Cytokine Regulation of Adult Human Osteoblast-Like Cell Prostaglandin Biosynthesis

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Abstract   Prostaglandin (PG) biosynthesis by cytokine stimulated normal adult human osteoblast-like (hOB) cells was evaluated by thin layer chromatography, high performance liquid chromatography, and specific immunoassays. PGE_2 was the predominant PG formed under all incubation conditions tested. Control samples produced measurable amounts of PGE_2, and the measured level of this metabolite increased by 22-fold (from 7 to 152 ng/ml) following a 20 h treatment with the combination of TGFβ and tumor necrosis factor-α(TNF). The production of 6-keto-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin) and of $PGF_{2\alpha}$ were each increased by about five-fold (from about 0.5 to 2.5 ng/ml) in samples treated with the cytokines. Thus, TGFβ and TNF exerted a regulation of hOB cell PG biosynthesis that was principally directed towards an increased PGE_2 biosynthesis, with lesser effects on the production of other PG metabolites. COX-2 mRNA levels were increased within 2 h of cytokine stimulation, reached a maximum at 6–12 h, and levels had appreciably diminished by 24 h after treatment. Both TGFβ and TNF could independently increase COX-2 mRNA levels and PG biosynthesis. However, the increased production of PGE_2 resulting from TNF stimulation was blocked by the addition of an interleukin-1β (IL-1β) neutralizing antibody, suggesting that TNF regulation of hOB cell PG synthesis was secondary to its capacity to increase hOB cell IL-1β production. TGFβ regulation of PG production was not affected by the addition of the neutralizing antibody. These studies support the proposition that PGs can be important autocrine/paracrine mediators of bone biology, whose production by hOB cells is responsively regulated by osteotropic cytokines. J. Cell. Biochem. 64:618–631.   © 1997 Wiley-Liss, Inc.

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Prostaglandins (PG) are synthesized upon need and act locally [see Smith, 1992, for review]. Since PGs are not stored, their release represents de novo synthesis from precursor polyunsaturated fatty acids, typically from arachidonic acid. Most, if not all, mammalian tissues are capable of synthesizing PGs; the specific PG produced can vary in a tissue specific manner. For example, the vascular endothelium generates prostacyclin (PGI_2) while platelets produce thromboxane A_2, each in a nearly quantitative manner [Needleman et al., 1976; Moncada et al., 1976]. The utilization of arachidonic acid by a given tissue can change with respect to the type or amount of metabolite formed as a function of tissue pathology, development, or differentiation [Keeting et al., 1986; Lysz et al., 1991; DeWitt, 1991]. Following earlier biochemical and pharmacological leads [Smith and Lands, 1972; Lysz et al., 1988; Fu et al., 1990], investigators have now confirmed that there are two genes that code for isoforms of the cyclooxygenase (COX, or prostaglandin endoperoxide H-synthase) enzymes, which catalyze the initial reaction of PG biosynthesis [Kujuba et al., 1991; Xie et al., 1991]. COX-1 is constitutively expressed in most tissues and is localized predominantly to the endoplasmic reticulum. COX-2 is typically expressed following stimulation with growth factors or cytokines and is found, principally, in the nuclear and perinuclear membranes of the cell [Morita et al., 1995]. Some evidence suggests that COX-1

and COX-2 may be functionally coupled to the production of different members of the PG family of compounds [Lysz and Needleman, 1982; Lysz et al., 1988].

The involvement of arachidonic acid metabolites in regulating bone metabolism is well documented. The first recognized effect of the PGs on bone was to stimulate resorption [Klein and Raisz, 1970], an observation that has been repeatedly confirmed. Further evaluations of the effects of PGs in bone now indicate that they have roles in the mediation of cyclic nucleotide accumulation, calcium levels, proliferation, matrix protein synthesis, calcification, and protease release, among other actions [see Kawaguchi et al., 1995, for review]. Many of the polypeptide stimulators of bone resorption increase PGE_2 production in osteoblastic cell cultures and in bone organ cultures. In some studies, but not all, the inhibition of PGE_2 synthesis limited the resorption promoting effects of the polypeptides [Tashjian et al., 1985; Akatsu et al., 1991].

Since the PGs have important bioregulatory roles in bone physiology and can contribute to the pathology of bone diseases, the characteristics of PG biosynthesis by cultured human osteoblast-like (hOB) cells are of interest and have been examined. These studies revealed that synthesis of the PGs is limited in unstimulated samples and that PGE_2 synthesis is markedly increased following the application of transforming growth factor-β_1 (TGFβ), tumor necrosis factor-α (TNF), or interleukin-1β (IL-1β), all known cytokine products of osteoblastic cells [Robey et al., 1987; Gowen et al., 1990; Keeting et al., 1991a]. Larger increases in PG synthesis by the hOB cells were elicited in samples treated with TNF and TGFβ in combination than when either cytokine was used alone, and evidence of a synergistic regulation of hOB cell PGE_2 biosynthesis by the cytokines is presented. The effects of TNF on hOB cell PG production were, at least partially, abrogated by the addition of an IL-1β neutralizing antibody, suggesting that TNF regulation of hOB cell PG biosynthesis may be a secondary effect due to its capacity to stimulate IL-1β production by these cells. Distinctions between the results of radiochemical and immunochemical analyses of hOB cell PG biosynthesis indicate that different glycerophospholipid pools provided substrate for the formation of different PGs. Northern blot analyses provides evidence that the cytokine-induced increase in PGE_2 biosynthesis is the result of increased COX-2 levels in the hOB cells.

METHODS AND MATERIALS
Patient Population

Trabecular bone explants were obtained, after gaining informed consent, from the manubrium of the sternum of patients undergoing elective thoracic surgery or from surgical waste generated from the femoral head during routine bone grafting procedures. All procedures were approved by the West Virginia University Internal Review Board. Patient records were evaluated, and explants were not taken from patients with diagnosed osteoporosis or from those presenting with endocrine disorders that affect bone. Femoral explants were obtained from 15 women, aged 42 to 78 (median age of 61), and from 13 men, aged 23 to 86 (median age of 65). Stenal explants were from four women, aged 46 to 67 (median age of 48), and from seven men, aged 30 to 75 (median age of 59). Due to the limited sample sizes, the data in the Results section do not distinguish between hOB cells derived from different bone compartments, or by the explant donor's age or sex.

hOB Cell Cultures

Trabecular bone explants were prepared and placed into culture according to the method developed by Robey and Termine [1985] as previously described [Cissel et al., 1996]. The essential elements of this method include stripping the explants in a 2-h collagenase (1 mg/ml; Gibco, Grand Island, NY) digestion after which the explants are placed in a calcium-free, phenol red-free mixture (1:1) of DMEM:Ham's F12K (Biofluids, Rockville, MD) supplemented to 10% heat-inactivated fetal calf serum (FCS;Gibco). These culture methods yield nearly homogeneous cell cultures that display multiple aspects of the mature osteoblast phenotype [Borke et al., 1988; Cissel et al., 1996]. The hOB cell phenotype is stable through two passages [Keeting et al., 1991b; Marie, 1994]. All experiments described in this report were performed with hOB cells subcultured at the end of first passage.

