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OSTEOBLASTS MEDIATE INTERLEUKIN 1 STIMULATION OF BONE RESORPTION BY RAT OSTEOCLASTS

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Although IL-1 was originally defined (1) as a monocyte/macrophage product that stimulates lymphocyte proliferation in vitro, it has become clear that a closely related or identical substance is also produced by other cell types and has actions on a variety of nonlymphoid target tissues (2–4). One such target tissue appears to be bone, which is stimulated by a mononuclear cell factor with IL-1-like properties to increased osteoclastic resorption in organ culture (5, 6). Bone resorption is often a feature of inflammatory diseases such as rheumatoid arthritis and periodontal disease, and IL-1 production by inflammatory tissue may be the mechanism by which inflammatory osteolysis is effected. We postulated that IL-1 may also have a role in the physiology of bone resorption. We have found (7) that agents which stimulate osteoclastic bone resorption in intact bone appear to have no direct stimulatory effect on osteoclasts. This infers that resorption stimulators act primarily on another bone cell type, which is induced, by the presence of these agents, to stimulate osteoclastic resorption: IL-1 might represent the unknown mediator of osteoclastic stimulation. We therefore elected to analyze the mechanism by which IL-1 induces bone resorption, in an attempt to identify the target cell type and to clarify the role of IL-1 in the pathophysiology of bone resorption.

Materials and Methods

Porcine IL-1 of pI 5.0 (originally called catabolin because of its ability to cause cartilage degradation) was purified to homogeneity from pig mononuclear leukocytes as previously described (8, 9). Hepes-buffered medium 199 (Flow Laboratories, Irvine, United Kingdom) was used for cell isolation, and phosphate-free Eagle's MEM (Flow Laboratories), supplemented with 100 IU/ml benzyl penicillin (Glaxo, Middlesex, United Kingdom), 100 μg/ml streptomycin (Glaxo), and 10% heat-inactivated FCS (Gibco Laboratories, Uxbridge, United Kingdom) used for subsequent incubations.

Slices of human cortical bone (3 × 3 × 0.1 mm) were cut with a low-speed saw (Isomet, Buehler, IL) longitudinally from the femurs of patients who died without evidence of bone disease. Bone slices were ultrasonicated for 30 min, washed in acetone and ethanol, and stored dry at room temperature.

Calvarial cells were obtained by collagenase digestion of calvariae from neonatal female Wistar rats killed by decapitation within 48 h of birth. The calvariae were removed and

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dissected free of periosteal tissue before incubation at 37°C in medium 199 with collagenase (1 mg/ml; type II; Sigma Chemical Co., St. Louis, MO) for 15 min. The calvariae were washed in medium 199 and returned to fresh collagenase for 90 min. After mild agitation with a plastic pipette, the resultant cell suspension was washed, resuspended in MEM/FCS, and incubated in air at 37°C in 50-mm Falcon tissue culture dishes. Upon reaching confluence after 3–7 d, the cells were removed with 1 mM EDTA in Ca^{2+}-free PBS, washed, and resuspended in 3.0 ml of MEM/FCS at 10^6 cells/ml.

Samples of cell suspension settled onto 6-mm glass coverslips were fixed for 10 s in 10% formal calcium. Alkaline phosphatase histochemistry revealed that the majority of cells were alkaline phosphatase–positive (10). 500 μl of calvarial cell suspension were added to a 16-mm-diam well of a Linbro (Sterilin, Teddington, United Kingdom) plate containing four bone slices in 1.5 ml MEM/FCS, and the cultures were incubated for 24 h in 5% CO_2. The remainder of the cell suspension was used as described below.

Osteoclasts were disaggregated as previously described (11). Female Wistar rats were decapitated within 48 h of birth. Tibias and femurs from one animal were dissected free of adherent soft tissue and curetted into 1.5 ml of medium 199. The resulting fragments were vigorously agitated with a plastic pipettte. The cell suspension released was added to 16 bone slices in a well of a Sterilin 100 × 18-mm multiwell dish, and the cells were allowed to settle at 37°C in air for 15 min. The bone slices were then washed in Medium 199. Groups of four bone slices were transferred to each of four 16-mm-diam wells of a Linbro plate containing 1.5 ml of MEM/FCS. 500 μl of calvarial cell suspension was added to two wells, and 500 μl of MEM/FCS was added to the remaining two wells. The resulting cultures were incubated for 24 h in the presence or absence of IL-1 (2 ng/ml).

