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RECOMBINANT HUMAN LYMPHOTOXIN EFFECTS ON OSTEOBLASTIC CELLS

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Lymphotoxin, or tumor necrosis factor β, has been shown to be a potent bone resorbing cytokine. In the present study, the effect of recombinant human lymphotoxin on osteoblastic cell proliferation and prostaglandin synthesis was investigated. Lymphotoxin (10^{-10}-10^{-7} M) caused a significant, dose-dependent decrease of rat osteoblastic cell proliferation. This appeared to be an indirect, prostaglandin-dependent action, since in the presence of indomethacin (1 μM) the lymphotoxin effect was reversed. Subsequently, prostaglandin E_2 and prostacyclin (assayed as 6-keto-prostaglandin $F_{1\alpha}$) levels produced by the osteoblastic cells in response to lymphotoxin were measured. The cytokine caused a dose-dependent increase of these arachidonic acid metabolites, with the maximum effect at 10^{-8}M. These results suggest that lymphotoxin's mechanism of action on bone may involve increases in arachidonic acid metabolite synthesis and an indirect, prostanoid-mediated decrease in the proliferation rate of osteoblastic cells.

Lymphotoxin (LT, or Tumor Necrosis Factor β) was initially described as a cytotoxic factor (1, 2). It is now recognized as a potent cytokine involved in inflammatory reactions (3), having a variety of biological actions (4). Both the human and murine forms of this 18,600 molecular weight factor have been sequenced and cloned (5-7). Lymphotoxin has been shown to be primarily a T-cell product (4) which can cause tumour regression *in vivo* (8), inhibition of proliferation (9) and induction of differentiation (10, 11) in various cell lines *in vitro*, as well as activation of neutrophils (12).

Lymphotoxin appears to have several effects on bone tissue. It stimulates *in vitro* bone resorption (13-15) and has been implicated in bone resorption occurring *in vivo*, accompanying diseases such as myeloma (16). It appears that LT's effect on resorption is mediated by a direct action on the osteoblastic cells, which then stimulate the osteoclasts (17). Studies on calvaria have shown that LT inhibits *in vitro* bone formation and collagen synthesis (13, 18, 19). Bertolini et al (13) have reported that LT decreases alkaline phosphatase activity in rat osteoblast-like osteosarcoma cells. To date, there appears to be no other study in the literature on the effects of LT on isolated bone cells.

Abbreviations: DNA, deoxyribonucleic acid; HBSS, Hepes-buffered balanced salt solution; Hepes, N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid; IL-1, interleukin-1; LT, lymphotoxin; PG, prostaglandin; rHuLT, recombinant human lymphotoxin; TCA, trichloroacetic acid; TNF, tumor necrosis factor.

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In the present study we used normal rat calvarial osteoblastic cells to investigate the effects of recombinant human LT on isolated osteoblastic cells.

Materials and Methods

Chemicals. Recombinant human lymphotoxin (rHuLT; specific activity $1.2x10^8$ U/mg) was a gift of Genentech (South San Francisco, CA, Dr. H. M. Shepard). Indomethacin was kindly provided by Merck, Sharp and Dohme (Rahway, NJ). Collagenase (CLS II) was purchased from Cooper (Freehold, NJ). ^{3}H-Thymidine (specific activity 7 Ci/mmol; 1mCi/ml) was obtained from ICN (Irvine, CA). PGE_2 and PGF_{1a} radioimmunoassay (RIA) kits were purchased from New England Nuclear (NEN) (Boston, MA).Tissue culture media and supplements were obtained from GIBCO (Grand Island, NY).

Isolation of bone cells. The method for isolation of osteoclastic- and osteoblastic-enriched cell populations, a modification of the Luben, Wong, and Cohn (20) technique, has been previously described (21). Calvaria of 20 to 21-day-old fetal Sprague-Dawley rats were cleaned of adherent connective tissue and incubated (four calvaria/ml) in a Hepes-buffered pH 7.4 balanced salt solution (HBSS: 120 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, 3 mM K_2HPO_4, 30 mM mannitol, 30 mM Hepes, 5 mg/ml glucose and 1 mg/ml crystalline bovine serum albumin fraction V) containing 2mg/ml collagenase at 37°C for four 15-minute periods.
The cells released during each incubation period were washed by centrifugation with fresh buffer without collagenase. To remove contaminating extracellular matrix, the cells were incubated for 5 minutes with cold (4°C) HBSS titrated to pH 6.0 and then were incubated for 10 minutes at 4°C in hypotonic buffer (HBSS/H_2O, 1:1) to selectively lyse erythrocytes. Cell concentration was determined by counting the cells with a haemocytometer.
This procedure yielded four populations of cells designated I, II, III and IV. Populations I and II have been previously characterized as osteoclastic (21). Populations III and IV were characterized as osteoblastic by high alkaline phosphatase levels, lack of calcitonin response and of bone resorbing activity in vitro (21), ability to synthesize collagen in vitro (20), and their ability to form bone in vitro (22). All the experiments described in this paper were performed with population III cells.