Reagents

Recombinant human TNF, recombinant human TGFβ_1, recombinant human IL-1β, and an IL-1β neutralizing antibody were purchased

from R&D Systems (Minneapolis, MN). Stock solutins of each were prepared and stored at $-20°C$ for no more than 3 months. TNF and IL-1β were reconstituted in phosphate buffered saline containing 0.1% bovine serum albumin (BSA), and TGFβ was prepared in 4 mM HCl containing 0.1% BSA. Unlabeled PGs, PGE$_2$-Monoclonal Enzyme Immunoassay Kits, and a cDNA to human COX-2 were obtained from Cayman Chemical Co. (Ann Arbor, MI). 1-[^{14}C]Arachidonic acid (55 mCi/mmol), [^{3}H]6-keto-PGF$_{1\alpha}$(168 Ci/mmol), [^{3}H]PGF$_{2\alpha}$(207 Ci/mmol), and [^{3}H]PGE$_2$ (168 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Antisera to 6-keto-PGF$_{1\alpha}$ and to PGF$_{2\alpha}$ were the generous gifts of Dr. Thomas Lysz of New Jersey Medical School, Newark, NJ [McGinley et al., 1985].

hOB Cell Incubations

High performance liquid chromatography (HPLC) and thin layer chromatography (TLC) studies. hOB cells were subcultured into six-well plates at 250,000 cells/well in medium supplemented to 1 mM calcium and 10% FCS. The calcium concentration was thereafter maintained at 1 mM. Forty-eight h after subculturing, media were replaced and the FCS supplementation was reduced to 1% for an additional 48 h. Over the final 24-h of this preincubation period, cells were exposed to 0.55 μCi 1-[^{14}C]arachidonic acid/well. Previous work has found that each of the major glycerophospholipids of the hOB cells becomes radiolabeled during this period [Cissel et al., 1995]. The media were then aspirated, and the cell layers were washed twice with 0.1% BSA in phosphate buffered saline to remove unincorporated 1-[^{14}C]arachidonic acid. Media supplemented to 1% or 10% FCS were applied to the cells, and the experimental manipulations were initiated 30 min later by the addition of 20 nM TNF, 40 pM TGFβ, both cytokines, or vehicle for durations as indicated. Before cytokine addition, some samples were pretreated with 50 μM ibuprofen for 30 min to inhibit PG production. Following the incubation, the hOB cell conditioned media were collected on ice, microfuged to remove cell debris, and stored at $-80°C$ in siliconized glass tubes until analyzed.

Enzyme linked immunosorbent assay (EIA) or radioimmunoassay (RIA) studies. hOB cells were subcultured at 50,000 cells/well into 24-well plates and pretreated as described above, omitting the preradiolabeling of the cells

with 1-[^{14}C]arachidonic acid. Cytokine treatments were performed using dosages and durations of treatment as described in the individual figures. In some experiments, 60 pM IL-1β and/or an IL-1β neutralizing antibody were used independently or in conjunction with TGFβ and TNF.

HPLC Assays

hOB cell conditioned media were thawed, and 1 μg each of unlabeled PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, 6-keto-PGF$_{1\alpha}$, and 13,14-dihydro-15-keto-PGE$_2$ were added to serve as carriers. Samples were acidified and loaded onto Sep-Pak C$_{18}$ cartridges (Waters Associates, Millford, MA) for extraction as described by Powell [1987]. Samples were washed sequentially with 15% ethanol, water, and petroleum ether, and PGs were eluted in ethyl acetate. In 10 trials, the recovery of applied [^{3}H]PGE$_2$ from the cartridges was 91 ± 9%. The ethyl acetate was evaporated under a stream of nitrogen, and the residues redissolved into acetonitrile. Products in the samples were separated by reverse phase HPLC using a 3.9 × 300 mm octadecylsilane (ODS) column with a 4 μm pore size (Waters Associates) according to the method of Cyran et al. [1989] with modification. PGs were isocratically eluted with 36% acetonitrile, pH 3.0, at a flow rate of 1.0 ml/min for 15 min. A gradient of 36–90% acetonitrile was then used to elute less polar products and arachidonic acid. Eluted fractions were collected for 18 second intervals and scintillation counted. UV monitoring at 196 nm of authentic PGs established the following retention times in this laboratory: 6-keto-PGF$_{1\alpha}$, 3.2 min; PGF$_{2\alpha}$, 4.9 min; PGE$_2$, 5.5 min; PGD$_2$ 6.2 min; 13,14-dihydro-15-keto-PGE$_2$, 13.4 min.

TLC Assays

hOB cell conditioned media were acidified with formic acid and extracted twice with three volumes of ethyl acetate. The extracts were dried under a stream of nitrogen, the residues redissolved into chloroform:methanol (1:2), and spotted onto Silica Gel G preadsorbent TLC plates (Analtech, Newark, DE). The plates were developed in ethyl acetate:iso-octane:acetic acid:H$_2$O (55:25:10:50) and used to expose Biomax X-ray film (Fisher Chemical, Pittsburgh, PA) for 7 days. Authentic PGs and arachidonic acid were chromatographed in this system and visualized by iodine vapor staining. Following autoradiography, the R$_f$ values of the radiolabeled

bands were compared to the R_f's calculated for the authentic standards to identify the products of the incubation. Ethyl acetate extraction recovers $72 \pm 5\%$ (N = 4) of [^{3}H]PGE$_2$ from incubation media.

Immunochemical Assays for PGs

PGE$_2$ was measured in hOB cell conditioned media using an EIA according to the supplier's instructions (Cayman Chemical). Briefly, an aliquot was removed from the media and assayed in duplicate in at least two dilutions. The detection limit was 60 pg/ml at 80% B/B$_0$.

6-Keto-PGF$_{1\alpha}$ and PGF$_{2\alpha}$ were measured in the hOB cell conditioned media by specific RIAs using similar procedures based on the method of Reingold et al. [1981] as described by McGinley et al. [1985]. The detection limit for 6-keto-PGF$_{1\alpha}$ was 125 pg/ml and 6 pg/ml for PGF$_{2\alpha}$, each at 80% B/B$_0$.

Northern Blot Analysis

Total RNA was isolated from hOB cells by the method of Chomczynski and Sacchi [1987]. Total RNA (10 µg/lane) was fractionated on a 1% agarose-formaldehyde gel (Life Technologies, Gaithersburg, MD) containing 100 µg/ml ethidium bromide. Subsequent to electrophoresis, RNA was transferred onto a 0.2 micron Biotrans nylon membrane (ICN Biomedicals, Aurora, OH) by capillary action. The integrity and equal gel loading of RNA and the efficiency of transfer were assessed by visualizing the 28S and the 18S rRNA bands under UV light. The RNA was cross-linked to the membrane using the CL-1000 UV cross-linker (UVP, San Gabriel, CA) and hybridized with the ^{32}P-labeled cDNA to COX-2 (Cayman Chemical). The COX-2 cDNA was radiolabeled by the random hexanucleotide primed second strand synthesis method using [α-^{32}P]dATP, [α-^{32}P]dCTP (3,000 Ci/mmol; DuPont, Wilmington, DE), Klenow fragment (New England Biolabs, Beverly, MA), and hexanucleotide primers (Boehringer Mannheim Biochemicals, Indianapolis, IN) [Feinberg and Vogelstein, 1983]. Hybridizations were performed at 42°C in the presence of 50% formamide (Sigma). The final low stringency washes were performed at 65°C in SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0). Autoradiography was performed by exposing the membrane to Kodak XAR film (Eastman Kodak, Rochester, NY) in the presence of DuPont Lightning Plus intensifying screens.

Statistical Analyses

Data are presented as the mean $\pm$ SEM of N experiments using different hOB cell strains. When appropriate the data were analyzed by the paired t test (e.g., cytokine effects in the presence or absence of the IL-1β neutralizing antibody). In other cases, data were analyzed by ANOVA and the Tukey-Kramer comparisons of all means (e.g., the analyses of the time course study of cytokine effects), using the SAS-JMP statistical package. Regression analyses were performed by the least squares method in the dose-response studies.

