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EFFECTS OF INTERLEUKIN-1β AND TUMOR NECROSIS FACTOR-α ON OSTEOBLASTIC EXPRESSION OF OSTEOCALCIN AND MINERALIZED EXTRACELLULAR MATRIX IN VITRO

RUSSELL S. TAICHMAN[1,2] and PETER V. HAUSCHKA[3]

Departments of [1]Periodontology and [3]Oral Biology
Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, MA 02115

Department of Orthopaedic Surgery, The Children's Hospital Corporation
Enders-12, 300 Longwood Avenue, Boston, Massachusetts 02115.

Abstract—Osteoblasts play a pivotal role during the bioresponse of bone to agents that stimulate bone resorption and/or inhibit bone formation including hormones, polypeptide growth factors, and cytokines. We examined the cytokines interleukin-1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) for their effects on osteoblastic proliferation and development and expression of alkaline phosphatase and the osteoblast-specific protein osteocalcin in a mineralizing environment. Primary rat osteoblast-like cells (ROB) and osteoblastic cell lines derived from rat (ROS 17/2.8) and human (MG-63) osteosarcomas were studied. IL-1β and TNF-α were chosen because of their critical importance during the host response to local inflammatory stimuli. Qualitatively similar two- to threefold inhibition of osteocalcin synthesis by IL-1β and TNF-α were observed in all three postconfluent bone-forming model systems. Because of the readily measurable concentrations of osteocalcin produced in our culture protocol, it was not necessary to enhance osteoblastic synthesis of osteocalcin by supplementation with 1,25$(OH)_2$-vitamin D_3, a treatment which exerts pleiotropic effects on osteoblasts. Under the constraints of our protocol, where alkaline phosphatase and mineralization were already elevated at the 14-day onset of treatment, neither of these phenotypic properties was sensitive to a three-day cytokine exposure. Differences were noted in proliferation, where only TNF-α stimulated DNA synthesis in ROB cells, while both cytokines stimulated MG-63 cells. IL-1β and TNF-α failed to alter ROS 17/2.8 DNA synthesis except at the highest doses (25 pM IL-1β and 1 nM TNF-α) where inhibition was observed. These results further support the view that cytokine-mediated osteoblastic regulation can be relatively selective.

[2]Present Address: Department of Periodontics, Prevention and Geriatrics, School of Dentistry, University of Michigan, 1011 North University Ave., Ann Arbor, Michigan 48109-1078.

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INTRODUCTION

Cytokines are important determinants of osseous tissue homeostasis in health and disease. Investigation of interleukin-1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) in experimental bone models indicates that these cytokines depress osteoblastic bone formation (1, 2) and stimulate osteoclastic bone resorption (3). At the cellular level, IL-1α and TNF-α interact with osteoblasts (4, 5) and other cell types (6, 7) by surface receptors, and these interactions lead to profound alterations in osteoblast functions (8). For example, depending on the model system investigated, osteoblastic DNA and prostaglandin synthesis are stimulated by IL-1β and TNF-α (9–12). IL-1β and TNF-α are also known to depress osteoblast synthesis of collagen, alkaline phosphatase, and secretion and mineralization of the extracellular bone matrix (10, 13, 14), although alkaline phosphatase stimulation has also been reported (15). At relatively high concentrations, IL-1β and TNF-α cause osteoblasts to transmit a signal(s) that activates osteoclastic bone resorption (14, 16–18). The nature and mechanism of action of the osteoclast-activating factor(s) hypothesized in 1981 by Rodan and Martin (18) is not understood.

Osteocalcin is another key osteoblastic product for which the expression is modulated by IL-1β and TNF-α. Osteocalcin (bone Gla protein, BGP) is a vitamin K-dependent Ca^{2+}-binding protein and is the most abundant noncollagenous protein of the mineralized extracellular bone matrix where it resides on the surfaces of hydroxyapatite crystals (20, 21). While the exact role of osteocalcin in bone formation or destruction is not known, there is evidence that after placement in the extracellular matrix by osteoblasts, osteocalcin may serve as an activator or chemoattractant for osteoclasts and/or their presumed monocytic precursors (22–24). As a specific differentiated product of mature osteoblasts, osteocalcin exhibits a highly conserved amino acid sequence, and is expressed relatively late in the developmental sequence of bone coincident with the onset of mineralization (21, 25, 26). The phenotypic expression of this protein is sensitive to numerous stimuli, including inhibitors of bone formation (e.g., corticosteroids) and growth factors and agents with bone resorbing activity (parathyroid hormone, vitamin D) (21, 27).

