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PROINFLAMMATORY CYTOKINES TUMOR NECROSIS FACTOR-α AND IL-6, BUT NOT IL-1, DOWN-REGULATE THE OSTEOCALCIN GENE PROMOTER[1]

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A proinflammatory cytokine cascade, including IL-1α, IL-1β, TNF-α, IL-6, and IL-8, is activated in response to infection or immunologic insult. Besides their immunologic effects, several of these mediators stimulate bone resorption and inhibit bone formation. Osteocalcin, the most abundant noncollagenous protein present in bone, is an osteoblast-specific product whose production closely correlates with bone formation, and which has also been implicated in control of bone resorption. IL-1 and TNF have previously been shown to down-regulate osteocalcin production in vitro and in vivo, although the mechanism of this inhibition is unknown. In the present studies, IL-1β and TNF-α both inhibited 1,25-dihydroxyvitamin D3-stimulated production of osteocalcin protein and mRNA by ROS 17/2.8 osteosarcoma cells, whereas IL-6 had no effect on protein and only weakly inhibited mRNA. To determine if down-regulation was exerted at the transcriptional level, an osteocalcin promoter-chloramphenicol acetyltransferase (CAT) fusion gene was constructed (PHOC-CAT). After transient transfection of PHOC-CAT into ROS 17/2.8 osteosarcoma cells, reporter CAT activity was up-regulated by vitamin D at concentrations above 10^{-12} M. In screening studies, TNF-α (−57%) and IL-6 (−37%) inhibited vitamin D-stimulated osteocalcin transcription, whereas IL-1α, IL-1β, and IL-8 had no effect. Other immune cytokines and growth factors, including IL-2, IL-3, IL-7, and M-CSF, also failed to regulate osteocalcin transcription. Despite their lack of promoter regulation, IL-1α and IL-1β also stimulated PGE_2 production by ROS 17/2.8, further confirming the ability of the host cell to respond to these mediators. In dose-response experiments, down-regulation by TNF-α was significant at concentrations as low as 0.14 pM (0.1 U/ml), whereas approximately 10^4-fold higher concentration of IL-6 was required to exert a similar effect. TNF-α-mediated down-regulation was unaffected by indomethacin. These data demonstrate that of these cytokines, TNF-α alone potently down-regulates osteocalcin promoter function, whereas IL-1 acts post-transcriptionally, possibly by reducing mRNA stability. Heterogeneity therefore exists among the proinflammatory cytokines with respect to the level at which control of osteocalcin expression is exerted.

The host response to infection and immunologic insult results in the rapid induction of a cytokine cascade, which includes IL-1β, IL-1α, TNF-α, IL-6, and IL-8 (1). Many of the proinflammatory activities of IL-1, TNF-α, and IL-6 are overlapping, and include induction of fever, acute phase protein synthesis, stimulation of collagenase and PG synthesis, and bone and cartilage resorption (2). Besides stimulating resorption, IL-1 and TNF also inhibit bone formation in vitro (3–5). IL-8, also termed neutrophil-activating factor, stimulates chemotaxis and degranulation of neutrophils (6). The effects of the proinflammatory cytokines on connective tissue and bone have important pathologic consequences in diseases such as arthritis, periodontitis, and certain malignancies (7).

Osteocalcin is an osteoblast product that constitutes the most abundant noncollagenous protein present in bone (8). Serum levels of osteocalcin correlate closely with histomorphometric parameters of bone formation (9); this marker has therefore been widely used as an indicator of new bone formation in vivo (10). Although its precise function is unclear, osteocalcin may control hydroxyapatite crystal growth and, in addition, may constitute an important signal for osteoclastic resorption. Subcutaneously implanted bone particles that contain reduced amounts of osteocalcin are poorly resorbed (11), and there is evidence that osteocalcin is chemotactic for osteoclast precursors (12, 13).

Osteocalcin production correlates with the onset of bone mineralization (14). Osteocalcin gene expression is up-regulated by 1,25-dihydroxyvitamin D3 (vitamin D), vitamin A, and parathyroid hormone, and is down-regulated by glucocorticoids, fibroblast growth factor, and transforming growth factor β (15). TNF-α and IL-1β both down-regulate osteocalcin protein, as well as type I collagen and alkaline phosphatase production by osteoblasts (3–5, 16, 17). Recently, we have shown that infusions of IL-1β inhibit serum osteocalcin levels and bone apposition in vivo (18). However, at present, little is known concerning the mechanisms by which the proinflammatory cytokines exert their inhibitory effect. In the present studies, we have therefore investigated whether down-regulation of osteocalcin expression is exerted at the level of osteocalcin gene transcription, using an osteocalcin promoter-CAT[3] fusion gene construct.