RESULTS

The release of 1-[^{14}C]-radiolabeled PGs by cytokine treated hOB cells was evaluated by HPLC (N = 4–8) and by TLC (N = 3–4), each method producing generally similar results (Figs. 1 and 2). Cells maintained in 1% FCS-supplemented medium produced little 1-[^{14}C]PG. Replacement of the 1% FCS medium with medium containing 10% FCS increased 1-[^{14}C]PGE$_2$ production. The application of the cytokines TGFβ or TNF, or both in combination, to the specimens elicited additional increases in 1-[^{14}C]PGE$_2$ formation, and a modest elevation, perhaps two-fold, in the levels of 1-[^{14}C]-6-keto-PGF$_{1\alpha}$ (the stable metabolite of PGI$_2$) produced. The independent effects of the cytokines on the production of the radiolabeled PGs appeared to be at least additive when cells were simultaneously treated with both agents. The capacity of the cytokines to modulate 1-[^{14}C]PG biosynthesis was sensitive to the concentration of FCS in the medium, and the 10% FCS concentration supported a greater cytokine-induced increase in PG synthesis than did medium containing 1% FCS. The formation of 1-[^{14}C]PGE$_2$ in these experiments was completely inhibited in samples that were incubated with 50 µM ibuprofen. The inhibitory effect of the ibuprofen on 1-[^{14}C]-6-keto-PGF$_{1\alpha}$ formation was variable, and in some HPLC experiments the cpm of this metabolite was actually higher in the presence of the cyclooxygenase inhibitor than in its absence. The elevation in 1-[^{14}C]-6-keto-PGF$_{1\alpha}$ production in those samples could reflect an increase in substrate availability due to the ibuprofen-dependent blockade of 1-[^{14}C]PGE$_2$ synthesis. Evidence for the production of other PG metabolites by the
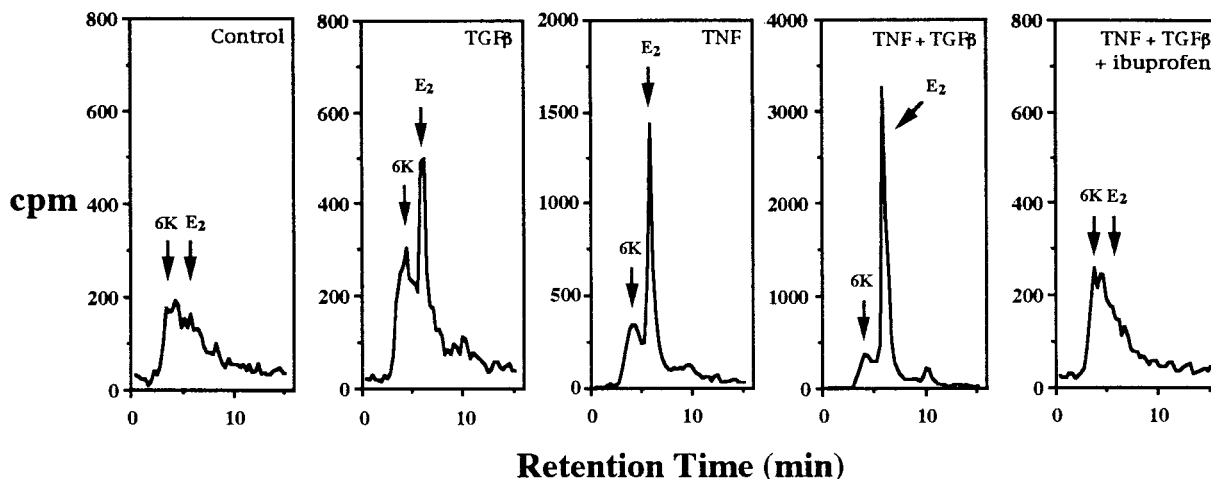
Retention Time (min)

Fig. 1. HPLC analysis of 1-[^{14}C]arachidonic acid metabolites produced by hOB cells. Pre-radiolabeled cells cultured in 10% FCS supplemented media were treated with (from left to right) vehicle, 40 pM TGFβ, 20 nM TNF, TGFβ + TNF, or TGFβ + TNF + the cyclooxygenase inhibitor ibuprofen. The 12-h conditioned media were collected, prepared as described, the peaks separated using an ODS column, and cpm determined. The experiment shown above used hOB cells from a 71-year-old male, and the results are representative of four to eight independent trials for each cytokine and two trials for the cytokine combination. Note that the Y-axis varies between graphs. Specific peaks are identified as 6K for 6-keto-PGF$_{1\alpha}$ and E$_2$ for PGE$_2$ based on the retention times of authentic PG standards.

hOB cells was not observed in these radiochemical evaluations.

A comparison of the effects of treatment of the cells with TGFβ, TNF, or both cytokines in combination, on radiolabeled PG production was made by calculating the ratios of the HPLC separated peaks for PGE$_2$ cpm:6-keto-PGF$_{1\alpha}$ cpm for the cytokine treated samples and their paired controls. A ratio of 0.68 ± 0.07 (N = 4) was calculated for the samples incubated in the 10% FCS supplemented medium. The ratio was 2.18 ± 0.66 following the addition of TNF (N.S.), and after the addition of TGFβ the ratio was increased to 1.30 ± 0.17 ($P < 0.05$). In two HPLC experiments cells were treated concurrently with both cytokines, producing a PGE$_2$:6-keto-PGF$_{1\alpha}$ ratio of 7.35 (8.37; 6.33). These calculations and the minimal changes in the cpm associated with the 6-keto-PGF$_{1\alpha}$ peak suggested that the cytokines functioned almost exclusively to stimulate increased PGE$_2$ production by the hOB cells.