Bone slices were fixed in 1% glutaraldehyde and stained with toluidine blue (10 mg/ml) for 45 s. Osteoclasts were identified as large multinucleate cells of complex morphology. All multinucleate cells were counted. Mononuclear cell density was estimated as the mean number of cells counted in 10 random fields at × 250 magnification using an eyepiece graticule.

To measure the volume of bone resorbed, the cells were removed from the bone slices by immersion in 10% NaOCl for 20 min. The slices were washed, dehydrated in ethanol, air dried, and sputter coated with gold. The specimens were inspected in a Cambridge S90B scanning electron microscope. The number of osteoclastic excavations were counted and the outline of each traced from the monitor. The area of each pit was calculated using a digitizing tablet input into an Apple IIe microcomputer. The depth of the first eight lacunae observed from each treatment and corresponding control group was measured by parallax change in the position of a point at the center of the lacunae in stereo paired photographs (tilt, 10°; magnification, × 2,000 [12]). The approximate total volume of bone resorbed was assessed from the total surface area of resorption lacunae and mean pit depth (13). Student's *t* test was used in tests for statistical significance.

For each of six experiments, the mean number and size of resorption lacunae produced on a group of four IL-1-treated bone slices were expressed as a ratio against a corresponding group of four control bone slices incubated in the absence of IL-1.

As additional controls, calvarial cells were settled onto bone slices as described above but without previous addition of osteoclasts, and were incubated with or without IL-1 for 24 h.

Effect of Other Cell Types on Osteoclastic Bone Resorption. Clonal osteoblast-like UMR 106 cells, grown to confluence on Nunclon 50-ml tissue culture flasks (Gibco, Uxbridge, United Kingdom) in MEM/FCS, were removed by 15 min incubation in 1 mM EDTA in Ca^{2+}-free PBS, washed, and resuspended (10^6 cells/ml) in MEM/FCS.

Skin fibroblasts were obtained by collagenase digestion of neonatal rat skin. 3 cm^2 of neonatal rat skin were incubated for 60 min in collagenase (1 mg/ml in medium 199), and the resulting cell suspension was washed twice and incubated in MEM/FCS in 60-mm Nunclon tissue culture dishes until confluent (3–7 d). Fibroblast suspensions were prepared by exposing these cells to 1 mM EDTA in Ca^{2+}-free PBS for 30 min before washing and resuspending in MEM/FCS (10^6 cells/ml).

For each of four experiments, osteoclasts were sedimented onto bone slices as above.

Groups of four bone slices were placed in three 16-mm-diam wells of a Linbro plate, each containing 1.5 ml MEM/FCS. 500 μl of UMR 106 cell suspension was added to one well, 500 μl of skin fibroblast suspension was added to the second well, and 500 μl of MEM/FCS to the third. The bone slices were incubated for 24 h, and cell numbers and bone resorption were quantified as above.

Effects of Other Cell Types on Osteoclastic Response to IL-1. For each of five experiments, osteoclasts were sedimented onto bone slices. Groups of four bone slices were placed into two 16-mm-diam wells of a Linbro plate containing 1.5 ml MEM/FCS. 500 μl aliquots of UMR 106 cell suspension were added to each well. IL-1 (1 ng/ml) was added to one 16-mm-diam well, while vehicle was added to the other well. As additional controls, 500 μl aliquots of UMR suspension were added to groups of four bone slices in the absence of osteoclasts, and in the presence or absence of IL-1 (1 ng/ml).

After incubation for 24 h, cell numbers and bone resorption were quantified as before. These experiments were repeated substituting skin fibroblasts for UMR cells, cultures being maintained for 24 h in the presence or absence of IL-1 (2 ng/ml for four experiments; 0.1 ng/ml in 6 experiments), with the numbers of cells and bone resorption quantified as before.

Sensitivity of Osteoclast–Calvarial Cell Cocultures to IL-1, and Effect of Indomethacin. Disaggregated osteoclast cultures were prepared by sedimenting osteoclasts onto bone slices as above. Groups of four bone slices with adherent osteoclasts were each placed into 16-mm-diam wells of a Linbro plate in 1.5 ml MEM/FCS, and 500 μl of calvarial cell suspension (10^6 cells/ml) were added. The plates were incubated for 24 h in the presence or absence of IL-1 (3 pg/ml to 2 ng/ml).