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[3] Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; M-CSF, macrophage CSF; VDRE, vitamin D-responsive element; GPDH, glyceraldehyde phosphate dehydrogenase; ROS, rat osteosarcoma; PHOC-CAT, human osteocalcin promoter CAT fusion gene.

MATERIALS AND METHODS

Cell culture and mediators. ROS 17/2.8, kindly provided by Dr. P. K. DenBesten, Department of Biochemistry, Forsyth Dental Center (Boston, MA) was maintained in F-12 medium supplemented with L-glutamine (4 mM), 1% penicillin-streptomycin, and 5% FCS. ROS 17/2.8 expresses the osteoblastic phenotype, including production of type I collagen, alkaline phosphatase, and 1,25-dihydroxyvitamin D3-regulated osteocalcin synthesis (19). Human rIL-1α, rIL-2, rIL-3, rIL-6, rIL-7, rIL-8, rTNF-α, and M-CSF were kindly provided by Genzyme Corp., Boston, MA. Human and mouse rIL-1β were generously donated by Dr. Robert Newton, Dupont, Glenolden, PA. Mouse IL-1α was the gift of Dr. P. LoMedico, Hoffman LaRoche, Nutley, NJ. Specific activities of the cytokines were as follows: IL-1α, IL-1β: 10^8 U/mg (conversion assay); IL-2: 2.5×10^6 U/mg; IL-3: 5×10^7 U/mg; IL-6: 10^7 U/mg; IL-7: 10^7 U/mg; IL-8: 10^6 U/mg; TNF-α: 2×10^7 U/mg; M-CSF: 2×10^5 U/mg. 1,25 dihydroxyvitamin D3 was purchased from Hoffman-LaRoche.

Construction of an osteocalcin promoter-CAT fusion gene. A vector was constructed to examine the regulation of osteocalcin promoter function, where expression of the bacterial CAT gene reflects the in vitro level of osteocalcin transcription. The phBGP/R1b plasmid, containing a 15-kb insert that includes the human osteocalcin gene, was generously provided by Dr. J. M. Wozney, Genetics Institute, Cambridge, MA (see Reference 20). A 5-kb *Xba*I fragment containing the coding region of the osteocalcin gene and the entire 5′-flanking region, was separated from phBGP/R1b, and was subcloned into the PGEM3Z polylinker *Xba*I site (PHOC1) by standard procedures (21). A 2.0-kb *Ava*I fragment (−1700 to +299 numbered relative to the cap site) from PHOC1 was made blunt-ended using the Klenow fragment of DNA polymerase I (BRL, Bethesda, MD), and was inserted into the *Sma*I site of PGEM3Z (PHOC2). The *Hin*dIII-*Bam*HI fragment of PSV2CAT (22) containing the entire CAT gene and the small t intron and polyadenylation site of SV40 was blunt ended, ligated to *Hin*dIII linkers, and inserted into the *Hin*dIII site of $PHOC_2$ to yield PHOC-CAT (Fig. 3).

DNA transfection and CAT assay. Transfection of plasmid DNA into ROS 17/2.8 was performed using the calcium phosphate co-precipitation technique. In brief, 24 h before transfection, 3×10^5 cells were plated in 60-mm culture dishes in supplemented F-12 medium. Cells were exposed to 10 μg of precipitated plasmid DNA/dish for 4 to 6 h. After a 3-min glycerol shock, the cells were cultured thereafter in F-12 medium containing 0.5% FCS, and were maintained in a 5% CO_2/95% air atmosphere at 37°C. In all experiments, the stimulatory signal (10^{-8} M vitamin D) and cytokines were added simultaneously, and combinations were tested in duplicate or triplicate. After 48 to 60 h, cells were harvested by scraping into a buffer consisting of 150 mM NaCl, 40 mM Tris-HCl, and 1 mM EDTA, pH 7.5. Cells were lysed by three cycles of freezing and thawing, and CAT assays were performed on lysates as described by Neumann et al. (23). The reaction mixture was overlaid with water-immiscible scintillation fluid (Econofluor, New England Nuclear, Boston, MA). Acetylated forms of chloramphenicol produced by the action of CAT in the aqueous phase diffuse rapidly and are trapped in the scintillant. CAT activity was quantitated by scintillation counting. CAT activity in transfected cultures was standardized by reference to the protein concentration of cell extracts, using a protein assay kit (BioRad, Richmond, CA).

Osteocalcin protein and mRNA determinations. The effect of cytokines on endogenous osteocalcin production by ROS 17/2.8 was assessed. Cells were cultured in 24-well culture plates in F-12 medium containing 5% FCS in duplicate, supplemented with vitamin D (10^{-8}M) in the presence or absence of cytokines as indicated. After 48 h, culture supernatants were collected and stored at −70°C. Osteocalcin levels were assayed using an RIA as previously described (24).