The radiochemical studies guided related investigations into hOB cell PG synthesis that used immunochemical based assays to determine quantitatively the effects of the cytokines on PGE$_2$ and on 6-keto-PGF$_{1\alpha}$ production. As shown in Table I, hOB cells stimulated with TGFβ and TNF in combination over a 20 h incubation produced 58 times as much PGE$_2$ as they did 6-keto-PGF$_{1\alpha}$ (N = 15). The formation of PGE$_2$ in the unstimulated (other than the 10% FCS supplementation) control hOB cell cultures exceeded that of 6-keto-PGF$_{1\alpha}$ by only 14-fold, indicating that the cytokines did exert a selective regulation of PGE$_2$ synthesis. The stimulation of the hOB cells by the TGFβ and TNF combination increased PGE$_2$ formation by about 22-fold above control production ($P < 0.01$) while the cytokine-dependent increase in 6-keto-PGF$_{1\alpha}$ formation was only 5.4-fold ($P < 0.01$). The interpretation of the effects of the cytokines on 6-keto-PGF$_{1\alpha}$ biosynthesis may be somewhat complicated by the ability of these cytokines to increase phospholipase activity, at least in some tissues [Jackson et al., 1993; Kawaguchi et al., 1996]. Therefore, the 5.4-fold increase in 6-keto-PGF$_{1\alpha}$ production may be a consequence of increased substrate availability, rather than a consequence of a cytokine dependent increase in the capacity of the cells to produce that metabolite. Also supporting the prospect that the cytokines specifically increased hOB cell PGE$_2$ production were the measurements made of an additional PG product, PGF$_{2\alpha}$, in some experiments (N = 8). For that metabolite, levels were increased by 4.7-fold ($P < 0.01$) following cytokine stimulation, an effect consistent with an increase in substrate availability, rather than a specifically increased capacity for PGF$_{2\alpha}$ biosynthesis.
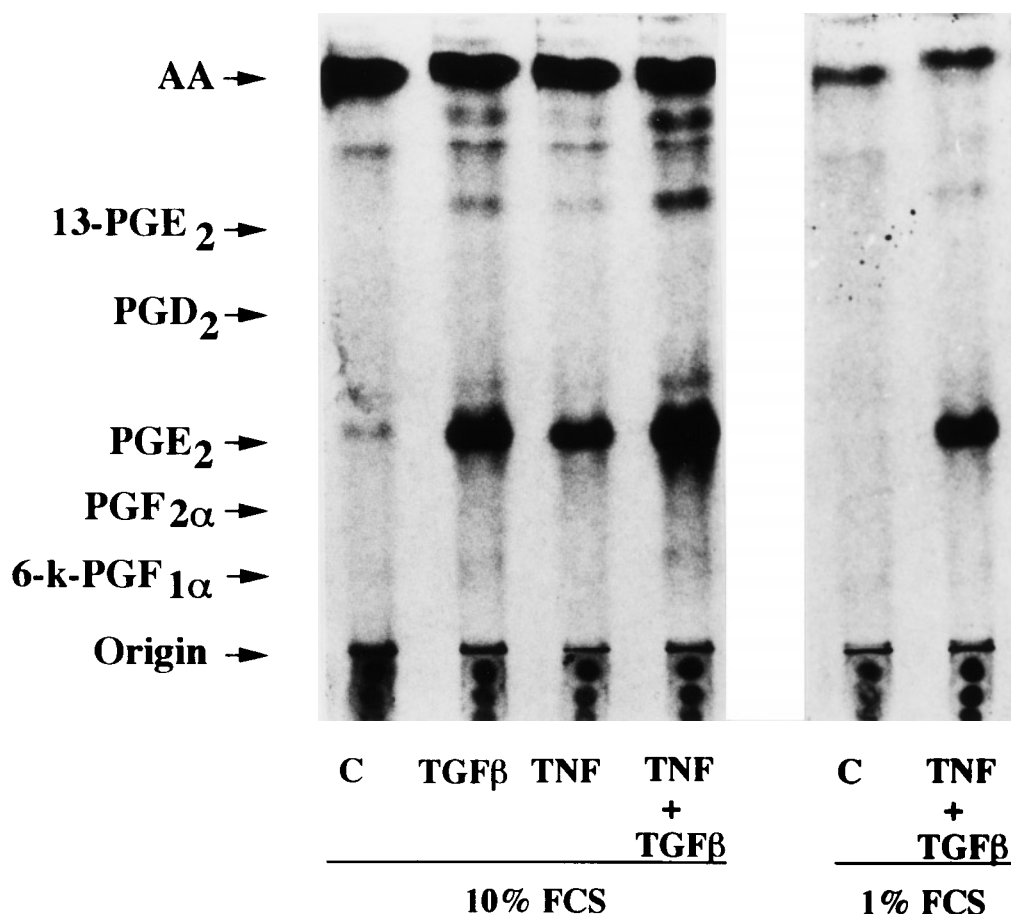
Fig. 2. TLC analysis of 1-[^{14}C]arachidonic acid metabolites produced by hOB cells. Pre-radiolabeled cells were treated with cytokines as indicated beneath each lane in media supplemented to either 1% or 10% FCS. The 20-h conditioned media were collected, prepared as described, developed, and the plates used to expose X-ray film. Shown above are the results from an experiment using hOB cells derived from a 42-year-old woman; the results are representative of four independent trials with different cell strains. The migration of authentic PG standards in this system are shown to the left of the autoradiograph. 13-PGE$_2$ = 13,14-dihydro-15-keto-PGE$_2$.

TABLE I. PGE$_2$ is the Predominant Product in Cytokine-Stimulated hOB Cells[†]

Treatment	PGE$_2$	6-k-PGF$_{1\alpha}$	PGF$_{2\alpha}$
	ng/ml[a]		
Control	7.1 ± 1.9	0.5 ± 0.14	0.3 ± 0.07
TNF + TGF$_\beta$	156.4 ± 32.3*	2.7 ± 0.4*	1.4 ± 0.26*

[†]hOB cells were treated with 20 nM TNF and 40 pM TGFβ in combination, or with vehicle for 20 h in 10% FCS supplemented medium. PGE$_2$ was assayed by EIA (N = 15) while 6-k-PGF$_{1\alpha}$ (N = 15) and PGF$_{2\alpha}$ (N = 8) were assayed by RIA.
[a]Data represent the mean ± SEM.
*Significantly ($P < 0.001$) different from control.

The effects of TGF$_\beta$ and TNF were time-dependent (Fig. 3), and the levels of PGE$_2$ rose significantly ($P < 0.05$; N = 5) within 12 h of cytokine addition. In the samples treated with both cytokines, levels continued to rise through the 20-h time course. 6-Keto-PGF$_{1\alpha}$ production in seven cell strains was significantly ($P < 0.05$) increased within the initial 4 h of cytokine treatment and continued to rise in the samples treated with both cytokines through the 12 h and 20 h time points (data not shown). Cytokine dose-response profiles were examined (Fig. 4, Panels A and B) in 5 hOB cell strains for TGFβ (r^2 = 0.17; $P < 0.01$) and for TNF (r^2 = 0.03; NS, $P = 0.29$). The responses of these cell strains to the treatments revealed a high degree of cell strain specific heterogeneity. For example, in two of the tested cell strains (Fig. 4, Panel A insert), treatment with 0.1 nM TNF was sufficient to increase PGE$_2$ production to nearly maximal levels with little further increase apparent as the TNF concentration was
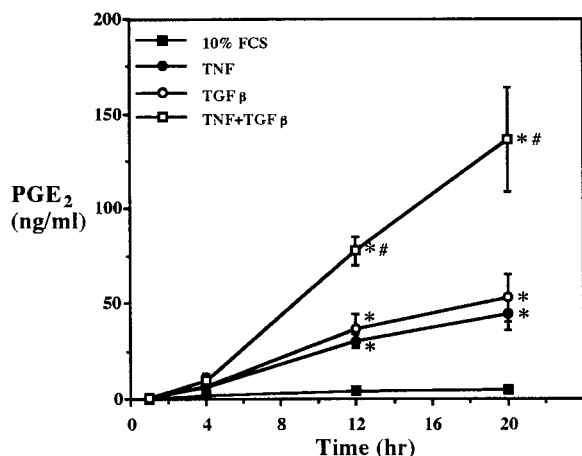
Fig. 3. Time course for PGE_2 production by hOB cells. Cells were incubated in 10% FCS supplemented media and treated with vehicle (solid square), 20 nM TNF (solid circle), 40 pM TGFβ (open circle), or TNF + TGFβ (open square). PGE_2 release was determined by EIA at 1, 4, 12, and 20 h of treatment. Data represent the mean ± SEM for five hOB cell stains. *$P < 0.05$ vs. 10% FCS alone. #$P < 0.05$ vs. all other treatments.

raised to 100 nM. The other 3 hOB cell strains appeared to exhibit a more typical TNF dose-dependency. The heterogeneity of these human cell strains was evident in the absolute amounts of PGE_2 produced in the TGFβ dose-response curve by the different strains (Fig. 4, Panel B inset).