Thus, studies on osteocalcin synthesis may be useful in assessing the overall effects of cytokines and hormones on osteoblast metabolism. Unfortunately, progress in this area has been restricted because mammalian primary osteoblasts usually express relatively low levels of this marker protein under conventional culture conditions (1, 11, 13, 28–31). Recent improvements in rat osteoblast culture (32) (R. S. Taichman, S. E. Doleman, P. V. Hauschka, unpublished), patterned after the chick model system (26), have allowed sensitive monitoring of inhibitory effects on osteocalcin expression by osteoblasts in a mineralizing

environment. In the present investigation, we assessed the effects of IL-1β and TNF-α on osteocalcin formation in three osteoblast cell culture systems where relatively high levels of osteocalcin are produced in vitro.

MATERIALS AND METHODS

Cytokines. Recombinant human IL-1β (bioactivity: 5.0×10^8 units/mg) and human TNF-α (bioactivity: 1.0×10^7 units/mg) were obtained from Amgen Biologicals (Thousand Oaks, California). These particular cytokines were chosen for their relevance to bone biology in that IL-1β and TNF-α coincide with the majority of activity chromatically isolated from supernatants containing osteoclast activating activity (43). The concentrations of cytokines employed (IL-1β: 0.25–25 pM; TNF-α 0.01–1.0 nM) are approximately equivalent to the bone-resorbing activity isolated from myeloma cells detected in bioassay (33) and below a concentration that, in preliminary investigations, caused the cells to round up and fail to exclude trypan blue stain (0.25 nM IL-1β and 10.0 nM TNF-α).

Cell Lines and Culture Methods. Rat osteoblast-like (ROB) cells were isolated by enzymatic digestion of newborn rat calvaria as outlined by Cohn and Wong (34). Six sequential 20-min digests were performed on suture-free dissected calvarial fragments at 37°C in minimum essential medium (MEM) containing 2 mg/ml collagenase A (Boehringer Mannhein Biochemicals, Indianapolis, Indiana) and 0.005% trypsin (Gibco Laboratories, Grand Island, New York). Cells obtained from the third through sixth digests were pooled and plated in 24-well tissue culture plates (1.0×10^5 cells/2 cm^2 well) in 0.5 ml of α-MEM (Gibco) containing 10% fetal bovine calf serum (FBS) with penicillin and streptomycin (day 0). The medium was changed on days 5, 8, 11, and 14 and was additionally supplemented with fresh β-glycerolphosphate and L-(+)-ascorbate to final concentrations of 10 mM and 50 μg/ml, respectively. On day 14 the cells were washed twice with warm phosphate-buffered saline (PBS) and fresh culture medium containing 1% FBS and appropriate concentrations of IL-1β and TNF-α were added to quadruplicate wells. Cultures were maintained as before until day 17, at which time cell-free culture media were collected, centrifuged, and stored at −20°C until assayed for osteocalcin. The adherent cells were washed twice with cold PBS and prepared for analyses of total DNA, extracellular matrix-bound osteocalcin, mineralization of the extracellular matrix, and alkaline phosphatase activity.

The MG-63 human osteosarcoma cell line (ATCC #CRL 1424) (1.0×10^5 cells/well) was cultured in 0.5 ml α-MEM with Earle's salts and 10% heat-inactivated FBS. The rat osteosarcoma cell line ROS 17/2.8 was generously provided by Dr. Robert Majeska (Mount Sinai Medical Center, New York, New York) and maintained in Ham's F12 medium with 5% FCS. Osteosarcoma cells were maintained as described above except that the serum concentrations were not altered during the 14- to 17-day experimental period.