Culture of bone cells. After isolation, the osteoblastic cells (population III) were resuspended in BGJb medium (Fitton-Jackson modification, containing 1 mg/ml crystalline bovine serum albumin fraction V) at $1x10^6$ cells/ml. Five-tenths (0.5) of a milliliter of the cell suspension was placed in each well of a 24-well plate (Flow Labs, McLean, VA). The cells were then cultured for 48 hours at 37°C in a 5% CO_2 atmosphere. At the end of the 48-hour period, the cells were washed once with BGJb, and then 0.5 ml of medium with the appropriate agent was placed in each well. In experiments where indomethacin was used, all controls received ethanol, the indomethacin solvent, which never exceeded 0.05%. Cells were then cultured for 72 hours. At the end of the 72-hour period, the supernatants were collected and immediately extracted for prostaglandins.

Cell proliferation assay. This was according to Puzas et al (23) with minor modifications. After removal of the supernatants for determination of prostaglandins in the medium, the cells were incubated with medium containing 2μCi/ml ^{3}H-Thymidine for 24 hours. At the end of this period the cells were washed with medium. The cells were then removed with a cotton tip applicator moistened in cold 12.5% trichloroacetic acid (TCA). The cotton tip applicators were then washed twice in 12.5% TCA and then once in 95% ethanol (10 minutes each wash). The applicators were then air-dried and the radioactivity associated with the tips was measured by liquid scintillation spectrometry.

Prostaglandin extraction and assay. Supernatants were extracted for prostaglandins according to the method of Jaffe and Behrman (24). Supernatants were first extracted with petroleum ether, then with a mixture of ethyl acetate: isopropanol: 0.2 M HCl (3:3:1 by volume), and then with ethyl acetate and water. The organic phase was evaporated overnight and the residues were reconstituted with 0.5 ml of RIA assay buffer. The recovery of the extraction procedure, using ^{3}H-PGE_2 (NEN), was 70%. An aliquot (0.1ml) of each reconstituted sample was then tested in the RIA. Tissue culture media with the appropriate agent were also extracted and used as blanks in the RIA. The RIA kits used ^{125}I-labeled PGE_2 and 6-keto-$PGF_{1\alpha}$ derivatives as tracers. The antibodies used had the following cross-reactivity when calculated at the 50% B/Bo point; the anti-PGE_2 antibody: PGE_2-100%, PGE_1-3.7%, PGA_2-0.4%, 6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGA_1, PGB_2 and PGD_2< 0.04%; the anti-6-keto-$PGF_{1\alpha}$ antibody: 6-keto-$PGF_{1\alpha}$-100%, $PGF_{2\alpha}$-2.6%, PGE_1-1.9%, PGE_2-1.1%, $PGF_{1\alpha}$-0.8%, PGA_1, PGA_2, PGB_2 and PGD_2 < 0.3%.

Statistical Analysis. All the determinations were done in quadruplicates or triplicates. Data were analysed using the Student's t test.

Results

Effect of rHuLT on proliferation. Lymphotoxin caused a significant reduction in DNA synthesis, as indicated by the decrease in ^{3}H-Thymidine incorporation. This inhibitory effect appeared to be prostaglandin-mediated, since it was reversed in the presence of indomethacin (Table 1). This evidence was strengthened when the effect of LT on proliferation and PGE_2 production was simultaneously followed in a single large experiment, which has been repeated twice, where the dose-response curves for ^{3}H-Thymidine incorporation and PGE_2 levels are almost the mirror image of one another (Figure 1). The maximum effect was seen at a concentration of $5x10^{-8}$ M.

Effect of rHuLT on prostaglandin production. Addition of rHuLT to the normal rat osteoblastic cells resulted in a significant and dose-dependent increase in PGE_2 production (Table 2). The effect of rHuLT on prostacyclin production, measured as 6-keto-$PGF_{1\alpha}$, was similar but quantitatively much lower (Table 3). Both basal and LT-stimulated PGE_2 and 6-keto-$PGF_{1\alpha}$ production was inhibited by indomethacin, the cyclooxygenase inhibitor (Tables 2 and 3).