The ability of TNF to regulate hOB cell PG biosynthesis could have been a direct action of the cytokine. Alternatively, this regulation could have been an indirect consequence, which was secondary to TNF's capacity to stimulate hOB cell IL-1β production [Keeting et al., 1991a], a cytokine that itself regulated hOB cell PGE_2 biosynthesis in a dose-dependent manner ($r^2 = 0.53$; $P < 0.001$) (Fig. 5). A neutralizing antibody specific for IL-β was used to examine this issue in 8 hOB cell strains (Fig. 6). IL-1β stimulation of PGE_2 biosynthesis by the hOB cells was blocked by the addition of the neutralizing antibody. TGFβ regulation was unaffected by the presence of the antibody, but the TNF effect was abolished by antibody addition ($P < 0.05$ vs. TNF treated; NS vs. Control). The antibody was unable to significantly oppose the increase in PGE_2 production due to treatment of the cells with the combination of TNF plus TGFβ (cytokines added vs antibody + cytokines: NS, $P = 0.26$), a result that many reflect, in part, TNF actions at the level of phospholipase activity, rather than to a TNF-enhanced PGE_2 biosynthetic capacity. The effects of the

combined cytokines on hOB cell PGE_2 biosynthesis typically exceeded the additive responses of the cells to treatment with the individual cytokines in the eight experiments performed to test that possibility (Fig. 7).

Northern blot analyses of the mRNA for COX-2 was performed in 2 hOB cell strains and demonstrated a cytokine-induced, time-dependent increase in the steady-state levels of the message (Fig. 8). Very low levels of the message were seen in the 6 h control samples. Within 2 h of treatment with TGFβ plus TNF an increased level of the COX-2 message was evident. Additional increases in the steady-state mRNA level for COX-2 were apparent at the 4 h to 12 h points of the incubations, and levels in each experiment had declined between 12 and 24 h of treatment. Although each cytokine could independently elevate the levels of the COX-2 mRNA over a 6 h incubation, neither was as effective as when they were added in combination.

DISCUSSION

Treatment of the hOB cells with TNF and TGFβ in combination resulted in increased PGE_2 synthesis. The effect of these combined cytokines was gradual in onset with a significantly increased accumulation of PGE_2 not evident within the initial 4 h of treatment. The response of the cells to the combined cytokines was prolonged, and the rate of PGE_2 synthesis was higher between 12–20 h after treatment than it was at the earlier intervals of 1–4 h or 4–12 h. Cytokine regulation of PG biosynthesis can show tissue specific effects, and TGFβ has been reported to promote, and to oppose, increased PG biosynthesis in various tissues [reviewed by Goppelt-Struebe, 1995]. IL-1 regulation of COX-2 mRNA levels occurs by an initial increase in transcription and a following stabilization of the message [Jones et al., 1993; Ristimaki et al., 1994]. Although in some tissues COX-2 is considered an early response gene that is rapidly, but transiently, increased by treatment over a 1–4 h span, IL-1 regulation can exceed 24 h [De Witt and Meade, 1993]. TNF can mediate PG biosynthesis by activating extant cytosolic phospholipase A_2 and by increasing the rate of phospholipase A_2 synthesis [Sato et al., 1987; Yanaga et al., 1992; Kawaguchi et al., 1996]. The present report demonstrates that an IL-1β neutralizing antibody completely blocked the independent effects of TNF
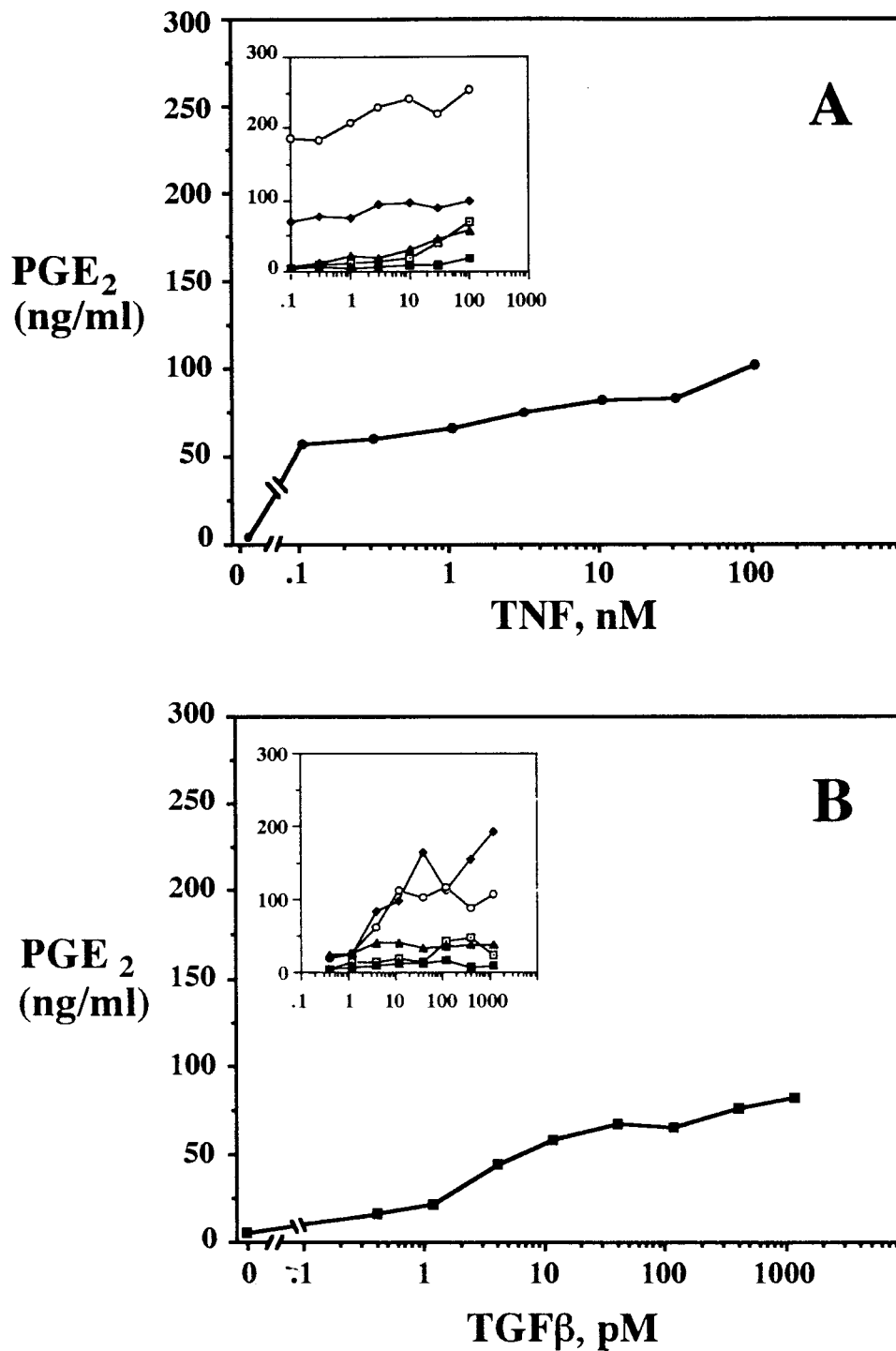
Fig. 4. Dose-response profiles for TNF and TGFβ on hOB cell PGE_2 production. hOB cells were treated with varied doses of TNF (**A**) or TGFβ (**B**) and the 20-h conditioned media assayed for PGE_2 by EIA. Insets within the panels show the responses of the five individual cell stains tested in these experiments, while the mean responses of the strains are shown in the main panel. TGFβ dose-dependently regulated PGE_2 production ($P < 0.01$) while TNF did not exhibit dose-dependent effects in these experiments.
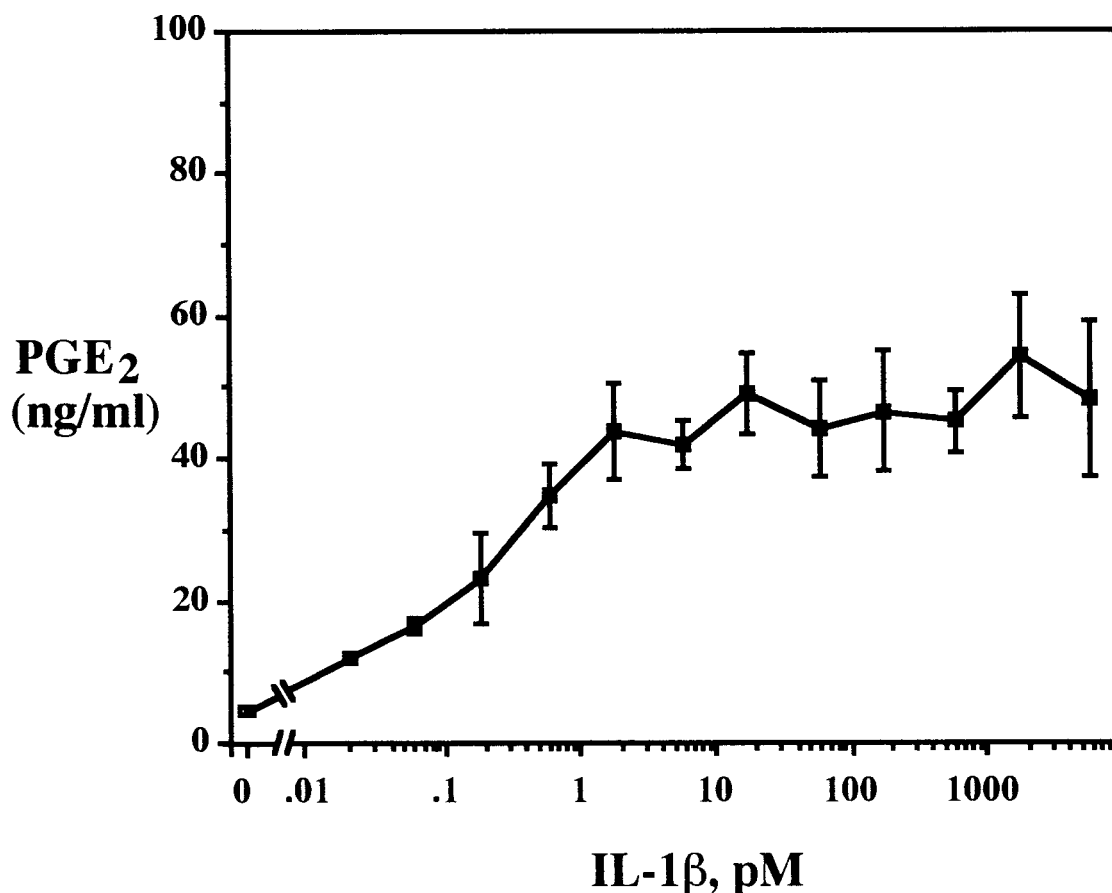
Fig. 5. PGE$_2$ production by hOB cells stimulated with varied doses of IL-1β. The 20-h conditioned media of three hOB cell stains stimulated with IL-1β were assayed for PGE$_2$ by EIA. PGE$_2$ production was dose-dependently regulated by the cytokine ($r^2 = 0.53$; $P < 0.001$).