Osteocalcin Radioimmunoassay. Osteocalcin levels in culture supernatants and cell-associated extracellular matrix were determined by a three-day nonequilibrium radioimmunoassay as previously described (35), employing the following antisera: goat anti-rat osteocalcin (first antibody) and donkey anti-goat (second antibody) for determinations from ROB and ROS 17/2.8 cell cultures; rabbit anti-bovine osteocalcin (first antibody: recognizes human osteocalcin) and goat anti-rabbit (second antibody) for the MG-63 cell line. Nonspecific binding of [^{125}I]osteocalcin (rat or bovine) was determined for each assay by omitting the primary antiserum from the reaction mixture. Data are reported either as total nanograms osteocalcin per culture, or as nanograms osteocalcin per microgram DNA. Because human and bovine osteocalcin are equally cross-reactive with our rabbit anti-bovine first antibody, the osteocalcin levels reported for the MG-63 cell line represent human

osteocalcin above the (approximately 15 ng/ml) bovine osteocalcin background originating from the 10% FBS in the medium. To detect osteocalcin associated with the extracellular matrix, the cell layers were extracted with 0.5 M EDTA containing protease inhibitors (EPIC buffer) (35).

Fluorometric Assay for Total DNA. *m*-Diaminobenzoic acid dihydrochloride (Sigma) was used to assay deoxyribose liberated by perchloric acid added to cell lysates (36). Samples were read on an Perkin-Elmer LS-5 fluorescence spectrometer at 408 nm (excitation) and 500 nm (emission) against a standard curve prepared with calf thymus DNA (Sigma) (assuming A_{260} = 0.025 for 1 μg/ml). Results are expressed as micrograms DNA per culture well.

In Vitro Mineralization. Mineralization of ROB cell extracellular matrix was determined by the Von Kossa staining technique (3% $AgNO_3$) for matrix-associated phosphate (37). Individual black-staining mineralized nodules were counted under a dissecting microscope at 10× and expressed as nodules per culture well.

Alkaline Phosphatase Activity. Alkaline phosphatase activity in cultured cells was determined by the method of Reddi and Huggins (38). Following removal of culture media, cell layers were subjected to three freeze–thaw cycles, and sonicated in 200 μl of assay buffer (0.15 M NaCl, 3 mM $NaHCO_3$, pH 7.4). Recovered samples (10 μl) were assayed for enzyme activity in 96-well plates with *p*-nitrophenylphosphate (Sigma) as a substrate in a total of 100 μl; after 15 min at 37°C the reaction was stopped with 100 μl of 1 M NaOH and the absorbance was measured on an Dynatec MR700 ELISA plate reader with *p*-nitrophenol as a standard. Results are presented in units per microgram DNA, where 1 unit = 1 nmol *p*-nitrophenol liberated/15 min at 37°C.

Northern Hybridization Analysis. ROB cell cultures were initiated on day 0 by plating 3.75 × 10^6 cells/75-cm^2 tissue culture flask containing 18.75 ml of medium. As described above, experimental conditions were established on day 14. Eighteen hours later, the ROB cell layers were washed twice with ice-cold PBS, solubilized in 7.5 M guanidine HCl (Sigma), and processed for total RNA as described by Sambrook et al. (39). Samples containing 20 μg total cellular RNA (quantified by A_{260}/A_{280} and the orcinol reaction) were separated electrophoretically on formaldehyde–agarose gels after the method of Thomas (40) and transferred to nitrocellulose (Schleicher and Schuell, Keene, New Hampshire) by capillary action. The cDNA probe for rat osteocalcin originally described by Celeste et al. (41) was generously supplied by Dr. A. J. Wozney (Genetics Institute, Cambridge, Massachusetts). The probe was labeled with [^{32}P]dCTP by the random primer method (42). The blots were prehybridized and hybridized in 50% formamide; 5× SSC; 50 mM sodium phosphate buffer, pH 6.5; 1% sodium dodecyl sulfate (SDS) at 45°C. Following an overnight incubation, the membranes were washed for two 30-min washes at 65°C in 2× SSC–0.1% SDS, 0.5× SSC–0.1% SDS, 0.1× SSC–0.1% SDS and prepared for autoradiography. The resulting autoradiographs were quantified by scanning laser densitometry (LKB Ultrascan).

Statistics. The Student's *t* test was utilized for the statistical analysis.

