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Interleukin 4 Inhibits Murine Osteoclast Formation In Vitro

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Abstract Interleukin 4 (IL-4) is a product of activated T cells and mast cells with effects on immunologic and hematopoietic processes. We now report that IL-4 inhibits the formation of osteoclasts from murine bone marrow cells cocultured with stromal cells. Numerous (3,000–4,000 cells/2 cm^2) tartrate-resistant acid-phosphatase-positive multinucleated cells with the capacity to generate cAMP in response to salmon calcitonin (ED_{50} = 10^{-10} M) developed within 10–12 days of culture. IL-4 (ID_{50} = 10 U/ml) inhibited osteoclast generation in doses similar to those that induce proliferation of IL-4-responsive T cells. Additionally, the rat antimurine IL-4 monoclonal antibody 11B11 antagonizes the IL-4-inhibitory effect on osteoclast formation. These results suggest that IL-4 impedes agonist-induced in vitro bone resorption by inhibiting osteoclastogenesis.

Key words: calcitonin, TRAP, cAMP, bone marrow cells, ST-2 cells, 11B11

Interleukin 4 (IL-4) is a 20 kDa glycoprotein produced by activated T cells and mast cells which, like other cytokines, impacts on many biological events. Originally isolated based on its capacity to induce proliferation of B cells costimulated with anti-IgM antibodies (Howard et al., 1982; Rabin et al., 1985), it is now recognized that IL-4 also influences B cell antibody isotype expression (Coffman et al., 1986; Vitetta et al., 1985), T cell activation (Kurt-Jones et al., 1987; Kupper et al., 1987), mast cell proliferation (Smith and Rennick, 1986; Mosmann et al., 1986), monocyte activation (Hart et al., 1989; Essner et al., 1989; Littman et al., 1989; Standiford et al., 1990; Abramson and Gallin, 1990; Gibbons et al., 1990), and hematopoiesis (Peschel et al., 1987, 1989; Rennick et al., 1987). While these studies have focussed primarily on IL-4's influence on immune responses and hematopoiesis, recent data suggest that this immunomodulatory cytokine may play a role in skeletal metabolism. Specifically, Watanabe et al. documented that IL-4 is a potent inhibitor of in vitro bone resorption (Watanabe et al., 1990). Utilizing a bone rudiment organ culture system, these authors observed that IL-4 antagonized the resorption-inducing effects of $1,25(OH)_2D_3$, prostaglandin E_2, tumor necrosis factor α, and interleukins 1α and β without influencing basal levels of bone resorption. While this observation is certainly novel, the mechanism by which IL-4 dampens bone degradation has remained enigmatic.

Because IL-4 can inhibit the resorption-inducing effects of such a diverse group of osteolytic substances, we reasoned that the IL-4-affected process(es) in these organ cultures would likely a) occur early in the bone-remodeling sequence and b) be essential for the resorptive process. Based on the fact that matrix resorption can only be affected by metabolically competent osteoclasts and, further, that few of these cells are present in quiescent bone, inhibition by IL-4 of osteoclast formation (osteoclastogenesis) would be a logical candidate mechanism. To address this possibility, we have examined the effect of IL-4 in an in vitro model of osteoclastogenesis involving the coculture of bone marrow cells and a stromal cell line. As will be shown, IL-4 inhibits formation of osteoclasts in a dose-dependent manner by utilizing concentrations of the cyto-

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kine which also effect the proliferation of an IL-4-responsive T cell line. Furthermore, when IL-4 and an antimurine IL-4 monoclonal antibody are coadded to these cultures, the inhibitory effect of IL-4 on osteoclastogenesis is significantly antagonized. These results suggest that IL-4 disrupts the resorption-inducing effects of osteolytic mediators in vitro at least in part by inhibition of osteoclast formation.

