-β secreted from osteoblasts. Although conflicting results of the effects of prostaglandins on TGF-β expression and bioactivity have been reported [52], the different sources of the cells (i.e. species of

experimental animal, cell fractions collected from enzyme digestions, etc.) or different incubation times with agents might reveal different responses of the cells to the agents. The findings of this study imply that 24-hour treatment with PGEs may not modulate the expression and bioactivity of TGF-$\beta_1$ in fetal rat calvarial osteoblasts.

NSAIDs have been reported to suppress cell proliferation and/or to induce apoptosis in several cell lines [53–57]. However, the possible mechanisms have yet to be elucidated. In our previous study, 24-hour treatment with ketorolac and indomethacin ($10^{-7}$–$10^{-4}$ M) significantly inhibited osteoblast proliferation [10]. In this study, we demonstrated that 24-hour treatment with NSAIDs did not alter the expression or bioactivity of TGF-β in osteoblasts, implying that the effects of NSAIDs on osteoblast function may not be mediated by TGF-β. A recent report indicated that NSAIDs, by reducing the expression of vascular endothelial growth factor, may suppress proliferation and angiogenesis, and induce apoptosis in gastrointestinal cancer cells [58]. Accordingly, other anti-proliferative or anti-apoptotic growth factors involved in bone metabolism may mediate NSAID effects on osteoblast function. Other reports show that NSAIDs suppress cell growth and/or induce apoptosis in normal or tumor cells by decreasing cell-cycle regulators (i.e. cyclins or cyclin-associated kinases) and/or increasing pro-apoptotic proteins (i.e. bak) [53–57]. Shiff et al reported that aspirin, indomethacin, piroxicam, and naproxen caused cell-cycle quiescence and apoptosis, and simultaneously reduced the levels of $p34^{cdc2}$ and $p33^{cdk2}$ in colon adenocarcinoma cells [59]. Arber et al indicated that the cell growth-inhibiting and apoptosis-inducing effects of sulindac sulfide on parental enterocytes may result from the reduction of bak expression [53]. Therefore, it may be worth investigating the mechanism of NSAID effects on osteoblast function by studying the possible pathways of NSAID effects on osteoblast function through altering the cell cycle and/or apoptosis cascades.

## ACKNOWLEDGMENTS

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