RESULTS

The effects of IL-1β and TNF-α on osteocalcin synthesis and the development of extracellular bone matrix were studied in 14- to 17-day-old osteoblast cultures. This period was chosen because it corresponds to the expression of the osteocalcin gene, secretion of osteocalcin into the culture medium, and osteocalcin incorporation into the cell-associated extracellular matrix. At this time, osteoblastic expression of other specific markers such as osteopontin, osteonectin, alkaline phosphatase, and mineralization is also elevated (32).

Effects of IL-1β on ROB Cell DNA and Osteocalcin Synthesis. Three concentrations of IL-1β (0.25, 2.5, 25 pM) were added to 14-day ROB cultures. Inhibition of osteocalcin synthesis was observed at all concentrations tested (Table 1). For example, control cultures produced a total of 228 ± 6.3 ng osteocalcin/culture during days 14–17, whereas cultures treated with 25.0 pM IL-1β produced 77.2 ± 11.6 ng osteocalcin/culture. The reduction in total osteocalcin was reflected in both the medium and cell-layer associated matrix fractions (Table 1, Figure 1). IL-1β had no significant effect on DNA synthesis (Table 1), suggesting that diminished osteocalcin synthesis is not merely a consequence of altered cellular replication.

Effects of TNF-α on ROB Cell DNA and Osteocalcin Synthesis. Table 1 shows results of four representative experiments where ROB cells were exposed to TNF-α. Increasing TNF-α concentrations reduced osteocalcin nanogram per microgram DNA levels at day 17 in both the medium (decreased 73%) and cell-layer associated extracellular matrix (decreased 59%) pools (Table 1, Figure 1). While inhibiting osteocalcin production, 0.01 and 0.1 nM TNF-α simulated ROB cell proliferation (Table 1). The total osteocalcin detectable in the cultures relative to control was reduced for each concentration of TNF-α tested (osteocalcin, nanograms per well: control, 227 ± 6.3; 0.01 nM TNF-α, 154 ± 28.9; 0.1 nM TNF-α: 148 ± 13.9; 1.0 nM TNF-α; 90.7 ± 2.4).

Effects of IL-1β and TNF-α on ROB Cell Osteocalcin mRNA. Total cellular RNA was isolated from ROB cells following an 18-h exposure to IL-1β and TNF-α. As negative controls, ROB cells that were withheld from the osteocalcin expression conditions [exposure to L-ascorbate (50 μg/ml) and β-glycerolphophate (10 mM) commencing on day 5] were included in the analysis. As shown in Table 1, message levels for osteocalcin were nearly four times greater in ROB cells grown in medium containing L-ascorbate and β-glycerolphophate than in the negative controls. Incubation of ROB cells with IL-1β decreased the message for osteocalcin detected by Northern hybridization analysis to levels below the basal levels of the negative control cells. Similar inhibition of the osteocalcin message occurred when ROB cells were exposed to 1.0 nM TNF-α (control, 2.58 densitometric units; 1.0 nM TNF-α, 1.66 units).

Effects of IL-1β and TNF-α on ROB Cell Alkaline Phosphatase Activity. Kinetic studies indicated that there was a progressive rise in alkaline phosphatase activity in control rat osteoblast cultures during the test period. At day 14 the total alkaline phosphatase activity was 456 units/μg DNA, increasing to 944 units/μg DNA at day 17, a 207% rise. At the concentrations tested, IL-1β and TNF-α failed to influence the expression of this enzyme by ROB cells (Table 2).

Effects of IL-1β and TNF-α on ROB Cell Mineralized Nodule Formation. Initial mineralization of the extracellular matrix by ROB cells occurs between days 11 and 14. These nodules begin as very small refractive inclusions

Table 1. Effects of IL-1β and TNF-α on ROB Cell Proliferation (DNA) and on Expression of Osteocalcin mRNA and Protein[a]