For analysis of steady state osteocalcin mRNA levels, ROS 17/2.8 was cultured in 100-mm dishes, in the absence or presence of vitamin D (10^{-8} M) plus various 10-fold dilutions of cytokines. After 48 h, total cellular RNA was isolated according to the method of Chomczynski and Sacchi (25). For slot-blots, 20 μg total cellular RNA was bound to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Osteocalcin mRNA was detected using a 0.5-kB *Eco*RI cDNA fragment of the plasmid pR22–11 (kindly provided by Dr. D. T. Wong, Harvard School of Dental Medicine). As an internal control, GPDH mRNA was detected using a 0.8-kb *Pst*I + *Apa*I fragment of plasmid pBGAPD, also provided by Dr. Wong. After purification through 1% low melting agarose gels, probes were radiolabeled with [^{32}P]αCTP by random priming to a specific activity of ~10^9 cpm/μg (21). Hybridizations were carried out using standard protocols (26), and the radioactivity present in each slot was quantitated using a BetaScope (Betagen, Waltham, MA). The amount of hybridization to the osteocalcin probe was standardized to the GPDH reactivity of replicate RNA samples.

PG and viability assays. The response of ROS 17/2.8 to cytokines was determined by the production and release of PGE_2 into the culture medium. Supernatants generated as for osteocalcin assays were collected at 24 and 72 h, and stored frozen at −70°C. In companion experiments, ROS 17/2.8 was cultured in 60-mm dishes, and exposed to calcium-phosphate-precipitated PHOC-CAT under transfection conditions. After 4 to 6 h, cultures were stimulated with vitamin D, in the presence and absence of cytokines. Supernatants were collected after 72 h. PGE_2 was measured in both culture supernatant sets using a commercially available kit (New England Nuclear, Boston, MA). Supernatants were diluted 1/10 or 1/50 in assay buffer, and were tested directly without extraction. PGE_2 content was calculated by reference to a standard curve that was linear over the range of 5 to 250 pg/ml.

For assessment of the effect of cytokines on ROS17/2.8 cell proliferation and viability, the relative number of adherent cells was determined using a microcytotoxicity assay (4). In brief, ROS 17/2.8 were plated at a density of 2×10^4 cells/well in a 96-well tissue culture plate, and cultured for 16 h in 100 μl of F-12 medium containing 0.5% FCS. Replicate cultures consisting of six to eight wells were exposed to cytokines for 24 h, at which time the medium was aspirated, and adherent cell nuclei were stained with a 1% solution of crystal violet for 3 min. The cell monolayers were washed extensively with distilled H_2O and air dried. The crystal violet was extracted in 95% ethanol containing 40 mM HCl, and plates were read on a Multiskan Plate reader at 580 nm.

Statistics. The significance of inhibitory treatments vs the vitamin D-stimulated positive control were assessed by one-way analysis of variance and *t*-tests with Bonferroni's correction for multiple comparisons.

RESULTS

Effect of pro-inflammatory cytokines on osteocalcin protein and mRNA levels. A number of mediators that comprise the proinflammatory cytokine cascade, including IL-1β, IL1-α, TNF-α, and IL-6 may exert regulatory effects on bone. In previous studies, IL-1 and TNF have been shown to down-regulate osteocalcin production by osteoblasts in vitro (5, 16, 17). To confirm those findings, the effect of these cytokines on the production of osteocalcin protein by ROS 17/2.8 cells was determined. Cells were cultured in medium containing 10^{-8} M vitamin D, in the presence or absence of various concentrations of cytokines. Supernatants were collected after 48 h and assayed by RIA. As shown in Figure 1, cells that were stimulated with vitamin D showed a 10-fold increase in osteocalcin production. This increase was inhibited in the presence of both TNF-α (1.4 to 1400 pM) and IL-1β (1 to 1000 pM), whereas IL-6 had no effect even at very high concentrations (20,000 pM). TNF-α appeared to be more potent than IL-1β, reducing osteocalcin to near basal levels at the maximum concentration tested (1400 pM). By comparison, the inhibition mediated by IL-β plateaued at about 40% at concentrations above 1 pM.

The effect of cytokines on steady state osteocalcin mRNA levels in ROS 17/2.8 cells was also determined. Hybridization to the osteocalcin probe was standardized to an internal control, GPDH. As shown in Figure 2, TNF-α and IL-1β both strongly inhibited steady state mRNA levels (>70% at 1400 and 1000 pM, respectively), and exhibited very similar dose responses. In contrast, IL-6 inhibited only at the highest concentration tested (44% at 20,000 pM). These results therefore indicate that the effect of these cytokines on mRNA levels closely parallel findings with osteocalcin protein production.