METHODS

Biologic Mediators and Antibodies

Recombinant interleukin 4 was expressed and purified from baculovirus-transfected SF-9 (*Spodoptera frugiperda*) cell culture supernatants as described (Ohara et al., 1987; Ohara, 1989). Stage 1 colony-stimulating factor 1 (CSF-1) was prepared from supernatants of L929 cells as described (Stanley and Heard, 1977). Salmon calcitonin (CT) was obtained from Peninsula Laboratories (Belmont, CA). Dexamethasone was obtained from Sigma (St. Louis, MO) and $1,25(OH)_2D_3$ was the kind gift of Milan Uskokovic (Hoffman-La Roche, Nutley, NJ). The rat antimurine IL-4 monoclonal antibody (11B11) (Ohara et al., 1987) was purified from caprylic-acid-treated hybridoma-conditioned medium by antirat IgG affinity chromatography (Sigma) (Harlow and Lane, 1988). In experiments where this antibody was employed, affinity-purified rat IgG (Sigma) was utilized as a control.

Bone Marrow Cell Preparation

Murine bone marrow cells were prepared by a modification of a method described previously (Tushinski et al., 1982). Briefly, the bone marrow of A/J (Jackson Laboratory, Bar Harbor, ME) mice was flushed from femurs and tibiae with ice-cold α-minimal essential medium (α-MEM). The cells were collected, pelleted, resuspended in α-MEM supplemented with 10% heat-inactivated fetal calf serum (HIFCS) containing 500 U/ml of stage 1 CSF-1 (50 ml medium/8 bones/150 mm tissue culture dish), and subsequently incubated for 24 h (37°C, 5% CO_2). The nonadherent cells were then collected, pelleted (1,000 rpm, 7 min, 4°C), and resuspended (1×10^7 cells/ml) in Pronase solution [0.02% Pronase (Boehringer Mannheim, Indianapolis, IN), 1.5 mM EDTA in phosphate-buffered saline]. After this incubation (15 min, 37°C), the suspension was layered onto 15 ml of horse serum (4°C) and sedimented (15 min). The cell suspension from the top of the gradient was carefully transferred onto fresh horse serum gradients (15 ml, 4°C) and centrifuged (2,000 rpm, 10 min, 4°C), and the cell pellet was suspended in α-MEM containing 10% HIFCS.

Bone Marrow/Stromal Cell Coculture

ST-2 cells (Nishikawa et al., 1988; *Ogawa et* al., 1988) were obtained from the RIKEN Cell Bank (Tsukuba, Japan), maintained in RPMI 1640 supplemented with 10% HIFCS, and passaged weekly following trypsinization (2×10^4 cell/ml, 25 ml/T75 flask). A modification of the method of Udagawa et al. was employed for the coculture experiments (Udagawa et al., 1989). Briefly, ST-2 cells (1×10^5 cells/ml, 1 ml/well, 24-well tissue culture plates) were cultured for 24 h. After this period, bone marrow cells (1×10^6 cells/ml, 1 ml/well) were seeded onto the ST-2 cells. Throughout the coculture period, the medium was α-MEM containing 10% FCS, $1,25(OH)_2D_3$ (10^{-8} M), and dexamethasone (10^{-7} M). The cultures were maintained for various periods with medium changed twice weekly.

HT-2 Cells

The murine IL-4-responsive T cell line HT-2 was obtained from Dr. T. Kupper (Washington University, St. Louis, MO) and was maintained as described (Lacey et al., 1987). Bioassays were performed as described (Lacey et al., 1987) by utilizing ^{3}H-thymidine incorporation (ICN, Irvine, CA) as an indicator of cellular proliferation.

Osteoclast Markers

TRAP staining. The number of formed osteoclasts was evaluated by cytochemical detection of tartrate-resistant acid phosphatase (TRAP, Sigma, St. Louis, MO). For the purpose of this study, any cell with three or more nuclei with TRAP-positive cytoplasmic staining was considered an osteoclast.

cAMP response to calcitonin. The cyclic AMP (cAMP) response to CT was examined as described (Takahashi et al., 1988). Briefly, the day 10 osteoclast cultures were preincubated with α-MEM containing 0.1% BSA and 1 mM isobutylmethylxanthine (IBMX) for 10 min. Subsequently various concentrations (10^{-13} to 10^{-7} M) of salmon CT were added in the presence of 1 mM IBMX. After incubation (15 min, 37°C), the medium was removed and the cell layer con-