Condition	DNA (μg/well)	Osteocalcin expression (ng osteocalcin/μg DNA)			OC mRNA (absorbance)	
		Medium	Cell layer	Total	Exp 1	Exp 2
Control	7.07 ± 0.5	10.7 ± 1.1	21.5 ± 1.0	32.1 ± 2.2	6.45	2.59
IL-1β 0.25 pM	7.66 ± 0.5	9.77 ± 2.3	10.7 ± 1.3[b]	20.5 ± 1.1[b]	4.45	
IL-1β 2.50 pM	7.57 ± 0.8	8.77 ± 1.5	15.1 ± 7.2	23.8 ± 5.0[b]	0.95	
IL-1β 25.0 pM	6.06 ± 0.8	6.14 ± 0.6[b]	5.50 ± 1.4[b]	11.7 ± 1.8[b]	0.80	
TNF-α 0.01 nM	8.92 ± 0.7[b]	6.64 ± 1.3[b]	10.6 ± 1.4[b]	17.2 ± 3.2[b]		
TNF-α 0.10 nM	9.22 ± 0.2[b]	5.54 ± 0.7[b]	10.6 ± 1.3[b]	16.1 ± 1.5[b]		
TNF-α 1.00 nM	7.82 ± 0.5[b]	2.85 ± 0.02[b]	8.80 ± 0.3[b]	11.6 ± 0.3[b]		1.66
Negative Control					1.68	1.20

[a] ROB cells were cultured as described in Materials and Methods, and test conditions initiated on day 14. Osteocalcin was measured by radioimmunoassay in media and cell layers harvested at day 17. Total osteocalcin is computed as the sum of medium and cell layers values from each well. RNA for Northern analysis was extracted on day 15 after 18 h of cytokine treatment in two independent experiments. Absorbance values were standardized for total cellular RNA and loaded in each lane. Data are means ($N = 4$) ± standard deviation.

[b] Significantly different from control: $P \leq 0.05$.

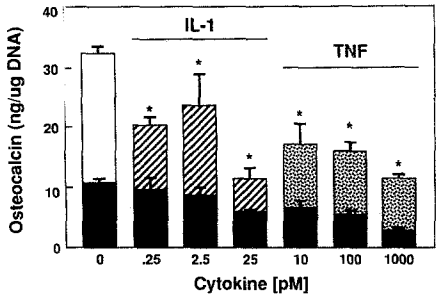

Fig. 1 Effects of IL-1β and TNF-α on osteocalcin expression by rat osteoblastic cells (ROB) in vitro. Primary cultures were established on day 0 from neonatal rat calvaria cells as described. From day 5 onward, cells were cultured under mineralizing conditions in the presence of L-ascorbate and β-glycerolphophate, with medium changes every three days. Cytokines were added at the indicated concentrations on day 14: IL-1β (0.25, 2.5, 25 pM); TNF-α (10, 100, 1000 pM). Media and cell layers were harvested on day 17 for analysis of osteocalcin and DNA. Values are expressed as ng osteocalcin/μg DNA. Solid bar = medium osoteocalcin; open bar = cell layer osteocalcin; Total bar height = total osteocalcin per culture; error bars shown separately for medium and total osteocalcin indicate means ± standard deviation ($N = 4$); *significant difference from control, $P \leq 0.05$.

Table 2. Effects of IL-1β and TNF-α on ROB Cell Alkaline Phosphatase Activity[a]

Condition	Alkaline phosphatase (units/μg DNA)
Control	943.7 ± 120
IL-1 0.25 pM	752.5 ± 120
IL-1 2.50 pM	1114.0 ± 164
IL-1 25.0 pM	1072.7 ± 164
TNF 0.01 nM	912.8 ± 49.0
TNF 0.10 nM	1073.1 ± 120.0
TNF 1.00 nM	980.6 ± 126.0

[a] ROB cell cultures were established and treated as in Table 1. Data are means ($N = 4$) ± standard deviation representing the *p*-nitrophenol liberated by homogenates from individual 4-cm^2 wells on day 17 (1 unit = 1 nmol/15 min at 37°C). There were no significant differences from the control.

in dense regions of the cell layer. Silver staining converts these nodular calcium phosphate deposits to silver phosphate, which become black upon exposure to light. Such nodules are readily counted. During the 14- to 17-day period, the number of mineralized nodules increase from 104 ± 9 nodules/culture to 163

Table 3. Effects of IL-1β and TNF-α on ROB Cell Mineralized Nodule Formation[a]