Vitamin D regulation of the osteocalcin promoter-CAT fusion gene. To determine if the observed down-regulation was exerted at the transcriptional level, a human osteocalcin promoter-CAT fusion gene (PHOC-CAT)

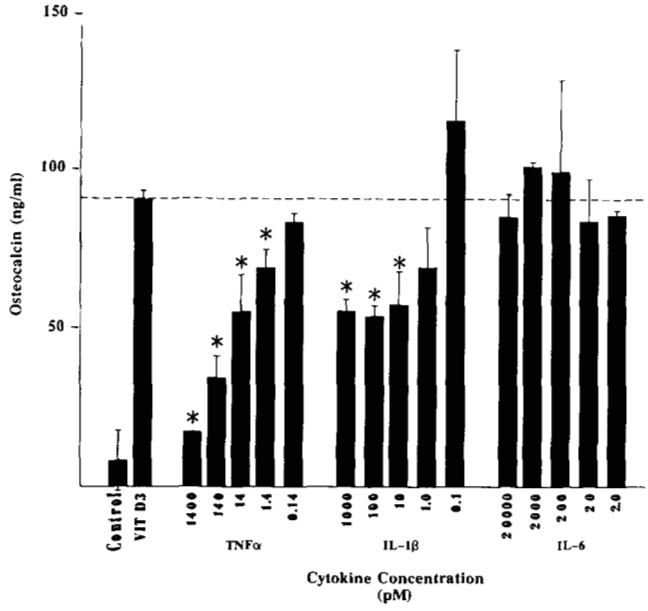

Figure 1. Effect of cytokines on vitamin D3-stimulated osteocalcin production by ROS 17/2.8 osteosarcoma cells. Cells were stimulated concomitantly with vitamin D (10^{-8} M) and cytokines, and 72-h supernatants were assayed for osteocalcin levels by RIA. *, Significant reduction ($p < 0.05$) compared with vitamin D alone.

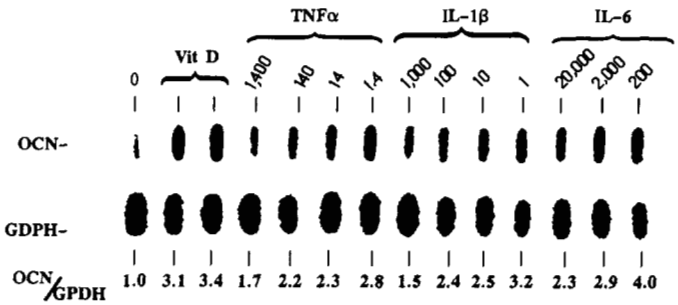

Figure 2. Slot-blot analysis of steady state osteocalcin mRNA levels in ROS 17/2.8 osteosarcoma cells. Vitamin D (10^{-8} M) and cytokines were added simultaneously in the indicated concentrations (pM). Hybridization to osteocalcin (*OCN*) and glyceraldehyde phosphate dehydrogenase (*GPDH*) cDNA probes. *OCN/GPDH*, hybridization to OCN probe standardized to GPDH.

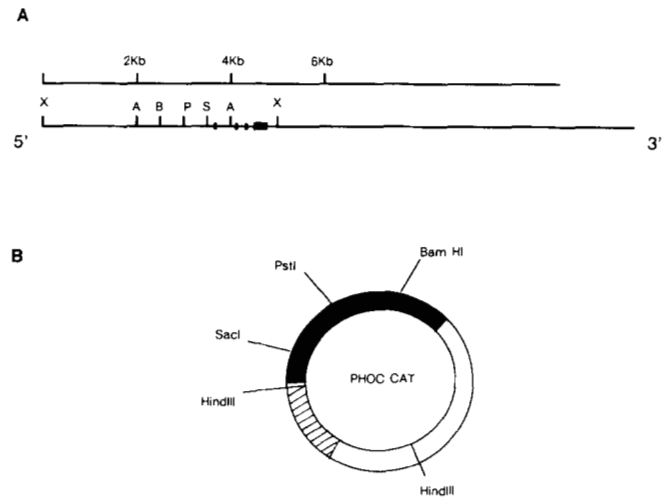

Figure 3. *A*, structure of the human osteocalcin gene (Celeste et al. [20]). Exons are indicated by dark boxes. *A*, *AvaI*; *B*, *Bam*HI; *P*, *PstI*; *S*, *SacI*; *X*, *XbaI*. *B*, PHOC-CAT. The osteocalcin 5′ regulatory region (*dark box*), CAT gene (CAT) (*hatched bar*), and SV40 small t intron and polyadenylation site (*open bar*) are indicated.