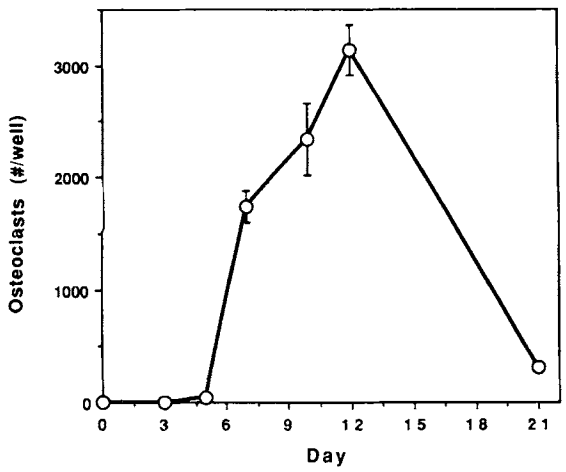

Fig. 1. Bone marrow and stromal cell cocultures generate osteoclasts in vitro. Bone marrow cells were seeded onto ST-2 cell cultures in the presence of 10^{-8} M $1,25(OH)_2D_3$ and 10^{-7} M dexamethasone as described (see Methods). At various times, parallel cultures were stained for TRAP and the number (mean (n = 4) ± standard deviation) of osteoclasts was determined.

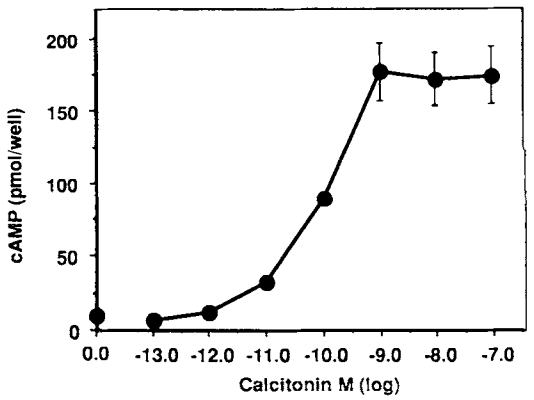

Fig. 2. Calcitonin treatment induces cAMP production by in vitro osteoclast cultures. Osteoclast cultures (day 10) were exposed to varying concentrations of salmon calcitonin and cell-layer-contained cAMP was evaluated as described (see Methods). The results are reported as the mean (n = 4) ± standard deviation of the cAMP generated after a 15 min treatment period.

taining cAMP was extracted with 500 μl of 5% trichloroacetic acid (TCA). Aliquots (100 μl) of these samples were washed (×3) with 5 volumes of water-saturated ether by vortexing and then dried. This extract was then analyzed for cAMP by utilizing a cAMP radioimmunoassay kit (Dupont—NEN, Wilmington, DE).

RESULTS

These experiments involved a modification of the method of Udagawa et al. which entails coculture of spleen cells or macrophages with a stromal cell line (ST-2 cells) (Udagawa et al., 1989, 1990). In the presence of $1,25(OH)_2D_3$ and dexamethasone, this combination leads to formation of osteoclasts. Because the osteoclast is a specialized bone-resorbing cell of monocytic lineage (Udagawa et al., 1990; Alvarez et al., 1991), we decided to utilize marrow cells optimally prepared for cultivation of macrophage precursors (Tushinski et al., 1982), cells ontogenetically related to the osteoclast. These cocultures result in formation of multinucleated cells (MNCs) which appear in a time-dependent manner (Fig. 1). Prior to day 5 after the initiation of coculture, no MNCs can be seen. By day 7, however, significant numbers of MNCs appear and their numbers continue to increase until approximately days 10–12. At this time, the yield of osteoclastic cells ranges from 3,000 to 4,000/24-well platewell. Thereafter, MNC numbers decline.

The generated MNCs are judged to be bona fide osteoclasts because they 1) express tartrate-resistant acid phosphatase, 2) specifically bind ^{125}I-calcitonin, 3) form resorptive pits on bone slices, and 4) efficiently degrade ^{3}H-proline-labeled devitalized bone particles (manuscript in preparation). In addition, these cultures respond to salmon calcitonin in a manner characteristic of osteoclastic cells (Nicholson et al., 1986; Takahashi et al., 1988). In these experiments, we assessed the effect of a range of calcitonin levels on cAMP production. As Figure 2 demonstrates, calcitonin induces cAMP production in a dose-dependent manner with the calcitonin ED_{50} approximating 10^{-10} M. This calcitonin concentration is clearly within the range of values reported for the calcitonin receptor Kd (Nicholson et al., 1986; Graves and Jilka, 1990). Taken together, these data support our contention that this MNC population represents osteoclasts.