Condition	Nodules/culture
Control	163.3 ± 14.6
IL-1β 0.25 pM	218.7 ± 23.9
IL-1β 2.50 pM	187.3 ± 22.8
IL-1β 25.0 pM	193.0 ± 15.6
TNF-α 0.01 nM	207.0 ± 20.2
TNF-α 0.10 nM	189.3 ± 31.7
TNF-α 1.00 nM	174.0 ± 16.5

[a]ROB cell cultures were established and treated as in Table 1. Data are means (N = 4) ± standard deviation representing the number of silver-stained mineral nodules per 4-cm^2 well on day 17 (1 unit = 1 nmol/15 min at 37°C); there were no significant differences from the control.

Table 4. Effects of IL-1β and TNF-α on ROS 17/2.8 Cells[a]

Condition	Osteocalcin (ng/ml medium)	DNA (μg/well)	Osteocalcin (ng/μg DNA)
Exp 1			
Control	96.4 ± 9.80	7.37 ± 0.58	13.0 ± 1.96
IL-1β 0.25 pM	99.7 ± 5.40	8.05 ± 0.60	12.4 ± 0.55
IL-1β 2.50 pM	58.3 ± 5.90[b]	7.45 ± 0.45	7.69 ± 0.95[b]
IL-1β 25.0 pM	45.0 ± 14.8[b]	6.35 ± 0.20[b]	7.17 ± 2.60[b]
Exp 2			
Control	96.0 ± 12.4	7.65 ± 0.49	12.4 ± 2.27
TNF-α 0.10 nM	57.0 ± 4.1[b]	8.12 ± 0.38[b]	7.14 ± 0.62[b]
TNF-α 1.00 nM	24.7 ± 9.5[b]	5.72 ± 0.49[b]	4.53 ± 0.22[b]

[a]Data are means (N = 4) ± standard deviation.
[b]Significantly different from controls: $P \leq 0.05$.

± 15 nodules/culture. IL-1β and TNF-α had no discernible effect on mineralized nodule formation during this interval (Table 3).

Effects of IL-1β and TNF-α on Osteocalcin and DNA Synthesis by ROS 17/2.8 and MG-63 cells. To confirm that the inhibitory effects of IL-1β and TNF-α on ROB cell osteocalcin synthesis is a generalized feature of the osteoblast phenotype, two osteosarcomas cell lines were studied. Both the rat 17/2.8 and the human MG-63 were found to produce moderate levels of osteocalcin using similar culture conditions, slightly modified for the particular growth

Table 5. Effects of IL-1β and TNF-α on MG-63 Cells[a]

Condition	Osteocalcin (ng/ml medium)	DNA (μg/well)	Osteocalcin (ng/μg DNA)
Control	46.1 ± 5.3	14.6 ± 1.6	3.20 ± 0.6
IL-1β 0.25 pM	40.5 ± 4.2	15.4 ± 5.0	2.81 ± 0.6
IL-1β 2.50 pM	39.4 ± 5.7	18.1 ± 1.6	2.19 ± 0.4[b]
IL-1β 25.0 pM	36.1 ± 3.3[b]	23.9 ± 0.7[b]	1.51 ± 0.1[b]
TNF-α 0.01 nM	41.7 ± 2.1	27.3 ± 1.6[b]	3.33 ± 0.4
TNF-α 0.10 nM	40.4 ± 5.5	24.9 ± 1.2[b]	2.55 ± 0.4
TNF-α 1.00 nM	31.7 ± 1.3[b]	14.6 ± 1.6	1.22 ± 0.1[b]

[a] Data are means ($N = 4$) ± standard deviation.
[b] Significantly different from controls: $P \leq 0.05$.

requirements of each tumor type. The medium was supplemented with L-ascorbate and β-glycerolphosphate, but no addition of $1,25(OH)_2$-vitamin D_3 was required to elevate osteocalcin levels into the measurable range. As presented in Tables 4 and 5, IL-1β and TNF-α inhibited the synthesis of osteocalcin by ROS 17/2.8 and MG-63 cells. The effects of the cytokines on DNA synthesis were variable. For ROS 17/2.8 cells, IL-1β and TNF-β failed to alter DNA synthesis except at the greatest concentrations of IL-1β (25 pM) and TNF-α (1 nM), where DNA declined 14% and 25% respectively (Table 4). For MG-63 cells, IL-1β at 0.25–25 pM and TNF-α at 0.01 and 0.1 nM stimulated DNA synthesis by up to 87% (Table 5).