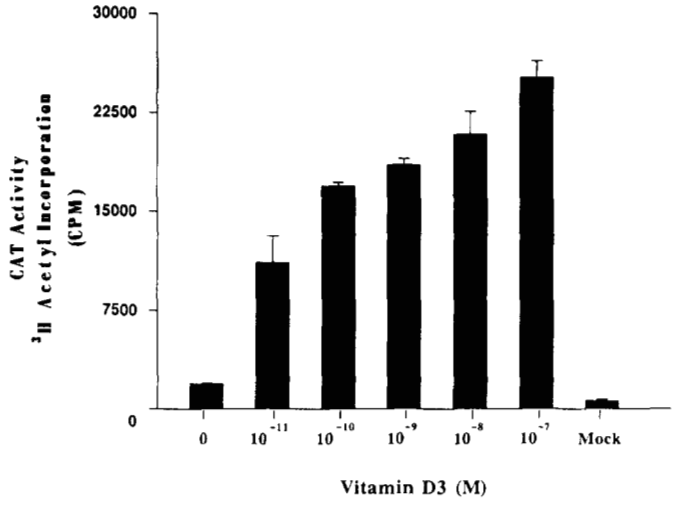

Figure 4. Dose response of ROS 17/2.8 cells transfected with PHOC-CAT to vitamin D, as determined by CAT activity. *Mock*, transfection with PGEM3Z-CAT construct lacking the osteocalcin promoter.

was produced (Fig. 3). ROS 17/2.8 rat osteosarcoma cells were transiently transfected with PHOC-CAT using standard calcium phosphate precipitation procedures, and reporter CAT activity was determined. In transfected cells, osteocalcin transcription, as determined by expression of CAT activity, was up-regulated by vitamin D as reported previously (27) (Fig. 4). Significant stimulation occurred at vitamin D concentrations above 10^{-12} M. For all experiments reported below, 10^{-8} M vitamin D-stimulated CAT activity was, on average, 10-fold higher than basal promoter activity in the absence of vitamin D. Mock transfection with a plasmid lacking the osteocalcin promoter (PGEM3Z-CAT) produced no significant CAT activity in the presence of 10^{-8} M vitamin D. Consistent with previous reports (28), transfection of a variety of other osteoblastic cells with PHOC-CAT, including the human osteosarcoma lines SaOS-2 and MG-63, as well as normal rat and human osteoblast-rich explants, failed to result in induction of significant vitamin D3-stimulated CAT activity. Consequently, all further studies were conducted with ROS 17/2.8.

Effect of pro-inflammatory cytokines on osteocalcin promoter function. A panel of human cytokines, including IL-1, IL-6, and TNF, was screened for regulatory effects on vitamin D-stimulated osteocalcin promoter function. In all studies, PHOC-CAT-transfected ROS 17/2.8 cells were stimulated with 10^{-8} M vitamin D alone (control) and in combination with individual cytokines at several $\log_{10}$ concentrations. Mean results from multiple experiments obtained with the highest tested concentration only of each cytokine are reported in Table I. All data were standardized to CAT activity stimulated by 10^{-8} M vitamin D alone (100% control response). As indicated, TNF-α exerted the strongest down-regulatory effect on osteocalcin promoter function, with mean inhibition of 57% at 1.4 nM (1000 U/ml). IL-6 also was inhibitory, albeit more weakly and at 15-fold higher concentrations (37% at 20 nM). Surprisingly, both IL-1α and IL-1β failed to affect osteocalcin gene transcription at doses as high as 10 nM, despite their ability to down-regulate osteoblast production of osteocalcin protein (Fig. 1) and mRNA (Fig. 2). IL-1β also did not affect promoter function when

TABLE I

Effect of cytokines on vitamin D-stimulated osteocalcin promoter activity

Cytokine	Concentration (nM)	No. of Experiments	% Control Response[a]
IL-1α	1.0	1	106 ± 17
IL-1β	1.0	3	97 ± 4
IL-2	2.6	2	102 ± 16
IL-6	20.0	3	63 ± 7[b]
IL-7	0.6	2	111 ± 13
IL-8	12.5	4	107 ± 23
TNF-α	1.4	6	43 ± 12[b]
M-CSF	2.0	2	98 ± 8

[a] Control: CAT expression in presence of 10^{-8} M vitamin D.
[b] $p < 0.01$.