Discussion

There is unequivocal evidence that cytokines, like IL-1 and TNFs originally associated with the immune system, have profound effects on bone and bone cells. Recombinant human LT can cause bone resorption in both a fetal rat long bone assay system (13, 15) and a murine calvarial assay system (14). There appear to be no studies on the possible inhibition of LT's bone resorptive action by indomethacin. However, the action of the closely related TNFα is inhibited by the cyclooxygenase inhibitor in the rat long bone (15) and the murine calvarial system (25), thus implicating the arachidonic acid metabolites in mediating this effect. Although LT has been recently shown to stimulate in a prostaglandin-independent manner the *in vitro* formation of osteoclastlike cells (26), it has also been shown that its action on osteoclastic resorption is indirect and dependent on its effect on the osteoblastic cells (17). LT causes a preferential inhibition of osteoblastic collagen synthesis in a rat calvarial system (13, 18, 19). This effect of LT has been determined to be inhibited by indomethacin (18).

Table 1
Effect of rHuLT on osteoblastic proliferation

Exp.	Treatment	^{3}H-Thymidine (cpm)
1	Control	14,618.0 ± 1,099.0
	LT 10^{-9}M	7,533.0 ± 1,363.0[a]
	LT 10^{-8}M	6,447.0 ± 612.0[a]
2	Control	23,584.0 ± 5,184.0
	Indomethacin 10^{-6}M	25,637.0 ± 3,372.0
	LT $5x10^{-8}$M	11,645.0 ± 2,503.0[a]
	LT + Indomethacin	18,800.0 ± 2,910.0[b]

Results are the mean ± standard deviation for triplicate or quadruplicate determinations.
a: $p<0.005$ from control; b: $p<0.01$ from LT alone.