Similarly, four experiments were performed in which groups of four calvarial cell–osteoclast cocultures, prepared as above, were incubated in the presence of indomethacin (10 μg/ml), IL-1 (1 ng/ml), indomethacin (10 μg/ml) plus IL-1 (1 ng/ml), and in the absence of both these agents to investigate the role of prostaglandin synthesis in IL-1's action upon bone.

To test for the existence of a stable diffusible factor produced by calvarial cells in response to IL-1, for each of five experiments, osteoclasts were sedimented onto 16 bone slices in the well of a Sterilin 100 × 18-mm multiwell dish. Eight of the bone slices were then transferred to each of two 16-mm-diam wells of a Linbro plate containing 1.5 ml MEM/FCS, and the osteoclasts were permitted to spread for 1 h. 500 μl of calvarial cell suspension (prepared as above) were added to one well, 500 μl MEM/FCS to the remaining well, and the calvarial cells were allowed to settle for 6 h. Four bone slices with adhering mixed osteoclast and calvarial cell cocultures, and four bone slices with adherent disaggregated osteoclasts were carefully placed into each of two wells of a Sterilin 100 × 18-mm multiwell dish containing 2.0 ml MEM/FCS and incubated in the presence and absence of IL-1 (1 ng/ml) for 24 h before cell counting and quantification of bone resorption as before.

Results

Bone slices incubated with preparations of disaggregated osteoclastic cells alone showed a sparse population of (multinucleate) osteoclasts (5 ± 0.8 osteoclasts per bone slice, mean ± SEM; 55 cells/cm^2) and mononuclear cells (mean cell density, 3 ± 0.5 × 10^3 cells/cm^2). Such populations showed no response to IL-1, as assessed by the numbers of resorption lacunae formed, their mean volume, or the total bone resorbed per slice (Fig. 1).

Calvarial cells, UMR 106 and fibroblastic cell suspensions settled onto bone slices adhered within a few hours and formed contiguous sheets of well-spread cells. Cell densities are given in Table I. None of these mononuclear cells altered the level of osteoclasis in mixed mononuclear cell–osteoclast cocultures as compared to that produced by disaggregated osteoclasts alone. However, in the

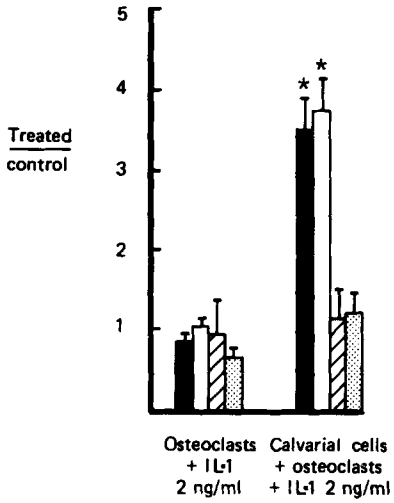

FIGURE 1. Effect of IL-1 on bone resorption by disaggregated osteoclasts and calvarial cell–osteoclast cocultures after 24 h of incubation. Results expressed as ratios (mean ± SEM) of bone resorption in the presence of IL-1 (2 ng/ml) to bone resorption in the corresponding IL-1-free control group (six experiments). Black bars, total volume of bone resorbed per bone slice; open bars, total plan surface area of excavations; crosshatched bars, mean excavation depth; stippled bars, mean plan excavation area. Asterisks denote groups with $p < 0.01$ Student's t test.

TABLE I

Dose- and Mononuclear Cell–dependence of IL-1-induced Bone Resorption by Disaggregated Osteoclast Populations

Cell types cocultured	IL-1 treatment (pg/ml)	Number of experiments	Number of excavations per bone slice (treated/control ± SEM)	Total plan area resorbed per bone slice (treated/control ± SEM)
Calvarial cells + osteoclasts	3	4	1.1 ± 0.13	1.2 ± 0.18
	10	4	1.5 ± 0.23	1.3 ± 0.24
	30	5	1.6 ± 0.1	1.8 ± 0.28*
	100	4	2.0 ± 0.23*	2.4 ± 0.12§
	1,000	4	2.8 ± 0.36‡	3.8 ± 0.38§
	2,000	5	2.7 ± 0.5*	3.8 ± 0.42§
UMR 106 + osteoclasts	1,000	7	3.9 ± 0.48‡	3.23 ± 0.65*
Fibroblasts + osteoclasts	100	6	0.82 ± 0.1	0.76 ± 0.17
	2,000	4	1.03 ± 0.49	1.24 ± 0.59