DISCUSSION

Effects of IL-1β and TNF-α on osteoblastic bone formation were studied in vitro by monitoring the osteoblast-specific protein osteocalcin and other differentiated functions. These particular cytokines were examined because they are of crucial importance during the hosts response to local inflammatory stimuli. IL-1β and TNF-α are relevant to bone biology in that they coincide with the majority of activity chromatographically isolated from conditioned media containing osteoclast activating activity (43).

IL-1β inhibits the synthesis of osteocalcin by normal rat osteoblastic cells (ROB) and by human (MG-63) and rat (ROS 17/2.8) osteosarcoma cell lines. For the range of concentrations investigated (0.25–25.0 pM IL-1β), ROB cell osteocalcin synthesis was inhibited up to about threefold, while the osteosarcoma lines were inhibited about twofold. In the case of ROB cells, the reductions in osteocalcin levels observed by radioimmunoassay of the secreted protein are

strongly correlated with decreased steady-state levels of osteocalcin mRNA from Northern hybridization analysis (Table 1). This is consistent with the possibility that TNF-α (44) and IL-1β (45) may regulate osteocalcin synthesis by transcriptional control. The depression of osteocalcin synthesis was not directly associated with an altered rate of division of the normal rat osteoblast-like cells. This is unusual in that many polypeptide growth factors (such as TGF-β, PDGF, aFGF, bFGF, IGF-I, and IGF-II) stimulate the proliferation of quiescent osteoblast populations in vitro. In most cases investigated, osteocalcin biosynthesis decreases in response to mitogens. (27, 46, 47).

TNF-α inhibits osteocalcin synthesis by primary ROB cells and the MG-63 and ROS 17/2.8 osteosarcoma cell lines. The reduction in osteocalcin synthesis is relatively selective, as the cytokine was without effect on other osteoblast phenotypic markers such as alkaline phosphatase activity and mineralized nodule formation. Similar specificity of TNF-α inhibition on osteocalcin synthesis was reported for primary human osteoblasts and may simply indicate the exquisite sensitivity of osteocalcin to perturbation by cytokines (29). Our Northern hybridization analysis of steady-state osteocalcin mRNA levels in ROB cells following exposure to IL-1β (0.25–25 pM) and 1 nM TNF-α suggests that the reduction in osteocalcin synthesis is due to reduced transcriptional rate and/or reduced stability of the osteocalcin mRNA. Nanes et al. (44) made similar observations using ROS 17/2.8 cells. Recent studies of the regulatory elements in the osteocalcin gene have shown AP-1 sites to which jun-fos protein complexes may bind (45, 48). IL-1β effects on T cells (49) and TNF-α effects on fibroblasts (50) reportedly involve AP-1 related sites in other genes.

IL-1β and TNF-α are known to stimulate bone resorption (2, 3, 33). This action may be accomplished by direct effects of IL-1β on osteoclast precursors and mature osteoclasts (43, 53, 54) or by indirect effects involving osteoblastic mediation by PGE_2, secreted proteinases, and other factors. (5, 11, 16–18, 31, 55–57). An additional regulatory loop requiring further investigation is osteocalcin–osteoclast interactions. Osteocalcin has been shown to be a chemoattractant for monocytic osteoclast precursors (22) and osteoclasts (24). Bone particles deficient in osteocalcin are poorly resorbed relative to normal bone (23). A hidden benefit for the host may be derived from the selective inhibition of osteocalcin by cytokines. Turning off osteocalcin synthesis, while associated with a reduction in bone formation, may limit the recruitment of osteoclasts to inflammatory sites (51).