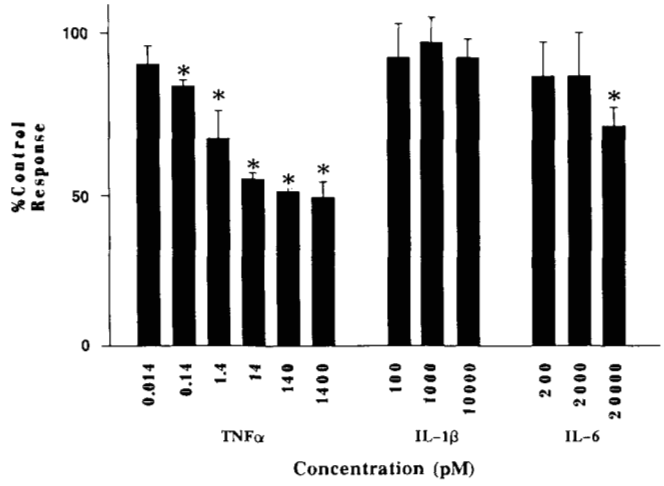

Figure 5. Dose response of inhibition of vitamin D-stimulated, PHOC-CAT-transfected ROS 17/2.8 cells by proinflammatory cytokines. Control response: stimulation with 10^{-8} M vitamin D alone (100%). *, Significant difference from control ($p < 0.05$).

vitamin D was reduced to 10^{-10} M, and murine rIL-1α and rIL-1β had no effect at concentrations as high as 10 nM (not shown). All other mediators tested, including the neutrophil activating and chemotactic factor IL-8, IL-2, and the hemopoietic growth factors IL-7 and M-CSF, also exerted no effect. These results indicate that some, but not all, cytokines that mediate inflammatory responses down-regulate the production of osteocalcin at the transcriptional level.

Dose responses and effect of indomethacin. Typical dose responses of transfected, vitamin D-stimulated ROS 17/2.8 cells to the proinflammatory cytokines are presented in Figure 5. As indicated, TNF-α exerted significant inhibition at very low concentrations (0.1 U/ml, 0.14 pM), and maximal inhibition was reached at 10 U/ml (14 pM). This dose response is closely similar to that observed above for inhibition of endogenous osteocalcin protein and steady state mRNA production by ROS 17/2.8 (Figs. 1 and 2). TNF-α also down-regulated basal expression of CAT in the absence of vitamin D to a similar extent, whereas all other cytokines tested had no effect (not shown). In contrast, IL-6 only showed consistent down-regulatory effects at approximately 10^4-fold higher concentration (8 to 20 nM), with variable inhibition observed at 4 nM. As reported above, IL-1β again failed to inhibit promoter function.

Effects of PG. Many of the effects of the proinflammatory cytokines, including TNF-α, are mediated via products of arachidonic acid metabolism (2). The effect of the PG synthetase inhibitor indomethacin on TNFα-mediated down-regulation of osteocalcin promoter function was therefore assessed. As shown in Figure 6, 1 μM indomethacin had no effect on TNF-α-mediated inhibition of osteocalcin gene transcription at 1 to 100 U/ml (1.4 to 140 pM). This result indicates that prostanoid-dependent pathways are not involved in down-regulation of the promoter.

Because IL-1β failed to affect promoter function despite inhibiting osteocalcin protein and mRNA, we determined whether transfected host cells were capable of responding to this mediator by an additional criterion, the production of PGE_2 (29). Cells were cultured under conditions identical to those utilized for transfection, and were exposed to the indicated concentrations of cytokines for 72 h. PGE_2 levels in culture supernatants were measured by RIA. As shown in Table II, ROS 17/2.8 does, in fact, respond by PGE_2 production to IL-1β, TNF-α, and IL-6. The multi-CSF IL-3, not known to stimulate osteoblastic cells, had no effect (not shown). In a second experiment, complete dose responses were carried out. As shown in Figure 7, ROS 17/2.8 responded similarly to both TNF-α and IL-1β, at concentrations of approximately 0.1 pM and above. In contrast, somewhat larger amounts of IL-6 were required for significant PGE_2 production (10 pM).

Lack of cytotoxicity of cytokines on ROS 17/2.8. TNF-α is a mediator of cytotoxicity on certain tumors and tumor cell lines (30). Although all CAT assay data were normalized to total ROS 17/2.8 cellular protein, and no significant decrease in cell protein was seen in TNF-α-stimulated cultures, the possibility that TNF-α adversely affected the host cells was examined directly using a microcytotoxicity assay. As shown in Table III, no significant decrease in ROS 17/2.8 cell number occurred at any dose of TNF-α tested. Similarly, neither IL-6, IL-1β, or IL-8 had any cytotoxic effects on ROS 17/2.8. Taken together with above findings, this result supports the