To assess the impact of IL-4 on this in vitro model of osteoclastogenesis, experiments were performed with bone marrow cell/ST-2 cell cultures in the presence of various concentrations of the cytokine which were added at coculture initiation and at every medium change. Figure 3 shows the results of a representative experiment where osteoclast number was determined at day 10 of coculture. Note that IL-4, in a dose-dependent manner, decreases the number

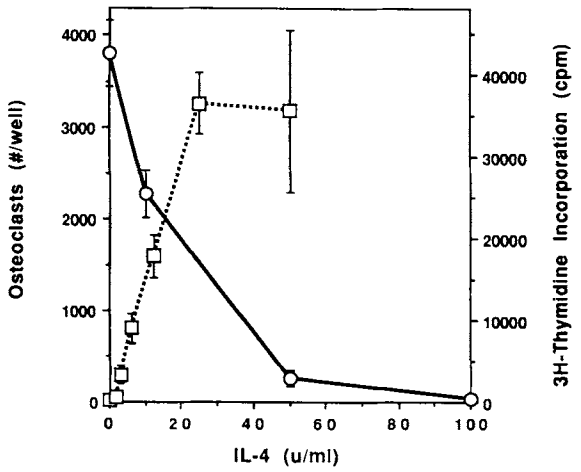

Fig. 3. IL-4 inhibits in vitro osteoclast generation. Bone marrow and ST-2 cell cocultures, established as in Figure 1, were treated with various concentrations of IL-4 as described (see Methods). Following 10 days of culture, osteoclast number (○—○) was determined as in Figure 1. As a means to compare IL-4 potency, similar concentrations of the same IL-4 stock were utilized in proliferation assays utilizing HT-2 cells (see Methods). As a measure of proliferation, the incorporation of ^{3}H-thymidine (□---□) was evaluated and is reported as the mean (n = 3) ± standard deviation.

TABLE I. Anti-IL-4 Antibodies Antagonize IL-4's Inhibition of Osteoclastogenesis

	Condition[a]	TRAP(+)MNC[b]
Experiment 1		
(IL-4 : 10 U/ml)	Control	3,038.3 ± 24.0
	IL-4	1,977.7 ± 311.5
	Rat IgG[c]	2,902.0 ± 305.3
	11B11	2,791.3 ± 207.9
	IL-4 + rat IgG	1,845.3 ± 382.2
	IL-4 + 11B11	2,579.3 ± 85.0
Experiment 2		
(IL-4 : 50 U/ml)	Control	3,157.3 ± 652.8
	IL-4	95.0 ± 52.9
	Rat IgG	2,820.0 ± 197.5
	11B11	2,879.7 ± 404.8
	IL-4 + rat IgG	68.7 ± 15.3
	IL-4 + 11B11	569.0 ± 83.4

[a]Bone marrow/ST-2 cocultures were established as described (see Methods and Results). Note that experiments 1 and 2 were performed with different IL-4 levels.
[b]The number of TRAP-positive MNC were determined after 10 days of coculture and represent the mean (n = 3) ± standard deviation.
[c]Rat IgG and the 11B11 monoclonal antibodies were added at 10 μg/ml where indicated.

of osteoclasts with an ID_{50} approximating 10 U/ml. At 100 U/ml IL-4, the generation of osteoclastic cells is virtually 100% inhibited. To evaluate the potency of our IL-4, we assessed its capacity to stimulate proliferation of the IL-4-responsive murine T cell line HT-2. As Figure 3 demonstrates, IL-4 induces proliferation of these cells at the range of concentrations which effectively inhibits osteoclastogenesis with an IL-4 ED_{50} of 10–20 U/ml.

To further confirm that the observed inhibition of osteoclastogenesis reflects the presence of IL-4, we examined the event in the presence of a purified rat anti-murine IL-4 monoclonal antibody. As Table I demonstrates, 11B11 (10 μg/ml) significantly antagonizes IL-4's inhibitory effect on osteoclastogenesis when employed with either 10 or 50 U/ml IL-4 (P values for IL-4 + 11B11 vs. IL-4 alone are <0.05 and <0.01, respectively). The 11B11 antibody alone exerts no stimulatory effect on osteoclast formation, implying that spontaneous IL-4 production, which could theoretically inhibit basal osteoclastogenesis, probably does not occur in this model. Additionally, an equivalent amount of purified rat IgG has no effect on either basal or IL-4-inhibited osteoclast formation.