Results are expressed as ratios (mean ± SEM) of bone resorption over a 24-h incubation period, in groups of four mixed mononuclear cell–osteoclast cocultures in the presence or absence of IL-1. Mononuclear cell densities: calvarial cell–osteoclast, $34 \pm 4 \times 10^3$ cells/cm^2; fibroblast-osteoclast, $49 \pm 3 \times 10^3$ cells/cm^2; UMR 106–osteoclast, 60×10^3 cells/cm^2

* $p < 0.05$.

‡ $p < 0.01$.

§ $p < 0.005$, Student's t test.

TABLE II

Number, Plan Area, Depth, and Volume of Excavations Formed by Mixed Mononuclear Cell–Osteoblast Cocultures During 24-h Incubation Period in Presence or Absence of IL-1

Incubation conditions	Excavations per slice (mean ± SEM)	Plan area (μm^2) of individual excavations (mean ± SEM)	Total plan area ($\mu m^2 \times 10^{-3}$) resorbed per bone slice (mean ± SEM)	Mean excavation depth (μm ± SEM)	Total volume ($\mu m^3 \times 10^{-3}$) of bone resorbed per bone slice (mean ± SEM)
Calvarial cells + osteoclasts + IL-1 (1 ng/ml)	23 ± 2.7*	928 ± 99	22 ± 3.4*	5.7 ± 0.8	84.4 ± 12.3*
Calvarial cells + osteoclasts + vehicle	7.5 ± 1.2	761 ± 86	5.6 ± 1.1	5.0 ± 0.8	18.5 ± 3.6
UMR106 + osteoclasts + IL-1 (1 ng/ml)	12.4 ± 1.6*	1,433 ± 109	17.5 ± 2.4*	5.1 ± 0.9	59.5 ± 8.3*
UMR106 + osteoclasts + vehicle	3.5 ± 0.7	1,463 ± 261	4.3 ± 0.9	5.3 ± 1.2	15 ± 3.0
Fibroblasts + osteoclasts + IL-1 (1 ng/ml)	5.0 ± 0.8	1,154 ± 344	3.4 ± 0.7	4.8 ± 2.9	10.9 ± 2.1
Fibroblasts + osteoclasts + vehicle	5.2 ± 1.1	1,025 ± 327	5.4 ± 1.8	3.8 ± 1.2	13.4 ± 4.6

* $p < 0.0125$; Student's t test.

presence of calvarial cells or UMR 106 cells, disaggregated osteoclasts responded to IL-1 at ≥30 pg/ml, showing a dose-dependent, statistically significant increase in the total area of bone resorbed (Table I). None of the mononuclear cell populations incubated without mechanically-disaggregated osteoclasts showed any morphological evidence of bone resorption. These results indicate that addition of such osteoblastic cells confers IL-1 responsiveness upon otherwise nonresponsive populations of disaggregated osteoclasts (Fig. 1 and Table II).

In contrast, osteoclast–skin fibroblast cocultures failed to respond to IL-1 with increased bone resorption when compared to IL-1-free control cultures (Tables I and II).

Indomethacin (10 μg/ml) did not alter the level of osteoclasis produced by mixed calvarial cell–osteoclast cocultures, nor did it modify the stimulation of bone resorption induced in these cultures by IL-1 (1 ng/ml) (Table III).

Mixed calvarial cell–osteoclast cocultures and disaggregated osteoclast cultures were maintained in the same culture volume, in the presence and absence of IL-1 (1 ng/ml), so that the disaggregated osteoclast cultures would be exposed to any stable diffusible factors released in response to IL-1 by calvarial cells. Calvarial cell–osteoclast cocultures treated with IL-1 responded with a fourfold stimulation of bone resorption when compared to non-IL-1-treated controls. In contrast, disaggregated osteoclast cultures from IL-1-treated wells (in the same dish with, but separated by 1 mm from bone slices upon which were calvarial cells and osteoclasts) displayed no increase in resorption compared to non-IL-1–treated controls (Table IV).