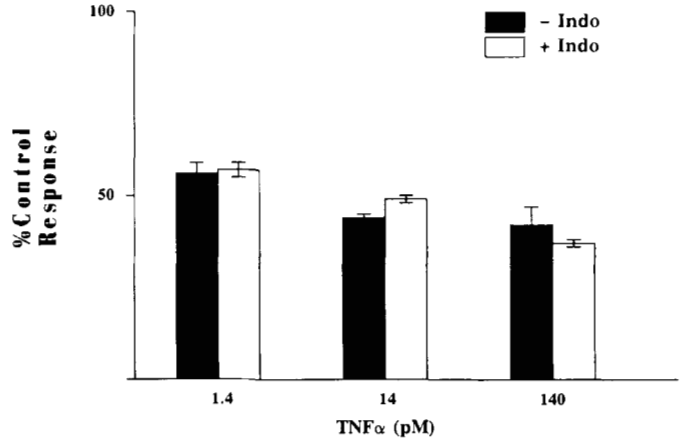

Figure 6. Effect of indomethacin on TNF-α-mediated inhibition of vitamin D-stimulated osteocalcin promoter activity. Transcriptional activity was determined by CAT assay. Indomethacin (*open bars*) was present at 1 μM.

TABLE II

PGE_2 production by ROS 17/2.8 in response to cytokines

Stimulant	Concentration (pM)	PGE_2 (pg/Culture)
Medium control		2,100 ± 400
IL-1β	100	10,200 ± 680[a]
IL-6	2,000	3,130 ± 450[a]
TNF-α	140	7,400 ± 220[a]

[a] $p < 0.05$.

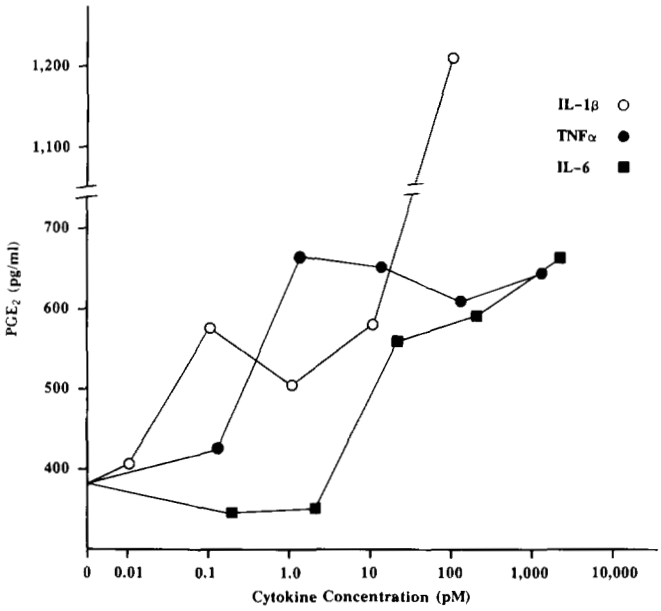

Figure 7. Production of PGE_2 by ROS 17/2.8 in response to cytokines. Cells were stimulated by cytokines in the presence of vitamin D (10^{-8} M). Supernatants were collected after 72 h and analyzed for PGE_2 by RIA. *, Significant stimulation vs cultures without cytokines.

TABLE III

Lack of cytotoxicity of cytokines on ROS 17/2.8

Stimulant	Concentration (nM)	Relative Cell No. (% of Control)
Medium control		100 ± 7[a]
TNFα	1.4	101 ± 4
	0.14	107 ± 7
	0.014	95 ± 5
IL-1β	10	98 ± 9
	1	98 ± 10
	0.1	98 ± 8
IL-6	20	97 ± 11
	2	101 ± 7
	0.2	98 ± 10
IL-8	12.5	97 ± 8
	1.25	98 ± 7
	0.125	110 ± 6

[a] Mean ± SD.

conclusion that TNF-α regulates osteocalcin expression primarily by effects on the gene promoter.

DISCUSSION

A considerable body of data has now demonstrated that the skeletal system is regulated by mediators that comprise the proinflammatory cytokine cascade. Several of these mediators, including IL-1 and TNF, stimulate bone resorption (31–33) and also inhibit bone formation (3–5, 18, 34), and may therefore induce an "uncoupled" state characterized by a net loss of bone mass (35, 36). Bone destruction is an important pathogenic consequence of a number of inflammatory and malignant disorders, including arthritis, osteomyelitis, periodontitis, and myeloma. The inhibition of bone formation by these mediators is mediated largely by down-regulation of the production of bone matrix proteins by osteoblasts, including the osteoblast-specific product osteocalcin (3–5, 16–18).