DISCUSSION

This report documents the capacity of IL-4 to significantly impair osteoclast formation, dose-dependently, in a concentration range which also effects T cell proliferation. These observations underscore the potential physiologic relevance of IL-4's inhibition of osteoclastogenesis. In a previous study of IL-4 effects on multinucleated cell generation, IL-4 was shown to induce granuloma-like MNC formation in bone marrow cultures treated with IL-3 in the absence of added stromal cells (McInnes and Rennick, 1988). Therefore, it appears that IL-4 possesses the capacity to influence, in a selective manner, the generation of phenotypically dissimilar MNCs of monocytic lineage which are specialized to perform distinct functions.

While the precise point in osteoclast ontogeny affected by IL-4 remains unknown, it likely targets an osteoclastic mononuclear precursor(s) because there is uniform suppression of MNC formation at all times in IL-4-treated cultures (unpublished observations). In addition to identifying the precise step(s) in the osteoclast maturation sequence, the cell type responsible for mediating this effect needs to identified. The coculture system employed is inherently com-

plex, containing a wide variety of cells at different densities. Despite this complexity, the most obvious IL-4 cellular targets would include the stromal cells, osteoclastic precursors, or both. In previous studies of IL-4 effects on in vitro hematopoiesis, a complicated picture has emerged. In some experimental conditions (particularly those not involving the presence of added stromal cells), IL-4 apparently has the capacity to increase colony-forming activity when coadded to lineage-specific colony-stimulating factors (Rennick et al., 1987; Peschel et al., 1987). Except for mast cell colony formation, IL-4 significantly antagonizes lineage-specific colony formation when combined with IL-3 (Rennick et al., 1987). In the presence of stromal cells, IL-4 inhibits lineage-specific colony formation even in the absence of exogenous IL-3 (Peschel et al., 1989). The mechanism involved in this latter event appears to be IL-4-induced stromal cell synthesis of a soluble inhibitory factor which is only produced if marrow cells are also present. Of particular relevance is the fact that dexamethasone, a glucocorticoid we employ in our coculture system, impairs production of this multilineage inhibitor. It is therefore unlikely that induction of this factor by IL-4 in our cocultures could explain the cytokine's inhibitory effect on osteoclast generation. Obviously, an IL-4 effect on the bone marrow osteoclast progenitor population cannot be excluded based on the information currently in hand.

Whether IL-4's effect on in vitro osteoclastogenesis serves to entirely explain the cytokine's effect on in vitro bone resorption is unknown. IL-4 could certainly target cells other than marrow stromal cells or hematopoietic osteoclast progenitors. In this regard, we have recently identified IL-4 receptors on murine osteoblastic cells (manuscript in preparation), raising the possibility that IL-4 could impair the capacity of this cell to mediate the effects of osteolytic factors or, perhaps, inhibit some other event integral to the bone remodeling sequence. Furthermore, IL-4 has bipotential effects on mature monocytes, suggesting that this cytokine may affect the fully differentiated osteoclast. While capable of inducing monocyte class II antigen expression, thereby potentially enhancing antigen presentation (Stuart et al., 1988), IL-4 also inhibits elaboration of activation-induced monocytic products (IL-1, tumor necrosis factor α, IL-6, and prostaglandin E_2) (Hart et al., 1989; Essner et al., 1989; Gibbons et al., 1990) which could play a role in the resorptive process. These observations suggest that IL-4 may also exert bipotential effects on cells of the osteoclast lineage.

While our observations are in keeping with an inhibitory effect on osteoclast progenitors, it remains possible that IL-4 can stimulate previously differentiated osteoclasts. The role of osteoclast cytokine production in skeletal metabolism remains essentially undefined. It is possible that osteoclasts secrete biologically active factors whose production may be modulated by IL-4. Regardless of the ultimate mechanism by which IL-4 inhibits osteoclast formation, it now presents itself as a candidate to treat hyperresorptive conditions such as hypercalcemia of malignancy and certain forms of osteoporosis.

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