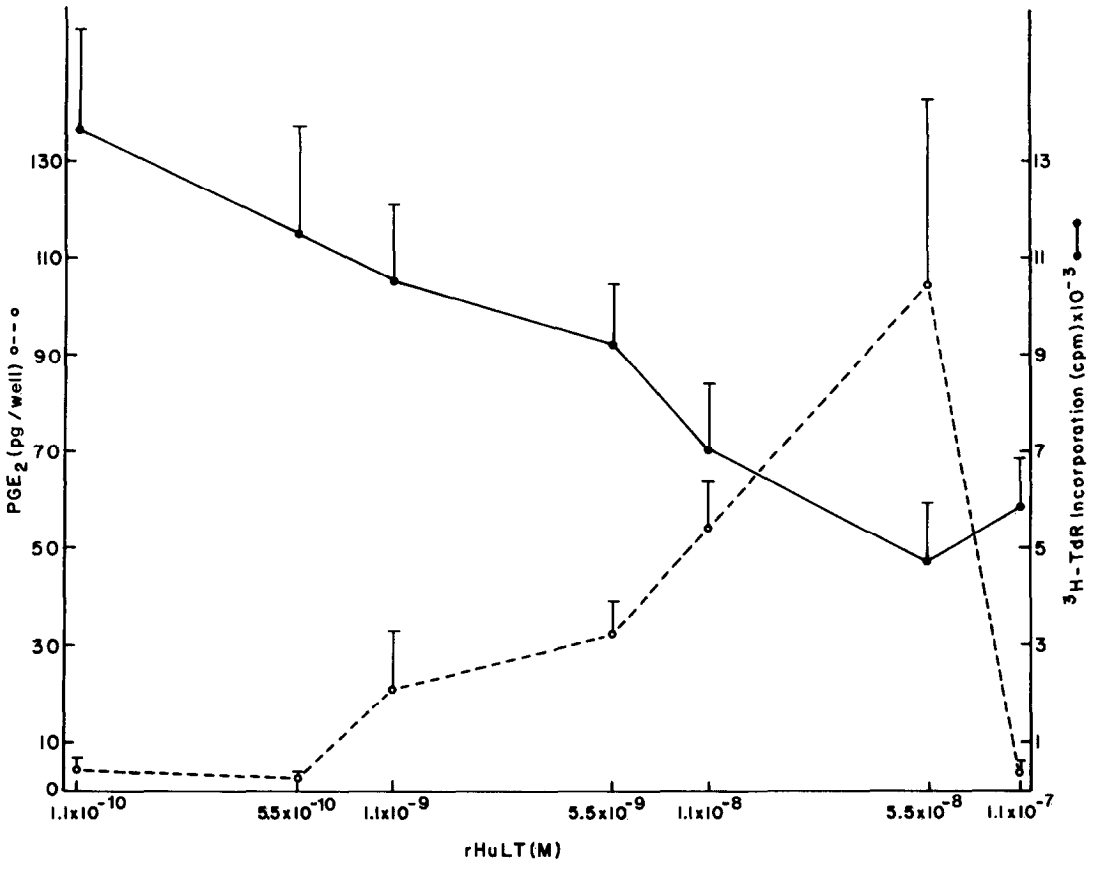

Fig. 1. The effect of rHuLT on osteoblastic cell proliferation and PGE_2 production. The data represent the mean ± standard deviation for triplicate or quadruplicate determinations, from a single experiment.

The results of the present study suggest that LT causes a prostaglandin-dependent inhibition of osteoblast-like cell proliferation. The observed maximum effective dose is in agreement with that of other studies, since lymphotoxin's action on bone resorption and collagen synthesis reaches a

Table 2
Effect of rHuLT on osteoblastic PGE_2 production

Exp.	Treatment	PGE_2 (pg/well)
1	Control	13.4 ± 7.2
	LT 10^{-9}M	140.0 ± 28.6[b]
	LT 10^{-8}M	343.1 ± 22.1[bc]
2	Control	15.5 ± 6.5
	Indomethacin 10^{-6}M	1.7 ± 0.8[a]
	LT $5x10^{-8}$M	2,215.0 ± 37.7[b]
	LT + Indomethacin	7.5 ± 5.9[d]

Results are the mean ± standard deviation for triplicate or quadruplicate determinations.
a: $p<0.01$ from control; b: $p<0.005$ from control;
c: $p<0.005$ from LT 10^{-9}M; d: $p<0.005$ from LT alone.

Table 3
Effect of rHuLT on osteoblastic 6-keto-$PGF_{1\alpha}$ production

Exp.	Treatment	6-keto-$PGF_{1\alpha}$ (pg/well)
1	Control	11.4 ± 1.5
	LT $5x10^{-8}M$	27.7 ± 4.9[a]
2	Control	30.2 ± 18.4
	Indomethacin $10^{-6}M$	28.5 ± 7.8
	LT $5x10^{-8}M$	122.3 ± 13.0[a]
	LT + Indomethacin	33.6 ± 10.9[b]

Results are the mean ± standard deviation for triplicate or quadruplicate determinations.
a: $p<0.005$ from control; b: $p<0.005$ from LT alone.

maximum at concentrations between 10^{-8} and 10^{-7} M, depending on the assay system used. In a whole calvarial system LT increases thymidine incorporation, despite its inhibitory effect on collagen synthesis (18, 19). The difference from the results of the present study probably reflect the fact that in a whole calvarium there are many different cell types that could respond to the agent. In addition, the interactions possible among the various cell types present, through the production of several mediators -including arachidonic acid metabolites- and growth factors, could alter the final response observed. TNFα has been shown to increase both thymidine incorporation and prostaglandin synthesis in human trabecular bone cells (27). This apparent discrepancy with the present results could simply reflect differences in the two biological systems and/or methodological differences, since in the afforementioned study the cells were cultured in the presence of serum. It should be noted that in the absence of serum there was no effect on thymidine incorporation. It is of interest that in isolated human synovial fibroblasts rHuLT increases DNA synthesis, and this increase is significantly elevated in the presence of indomethacin (28). In normal rat skin fibroblasts LT increases proliferation only in the presence of indomethacin (Tatakis and Dziak, unpublished observations). In contrast, human endothelial cell growth is inhibited by the cytokine (29).

The prostanoids have profound and complex effects on bone. PGE_2 has been demonstrated to be the most potent bone resorbing agent among the prostanoids (30), as well as being capable of both inhibiting (31) and stimulating bone formation (32) depending on the dose employed. PGE_2 can also have a direct action on osteoclast-like cells (33, 34) and, as shown recently, it may promote their formation (35). The studies reported here provide evidence that LT has direct effects on normal osteoblastic cells and that increases in endogenous prostanoid synthesis may be involved in the regulation of the proliferative ability of these cells. Thus, LT may affect bone metabolism at several different levels through the significant increase of prostanoid synthesis by osteoblast-like cells, as well as through its direct action on osteoclast-like bone cells.

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