We have observed similar inhibitory effects of IL-1β and TNF-α on osteocalcin synthesis in three osteoblastic cell types. Differences were noted for cytokine effects on DNA synthesis. Only TNF-α stimulated DNA synthesis in ROB cells (Table 1), while both cytokines stimulated DNA synthesis by MG-63 cells (Table 5). Under the constraints of the day 14–17 protocol, ROS 17/2.8 cells did not show increased DNA synthesis in response to IL-1β and TNF-α (Table

4). The healing response of osteoblasts to wounding frequently involves proliferation of osteoprogenitor (preosteoblastic) cells. Subsequent to the proliferative events, a period of deposition and maturation of new extracellular matrix ensues. It has been observed that both IL-1β and TNF-α inhibit markers of new bone formation (alkaline phosphatase and mineralized nodules), particularly if added early to developing osteoblast cultures (52). However, in our study of postconfluent normal ROB cells, the only formation marker inhibited is osteocalcin, and only TNF-α stimulates osteoblastic replication. This suggests that there may be different mechanisms by which the two cytokines IL-1β and TNF-α inhibit bone formation.

IL-1β and TNF-α are known to have similar effects on osteocalcin synthesis by ROS 17/2.8 cells and primary human osteoblast-like cells with in the first week of culture (1, 13, 29). The present study extends these observations to include the effects of the cytokines on postconfluent cultures of both normal and transformed osteoblasts. The time distinction is important, because elevated osteocalcin synthesis in vitro does not commence until the two- to three-week period when primary cells have elaborated an extensive extracellular collagenous matrix, which can be induced to mineralize with β-glycerolphosphate (32, 36, 37). Importantly, several commonly utilized osteoblast culture protocols actually diminish osteocalcin synthesis: (1) the use of passaged primary cells (32); (2) charcoal treatment of serum to remove osteocalcin and vitamin D backgrounds (39, 51); and (3) exposure of preconfluent osteoblast cultures to L-ascorbate (32, 51). In addition, certain lots of serum were nonpermissive for the inhibitory effects of IL-1β and TNF-α on osteocalcin synthesis by ROB, MG-63, and ROS 17/2.8 cells (51). In light of the relatively high levels of osteocalcin achieved in the osteoblast cultures in this study, it was not necessary to enhance osteocalcin synthesis by supplementing with $1,25(OH)_2$-vitamin D_3, a treatment that would cause pleiotropic effects including diminished matrix collagen synthesis. This reduces the complexity compared to human osteoblast culture systems where effects of cytokines on osteocalcin expression have been assessable only if studied in the presence of high concentrations of $1,25(OH)_2$-vitamin D_3 (11, 12, 29, 31). While these differences in culture methods are largely methodological, for osteocalcin synthesis the differences are hardly trivial, underscoring the distinctions between available models systems for studying bone formation. The present ROB cell culture model of bone formation in vitro would likely correspond to the effects IL-1β and TNF-α on mature osteoblasts in vivo at a site of inflammation, rather than the effects of these cytokines during initial bone development.

Tissue destruction in arthritis, osteomyelitis, and periodontal disease may occur as a direct result of bacterial attack and/or as a by-product of the response of immunocompetent cells to exogenous microbiological and pathological stimuli. In the past, immunocompetent cells have been defined as lymphocytes,

granulocytes, and macrophages; however, recent findings indicate that osteoblasts can synthesize cytokines (including IL-1, TNF, M-CSF, GM-CSF, IL-6) (30, 53, 55, 58–60) and may also function as antigen-presenting cells under appropriate conditions (61). These insights have begun to reshape the hypothesis regarding the role of various inflammatory and resident cells during local immunopathologic responses. In this context, the effects of injury to osteoblasts is of critical importance for our understanding of the mechanisms of bone destruction. Autocrine regulation of osteoblast metabolism by osteoblast-synthesized cytokines and growth factors undoubtedly plays an important role (27, 28, 46, 53, 62, 63). Clearly, the mitogenic and phenotypic response of osteoblastic cells will depend on the constellation of bioactive factors in their environment, as well as their membrane-receptor status for each of these multiple agents. Fundamental investigation of responses to endogenous and exogenous cytokines in the context of the inflammatory response is needed. Here we provide evidence for the ability of IL-1β and TNF-α to inhibit a major differentiated function of osteoblasts, osteocalcin synthesis, at a time when the cells are relatively quiescent and producing a mineralized matrix. This inhibition of osteocalcin appears relatively selective in that alkaline phosphatase expression and mineralized nodule parameters were unaffected during the test period.

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