on hOB cell PGE$_2$ production. However, the efficacy of the antibody treatment was less clear when used in incubations in which the TNF treatment was combined with a TGFβ treatment. Pilbeam and coworkers [Kawaguchi et al., 1996] reported that the application of IL-1 receptor antagonist could not completely block TNF effects on COX-2 and phospholipase A$_2$ expression in a murine osteoblastic cell model system. Thus, TNF may exert some effects directly, while other responses are secondary and are dependent on its induced production of IL-1.

Radiochemical analyses indicated that PGE$_2$ was the major prostanoid product of cytokine stimulated hOB cells. This indication was confirmed by the immunochemical studies performed. To our knowledge the characterization of PG metabolites produced by cytokine-stimulated normal adult human osteoblast-like cells by HPLC has not previously been reported. HPLC analysis of PG production by the human osteosarcoma G292 cell line indicated that

6-keto-PGF$_{1\alpha}$ was the major product of control cells, and that stimulation of these cells with IL-1 resulted in the production of increased amounts of PGE$_2$ [Zhang and Dziak, 1993]. 6-Keto-PGF$_{1\alpha}$, PGE$_2$, and PGF$_{2\alpha}$ were detected by HPLC analyses of conditioned medium from studies of neonatal rat calvarial cells or from a rat clonal osteosarcoma cell line [Nolan et al., 1983]. 6-Keto-PGF$_{1\alpha}$, PGE$_2$, and PGF$_{2\alpha}$ were also the major PG products detected in epidermal growth factor stimulated mouse calvarial cell cultures; that study also reported detecting thromboxane formation following the addition of radiolabeled arachidonic acid [Voelkel et al., 1980]. 6-Keto-PGF$_{1\alpha}$, PGE$_2$, PGF$_{2\alpha}$, PGD$_2$, and thromboxane were all identified in HPLC analyses of the media from osteoblast enriched neonatal chick calvarial cell cultures [Feyen et al., 1984]. TLC analysis of the conditioned medium from IL-1α and TGFβ stimulated murine clonal osteoblastic MC3T3-E1 cells revealed only PGE$_2$ formation [Marusic et al., 1991]. The radio-
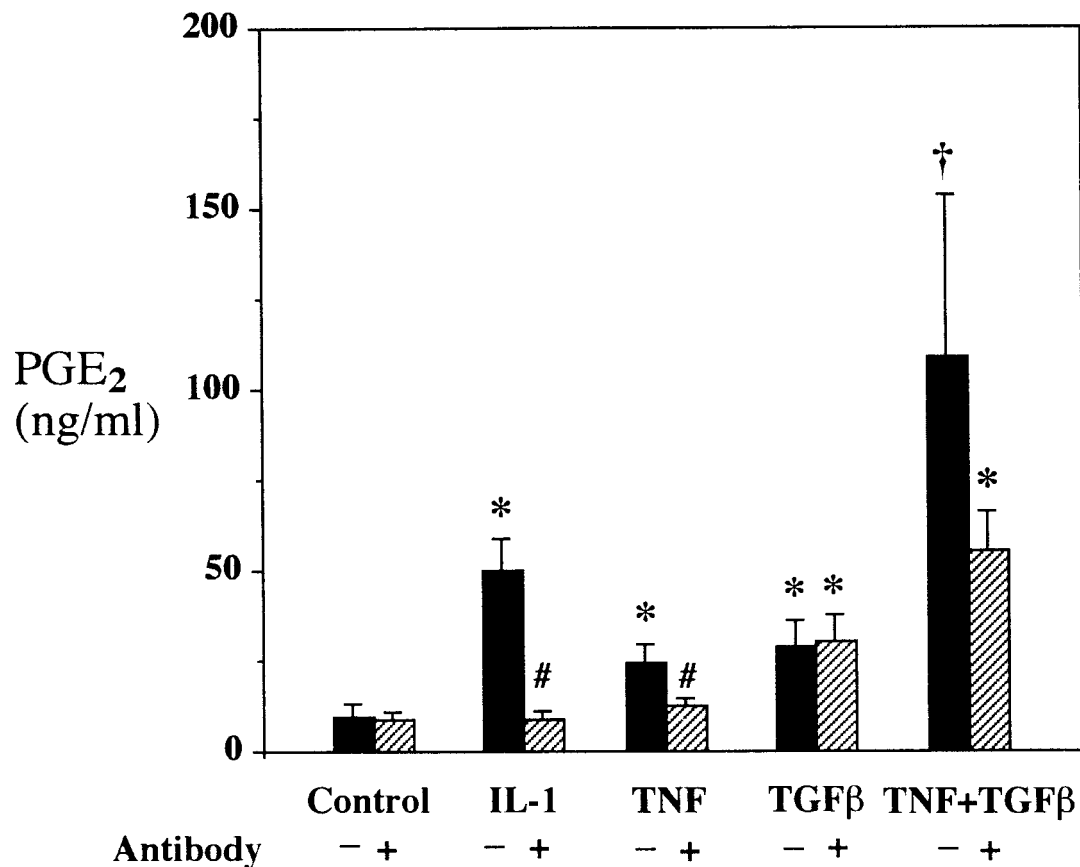
Fig. 6. Effects of an IL-1β neutralizing antibody on hOB cell PGE_2 production. hOB cells were incubated in 10% FCS supplemented media with vehicle (control), 60 pM IL-1β, 20 nM TNF, 40 pM TGFβ, or TNF + TGFβ added (solid bars). Paired samples were identically treated with the further addition of the IL-1β neutralizing antibody (cross-hatched bars). The 20-h conditioned media were collected and PGE_2 was assayed. Data represent the mean ± SEM from eight hOB cell stains. *$P < 0.01$ vs. control; +$P < 0.05$ vs. control; #$P < 0.05$ vs. paired cytokine only.