In the present studies, we have attempted to determine the level at which this inhibition is exerted. Transfection studies with an osteocalcin promoter-CAT fusion gene have demonstrated that two proinflammatory polypeptides, TNF-α, and to a marginal degree IL-6, down-regulate osteocalcin transcription. This finding confirms and extends a recent report by Nanes et al. (37), who found that TNF-α inhibits osteocalcin at a pretranslational level in ROS 17/2.8. Of note, the sensitivity of ROS 17/2.8 to TNF-α in the Nanes study was 1 to 2 orders of magnitude lower than we have observed here. It is possible that this difference may be attributable to the fact that vitamin D was added 1 day before TNF-α in the study by Nanes et al., thus favoring promoter up-regulation, whereas the two agents were added simultaneously in the present experiments. We also found that IL-1β and IL-1α had no effect on transcription, even though endogenous production of osteocalcin protein and mRNA by the ROS 17/2.8 cell host was down-regulated by IL-1β (Figs. 1 and 2). ROS 17/2.8 was furthermore shown to be responsive to IL-1β by PGE_2 production (Table II, Fig. 7), although TNFα-mediated down-regulation of promoter function was PG independent (Fig. 6). Maximal inhibition of transcription by TNF-α was observed at very low concentrations (14 pM), whereas approximately 1000-fold more IL-6 was required to exert a similar effect.

Although both TNF-α and IL-1β are important regulators of osteocalcin production by osteoblasts, our results strongly indicate that different mechanisms of control are operative. Because TNF-α as well as IL-1β reduce steady state mRNA levels (Fig. 2), (16, 37), it is likely that IL-1β may reduce mRNA stability (38) whereas TNF-α effects are primarily transcriptional and may operate via a TNF-α-responsive element. In this regard, an inhibitory glucocorticoid-responsive element has recently been described between coordinates −196 and +34 of the osteocalcin promoter (39). In addition, a preliminary report has indicated that a TNF-α-responsive element is present in the γ1(I) collagen promoter, although this is as yet uncharacterized (40). Studies of the vitamin D-responsive element (−513 to −493) within the osteocalcin promoter have shown that a binding site for the transcription factor AP-1 (41, 42) is located just distal to the vitamin D receptor site (43). Co-transfection of AP-1 components *c-jun* and *c-fos* suppresses basal and vitamin D-induced osteocalcin expression (28). Because TNF-α and IL-1β can induce AP-1 expression in fibroblasts and lymphocytes, respectively (44, 45), it has been suggested that inhibition of osteocalcin transcription in osteoblasts may operate via an AP-1-mediated mechanism. This may not be the case, however, given that IL-1 appears not to affect transcription. Alternatively, it is possible that TNF-α but not IL-1 stimulates AP-1 in osteoblasts. Mapping studies will be required to definitively resolve these issues.

Cytokine-mediated inhibition of osteocalcin protein production is reported to be partially reversed by the addition of indomethacin in human osteoblast-like cells, suggesting the existence of PGE_2-dependent and PGE_2-independent pathways (46). In the present studies, TNF-α-mediated inhibition was totally PGE_2 independent (Fig. 6), even though TNF-α did stimulate the production of PGE_2 by ROS 17/2.8 (Fig. 7; Table II). This is furthermore consistent with a report that ROS 17/2.8 may not respond to PGE_2 (19). Agents such as forskolin and cholera toxin which, like PGE_2, activate adenylate cyclase, also inhibit 1,25 (OH) 2D3 stimulation of osteocalcin synthe-

sis (47). However, TNF-α has no effect on cAMP levels in osteoblastic cells, ruling out the participation of a cAMP-mediated pathway (48).

IL-1, TNF, and IL-6 share an overlapping spectrum of biologic activities that mediate host inflammatory responses to infection (1, 2). In contrast to IL-1 and TNF, however, the effects of IL-6 on bone are somewhat controversial. IL-6 has been reported both to stimulate (49) and to have no effect on bone resorption in organ culture (50). Because resorption in response to cytokines and hormones is mediated via osteoblasts (51), the response of this cell to IL-6 is relevant. UMR-106–01 osteosarcoma cells were shown (46) to respond to IL-6 at 1000 U/ml (20 nM) by PGE_2 production, and inhibition of the synthesis of collagen and noncollagenous proteins (52). In contrast, Littlewood et al. (53) reported that IL-6 had no effect on PGE_2 production by human osteoblasts or ROS 17/2.8 at doses up to 0.2 nM. As shown above, ROS 17/2.8 responds to IL-6 at concentrations above 10 pM by PGE_2 production, and osteocalcin promoter function was inhibited by IL-6 at 8 to 20 nM, demonstrating the responsiveness of ROS 17/2.8 to relatively high concentrations of this mediator. The conflicting literature may therefore reflect the different doses of IL-6 utilized by various investigators, and the necessity for high concentrations of IL-6 to stimulate effects. Given the marginal effect of IL-6 on osteocalcin protein and mRNA production compared with TNF-α and IL-1β, IL-6 may play a minor role in osteocalcin regulation.

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