The osteoclasts in our cultures excavate clearly delineated resorption lacunae. Fig. 2 shows the distribution of the proportion of pits of a given surface area. This distribution was unaltered when total bone resorption was stimulated by IL-1. Fig. 1 and Table II indicate that the mean surface area and depth of resorption lacunae were uninfluenced by IL-1. The increased resorption induced by IL-1 appears therefore to result from increased numbers of resorption lacunae.

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TABLE III

Effect of Indomethacin (10 μg/ml) on IL-1 (1 ng/ml) Responsiveness of Calvarial Cell–Osteoclast Cocultures

Culture conditions compared	Number of excavations per slice	Plan area of individual excavations	Total plan area resorbed per slice
Indomethacin vs. vehicle	0.94 ± 0.1	1.04 ± 0.2	1.1 ± 0.25
IL-1 vs. vehicle	2.9 ± 0.34	1.19 ± 0.2	3.8 ± 0.46
IL-1 + indomethacin vs. vehicle	2.6 ± 0.33	1.42 ± 0.12	4.0 ± 0.5
IL-1 + indomethacin vs. IL-1	0.92 ± 0.06	1.37 ± 0.19	1.06 ± 0.15

Results expressed as ratios (treated/control, mean ± SEM) of bone resorption by groups of four bone slices to bone resorption by appropriate control groups of bone slices in four experiments.

TABLE IV

Number and Plan Area of Excavations Formed by Disaggregated Osteoclast and Mixed Calvarial Cell–Osteoclast Cocultures Incubated in the Same Volume in Presence and Absence of IL-1

Incubation conditions	Excavations per bone slice (mean ± SEM)	Plan area (μm^2) of individual excavations (mean ± SEM)	Total plan area (μm^2) resorbed per bone slice (mean ± SEM)	Number of bone slices
Osteoclasts + vehicle	5.6 ± 1.0	1,229 ± 207	5,451 ± 1,200	20
Calvarial cells + osteoclasts + vehicle	3.4 ± 0.55	1,334 ± 151	4,450 ± 875	20
Osteoclasts + IL-1 (1 ng/ml)	3.7 ± 0.7	1,201 ± 221	3,582 ± 796	20
Calvarial cells + osteoclasts + IL-1	21 ± 2.1*	1,484 ± 189	20,293 ± 4,086*	21

* $p < 0.001$ Student's t test.

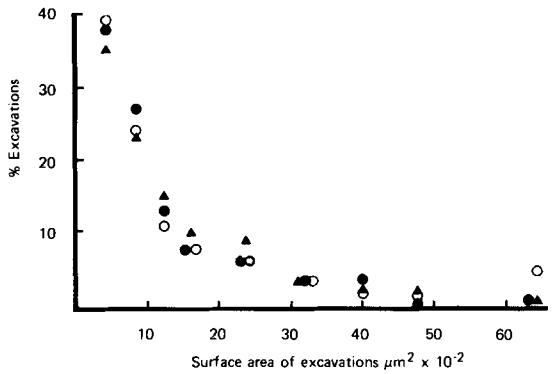

FIGURE 2. Effect of IL-1 (1 ng/ml; 3 pg/ml) on the size distribution of osteoclastic excavations formed by mixed calvarial cell–osteoclast cultures. ▲, IL-1, 1 ng/ml; ●, IL-1 3 pg/ml; ○, vehicle.

Discussion

We have found that IL-1 does not stimulate resorption by osteoclasts disaggregated from bone and widely dispersed at low cell density. However, in the presence of calvarial cells or cloned osteosarcoma cells, IL-1 effected a 3.8 ± 0.38–fold increase in osteoclastic bone resorption, with statistically significant enhancement at concentrations of ≥30 pg/ml. Since the osteoblast-containing populations themselves did not resorb bone, even in the presence of IL-1, these results imply that osteoblastic cells are induced by the presence of IL-1 to stimulate osteoclastic bone resorption.

The mechanism by which osteoblastic cells stimulated osteoclasts did not seem to depend on PG synthesis; nor could we detect a diffusible substance in the medium of stimulated cocultures. Osteoclastic stimulation was observed only on bone slices on which osteoblasts and osteoclasts were both present, and this indicates a short-range diffusible mediator or a contact-dependent interaction.