chemical data from the HPLC and the TLC experiments reported herein, indicate that PGE_2 and 6-keto-$PGF_{1\alpha}$ are the predominant prostanoid products of unstimulated hOB cells and that cytokine treatment of the cells specifically increases PGE_2 formation. The hOB cell cultures that are produced using the Robey and Termine method [1985], as employed in this laboratory, are at least 95% homogeneous for cells that stain for osteocalcin following 1,25-dihydroxyvitamin D_3 treatment [Borke et al., 1988] and express multiple additional aspects of the mature osteoblast phenotype [Cissel et al., 1996; Keeting et al., 1992]. Calvarial cell cultures include cells not of the osteoblastic lineage, which may contribute to the array of products identified in those studies. The different results reported for the various model systems may also reflect species or age related distinctions, or the various treatments employed to stimulate PG biosynthesis. While the present radiochemical data do not provide evidence of the synthesis of other PGs or thromboxane by hOB cells, the data cannot rule out the possibility that other metabolites are produced in limited amounts; indeed, our own measurements of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ by RIAs illustrate the limitations inherent in the exclusive use of radiochemical analyses to study PG biosynthesis.

Whereas the radiochemical data suggested that cytokine treatments had little effect on 6-keto-$PGF_{1\alpha}$ production, the RIA data revealed that its level rose by greater than five-fold following stimulation of the cells with the combination of TNF and TGFβ. The discrepant results produced by these complementary methods indicate that the arachidonic acid used by the cells for the production of PGE_2 and for 6-keto-$PGF_{1\alpha}$ must be derived from distinct glycerophospholipid pools that are differentially radiolabeled during the preincubation with
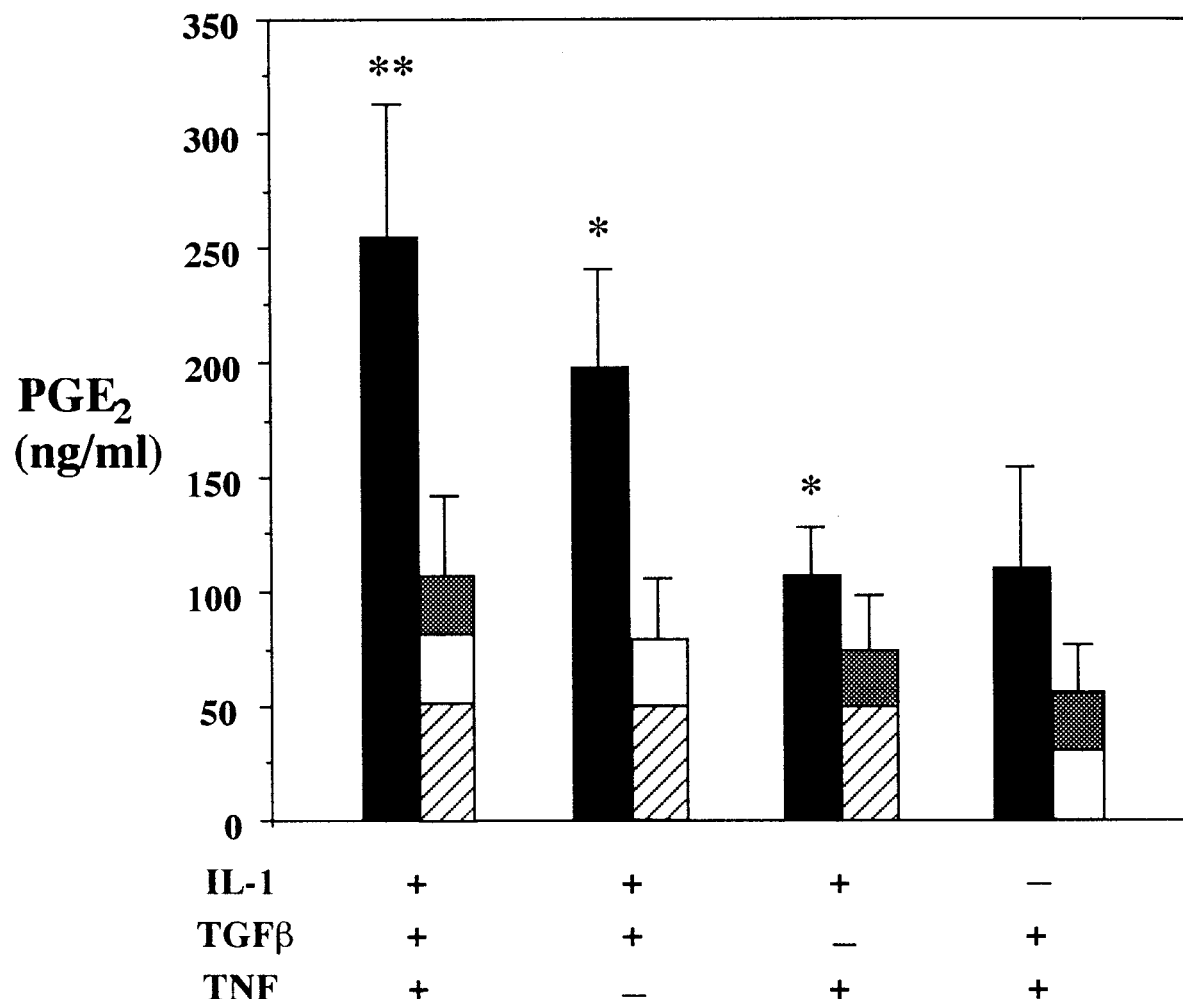
Fig. 7. Factorial examination of cytokine regulation of PGE_2 production. hOB cells were treated with 60 pM IL-1β, 40 pM TGFβ, 20 nM TNF, singly or in all possible combinations for 20 h in 10% FCS supplemented medium, PGE_2 release was measured by EIA. The solid black bars represent PGE_2 production for the samples treated simultaneously with two or all three cytokines evaluated. The paired complex bars reflect the additive product of PGE_2 synthesis in the samples treated singly with IL-1β (cross-hatched), TGFβ (white), and TNF (gray). Data represent the mean ± SEM from eight hOB cell strains. **$P < 0.001$; *$P < 0.01$.

1-[^{14}C]arachidonic acid and suggest a compart-mentalized formation of the different PG prod-ucts within the cells. The apparent utilization of distinct arachidonic acid pools by the dual cyclooxygenases has previously been reported for murine cells by Murakami et al. [1994] and by Reddy and Herschman [1994]. The observed effects of the cytokines on COX-2 mRNA levels was entirely consistent with the biochemical data and, in agreement with much of the recent literature, suggests that hOB cell PG synthesis is regulated in part by the de novo synthesis of the inducible form of the enzyme [Goppelt-Struebe, 1995; Kawaguchi et al., 1995]. The distinct reserves of arachidonic acid utilized for the synthesis of the different PGs, the apparent

requirement for COX-2 expression to permit an amplification of PGE_2 production, and the differ-ent subcellular locations of COX-1 and COX-2 that have been reported [Morita et al., 1995] provide an array of potential control points that should permit a fine regulation of hOB cell PG biosynthesis to be achieved.

The complexities inherent in bone cell PG biosynthesis are entirely consistent with the diverse biological roles ascribed to the PGs by various investigators as reviewed by Raisz and coworkers [Kawaguchi et al., 1995]. As the physiological roles of the PGs in bone are ex-plored and catalogued, the present data indi-cate the need to consider that exogenous PG application to bone cell and organ systems may

EXPT. 1

COX-2 →

28 S →

18 S →

EXPT. 2

COX-2 →

28 S →

18 S →

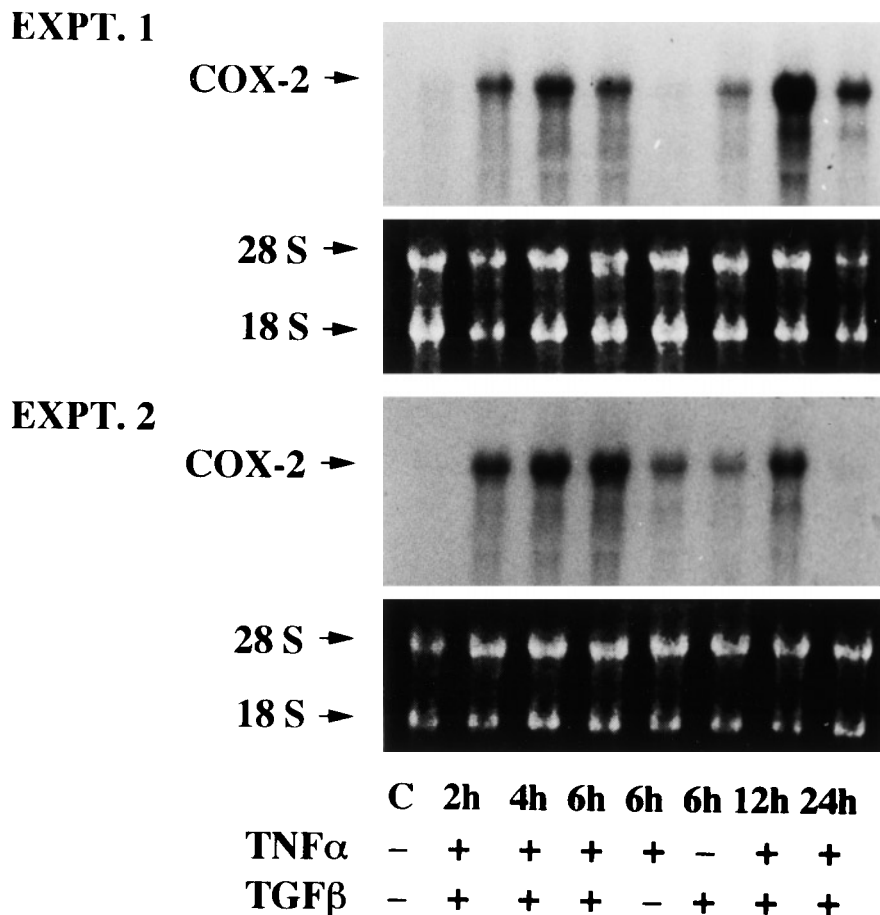|	C	2h	4h	6h	6h	6h	12h	24h
TNFα	–	+	+	+	+	–	+	+
TGFβ	–	+	+	+	–	+	+	+

Fig. 8. Northern blot analyses of COX-2 mRNA in cytokine treated hOB cells. The results of two experiments in which hOB cells were treated with vehicle (control), 40 pM TGFβ, or 20 nM TNF for 6 h, or with both cytokines for 2, 4, 6, 12, or 20 h, as indicated beneath each lane, are shown. Hybridization of total RNA with the ^{32}P-radiolabeled cDNA to human COX-2 was performed as described in the Methods. Photographs of the ethidium bromide stained 28S and 18S rRNA bands from each membrane are shown as an indication of equivalent loading of the lanes and transfer of the separated RNA species. Experiment 1 was performed using cells from a 70-year-old male and Experiment 2 used cells from a 66-year-old male explant donor.

not entirely reflect the actions of endogenously produced PGs. The present report demonstrates that while various osteotropic cytokines can increase PGE_2 biosynthesis by hOB cells, the cytokines also lead to increased synthesis of other PG products. Whether the more significant response of the cells to mediators is the increased amount of specific PG produced, or whether the relative ratios of the different PGs formed are as critical to their actions is not yet known. The present findings also suggest that released arachidonic acid is selectively directed towards COX isoforms or towards a compartmentalized COX-2, which may be functionally coupled to the formation of specific PGs. Therefore, exogenous arachidonic acid provided to variously treated bone cell or organ cultures may not be appropriately channeled to support the synthesis of a specific PG product.

COX-1 products seem to be directed for export to condition the local environment [Morita et al., 1995]. The activity of this enzyme is generally regulated by circulating hormones which activate cellular phospholipases. COX-2 products may serve first as intracellular regulators, although the products are also exported and do operate extracellularly as well [DeWitt and Smith, 1995]. COX-2 activity is regulated through its increased expression following stimulation with an array of growth factors or cytokines. The released PGs occupy plasma membrane associated, G protein-coupled receptors to elicit the production of various second messengers [Narumiya, 1995]. The functions of the intracellular PGs are less understood. It was recently shown that PGs of the J-series can directly activate the peroxisome proliferator-activated receptor γ (PPARγ) to mediate adipo-

genesis [Foreman et al., 1995; Kliewer et al., 1995]. 15-Deoxy-PGJ$_2$ may be the endogenous ligand of this nuclear transcription factor. Whether 15-deoxy-PGJ$_2$ is unique among PGs in activating a nuclear receptor, or if it represents an alternative mechanism for PG action in some cells is unknown. An EC$_{50}$ of 2 μM for 15-deoxy-PGJ$_2$ was reported in these studies for PPARγ activation. PGE$_2$ released into the medium of 20 h cytokine stimulated hOB cells frequently reached, or exceeded, 0.1 μM indicating that nuclear and perinuclear concentrations of PGs must reach rather high concentrations.

PG mediation of bone biology occurs at several levels. They are mitogenic for preosteoblasts, they influence components of mature osteoblast functions, they participate in the development of the bone resorbing osteoclasts from mononuclear cells, and they can directly limit osteoclast activity [Kawaguchi et al., 1995]. The PGs can increase protease and collagenase release thereby facilitating the initiation of the resorptive process. PGs can limit apoptosis in some cells, a feature that may support cytokine functions during inflammation and tissue repair [Tsuji and DuBois, 1995; Machwate et al., 1996]. The inhibition, or enhanced production, of specific PG biosynthesis in bone could represent a means to manipulate selected aspects of bone biology to produce a therapeutic outcome. The characterization of bone cell PG biosynthetic processes and pathways may advance the field towards such goals.

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