Skin fibroblasts were unable to duplicate the osteoblast-mediated IL-1 stimulation of osteoclastic bone resorption. We do not know whether this failure was in the afferent or efferent limb of the IL-1 response. However, osteoblasts and osteoclasts are closely associated on bone surfaces in intact bone, and in view of the short-range nature of the interaction we have demonstrated, the degree of specificity for the interaction between osteoblasts and osteoclasts in the presence of IL-1 may owe less to specificity of action on a given cell type and more to close spatial juxtaposition of the interacting cells.

Increased resorption in the presence of IL-1 was effected by an increase in the number of excavations, without significant increase in the volume of each excavation. One explanation for the increased number of excavations may be induction by IL-1 of an increased proportion of osteoclasts to resorb bone. Another, which would also account for the failure of IL-1 to increase mean pit volume, is that IL-1 may induce less mature, smaller or even mononuclear osteoclastic cells to resorb bone: some of the mononuclear cells in disaggregated osteoclastic populations do show phenotypic similarities with osteoclasts (11), but these cells are not at present readily distinguished from nonosteoclastic mononuclear cells. Alternatively, osteoclasts may be induced by IL-1 to undertake multiple episodes of bone resorption. We previously noted in time-lapse observations that osteoclastic motility tends not to be constant, but intermittent, with episodes of relative quiescence interspersed by bursts of cytoplasmic motility (14). This episodic behavior may account for the multiple discrete excavations frequently observed after incubation of osteoclasts on bone (15, 16), and IL-1 may act by reducing the time interval between episodes of osteoclastic resorptive activity. It is of interest that the dominant effect of inhibitors of bone resorption on disaggregated osteoclasts was also large accounted for by a change in the number of excavations induced, with little or no decrease in the volume of bone resorbed in each concavity (7).

The extreme sensitivity of osteoblast-osteoclast cultures to the presence of IL-1 suggests that the agent may play a role in bone resorption in vivo. Our results seem to exclude a role of IL-1 as a mediator of osteoblastic stimulation of osteoclastic resorption. Because IL-1 is produced by macrophages and induces bone resorption, it may be instrumental in the local osteolysis of inflammatory

disease. However, it is difficult to discern a clear adaptive role for local osteolysis in inflammation; this may not be its primary role. One role of IL-1 appears to be as an agent of the systemic host response to inflammatory and injurious stimuli: it is pyrogenic (17–19), and is associated with generalized skeletal muscle catabolism in fever (20). Enhanced bone resorption, by effecting a shift in systemic skeletal homeostasis towards catabolism, may be another aspect of the role of IL-1 in the adaptive host response to injury. Another theoretical possibility is that macrophages play a physiological role, through local IL-1 production, in the control of bone remodelling. However, cell types other than mononuclear phagocytes, and including periosteal fibroblasts, are known to produce cytokines with IL-1-like properties (21, 22), and a further possibility is that IL-1 may be produced by bone cells as one of the local mediators of the physiological interactions of bone cells. Inflammatory osteolysis may then be explicable as a perturbation of normal physiological mechanisms through the immigration into bone of inflammatory cells that similarly use IL-1 as a local mediator of cell-cell interactions. Resolution of these various possibilities requires identification of the bone cell type that produces IL-1, and of the control of its production in response to environmental agents.

Summary

A monocyte-derived factor with IL-1-like properties has recently been shown to cause resorption of bone in organ culture. We have investigated the action of IL-1 on disaggregated populations of osteoclasts, incubated alone or in the presence of osteoblastic cells, in an attempt to identify the target cell for IL-1 in bone, and to elucidate the mechanism by which IL-1 induces osteoclastic resorption. Osteoclasts were disaggregated from neonatal rat long bones and incubated on slices of human femoral cortical bone. Under these conditions, the majority of osteoclasts form distinctive excavations in the bone surface within 24 h, the volume of which can be quantified by computer-assisted morphometric and stereophotogrammetic techniques. IL-1 had no effect on bone resorption by osteoclasts alone, but when incubated in the presence of calvarial cells or cloned osteosarcoma cells, it induced a 3.8 (±0.38)–fold increase in osteoclastic bone resorption, with significant enhancement at concentrations of ≥30 pg/ml. The osteoblastic populations themselves did not resorb bone.

The mechanism by which osteoblastic cells stimulate osteoclasts did not appear to depend upon PG synthesis; nor could we detect a diffusible substance in the medium of stimulated cocultures. These results indicate that IL-1 stimulates bone resorption through a primary action on osteoblasts, which are induced by IL-1 to transmit a short-range signal that stimulates osteoclastic bone resorption.

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