Prostaglandin E₂, Interleukin 1α, and Tumor Necrosis Factor-α Increase Human Osteoclast Formation and Bone Resorption in Vitro*

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ABSTRACT

Prostaglandin E₂ (PGE₂) and the cytokines interleukin (IL) 1α and tumor necrosis factor (TNF)α increase bone resorption in vivo, but the effect of these agents on osteoclastic bone resorption has never been studied in an in vitro human system. Our recently described human bone marrow culture system, in which osteoclasts are generated (vitronectin and calcitonin receptor-positive cells which resorb bone), was used to study the effects of these agents. Addition of indomethacin to macrophage colony-stimulating factor (M-CSF)-treated cultures nearly abolished osteoclast parameters, indicating that prostaglandins are virtually essential for human osteoclast formation. Additionally, PGE₂ dose responsively increased osteoclast numbers and bone resorption. The effects of M-CSF and PGE₂ are independent, as demonstrated by unaltered PGE₂ concentrations in culture supernatants in spite of the dose-responsive increase in osteoclast parameters in response to M-CSF. The generation of osteoclasts in the presence of PGE₂ occurred in favor of CD 14-positive macrophage formation. IL 1α and TNFα increased osteoclast parameters in a dose-responsive manner. Maximum stimulation yielded culture supernatant levels of PGE₂ approximately the same as those concentrations of exogenous PGE₂ that dramatically induced osteoclast formation. This osteoclast-inducing effect was inhibited both by indomethacin and by the specific inhibitor of inducible prostaglandin G/H synthase, NS-398, and this was reversed by addition of exogenous PGE₂. These results demonstrate unequivocally that IL 1α and TNFα enhance human osteoclast formation and suggest that they mediate their effects through PGE₂. (Endocrinology 139: 3157–3164, 1998)
cytokines in our human BM assay, in the absence of M-CSF (17); and although others have reported increased numbers of osteoclast-like cells in response to these agents, bone resorption was not assessed in these experiments (26). The failure of these factors to enhance human bone resorption in vitro might be caused by the regulation of human bone resorption differing from that in the murine species. We therefore elected to study the effect of these cytokines in our human osteoclast-forming culture system.

Materials and Methods

Human BM cultures

Osteoclasts were generated from human BM in a two-phase culture system, as previously described (17, 18). In brief, BM was aspirated, under 1% lignocaine local anesthesia, from the posterior iliac crest of healthy volunteers between the ages of 30 and 75 yr (approved by St Mary’s Hospital Medical ethics committee) into preservative-free heparin (CP Pharmaceuticals Ltd. Wrexham, UK). Marrow mononuclear cells were isolated by centrifugation over ficoll/hypaque density gradients (Sigma Chemical Co., Poole, Dorset, UK) and resuspended in phenol red-free RPMI medium (Sigma) supplemented with 10% inactivated newborn calf serum (Harlan Sera Lab. Ltd., Sussex, UK), l-glutamine (2 mM), 100 IU benzylpenicillin per ml (Gibco Life Technologies, Paisley, Scotland, UK), 100 μg streptomycin per ml (Gibco), and 10⁻⁶ M hydrocortisone (Sigma Chemical Co.) and were incubated in tissue culture flasks in the presence of M-CSF (10 ng/ml; Genetics Institute, Boston, MA) at 37°C in a humidified atmosphere of 5% CO₂/95% air for 10–14 days (phase I). The BM cells were fed weekly by replacing half the medium. After this period, the cells were harvested from the flasks, using trypsin (Sigma), and 10⁷ mononuclear cells per well were sedimented onto devitalized bone slices (3 × 3 × 0.1 mm) in a 96-well plate (Corning Glass Works) and were cultured in the presence of M-CSF at 25 ng/ml for 10 days (phase II). The effect of PGE₂ (Sigma; 10⁻⁹–10⁻⁶ M), IL 1α, and TNFα (Genetics Institute; 0.01–10 ng/ml) were tested by adding them to phase II of the cultures in the presence and absence of indomethacin (10⁻⁶ M; Sigma) and PG endoperoxide synthase (PGHS-2) inhibitor, NS-398 (4 × 10⁻⁶ M; ICN Biomedicals Ltd, Thame, UK).

The cells on the bone slices were fed by replacing half the medium every 3–4 days. At the end of the experiment, the bone slices were fixed in acetone (Analar, Hayman Ltd., Essex, UK) and air dried. Osteoclasts were quantitated by counting the number of 23c6-positive cells, an antibody which preferentially stains osteoclasts (14, 15), kindly provided by Professor M. A. Horton, London. Macrophage numbers were assessed by labeling the cultures with an anti-CD 14 monoclonal antibody (Sigma). The entire surface of each bone slice was inspected, and the area of resorption was quantified by reflected light microscopy.

Labeling of cells with antibodies and iodinated (¹²⁵I) CT

Salmon CT (kindly provided by Rhone-Poulenc Rorer, Collegeville, PA) was iodinated using a modification of the chloramine T method (27). BM cultures were incubated in radiiodinated (¹²⁵I) CT (10 days after the cells were plated on bone slices, with or without excess unlabeled CT) and were washed, fixed, and stained with the antibody against the vitronectin receptor, 23c6, or against CD 14 (using conventional techniques) and were processed for autoradiography, as previously described (28). After development, the cultures were counterstained with toluidine blue.

Enzyme immunoassay (EIA) for PGE₂

PGE₂ concentration was determined using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI). Culture supernatants from all the cultures were collected onto ice at the end of the experiments. The supernatants from each treatment group were pooled, and the samples were subaliquoted before storage at -70°C. Samples were assayed in duplicate and at two dilutions, according to the manufacturer’s instructions. Briefly, the samples were added to the microtiter plates, which were supplied precoated with goat antinmouse polyclonal antibody.

Monoclonal anti-PGE₂ antibody and PGE₂ tracer were added to the wells, and the EIA plate was incubated at room temperature for 18 h, washed 5 times, and developed using Ellman’s reagent for 60–90 min. The plate was read at 420 nm, and PGE₂ concentration was calculated by reference to a standard curve. Values shown are the mean of the four values obtained for each sample. The inter- and intraassay coefficients of variance were less than 4%.

The results were analyzed using ANOVA, where significance was accepted as P < 0.05. Results are displayed as mean ± SEM

Results

As previously reported, the addition of M-CSF to human BM cultures induced osteoclast formation and bone resorption, the former being identified as cells expressing the α₁β₃ subunit of the vitronectin receptor using monoclonal antibody 23c6 (Fig. 1). This has been found to be a specific marker for the osteoclast phenotype (17, 18). The 23c6-positive cells were further confirmed as osteoclasts by labeling some experiments with [¹²⁵I]TsCT (double-labeled cells, 87 ± 0.9%) (Fig. 1, center panel). Bone resorption was also assessed (Fig. 1). In this study, we found that addition of the cyclooxygenase inhibitor, indomethacin (10⁻⁶ M) to M-CSF-treated human BM cultures completely suppressed osteoclast formation, and this was associated with suppression of bone resorption (Fig. 2). Taken together with the finding that indomethacin (10⁻⁶ M) reduced the concentration of PGE₂ in the culture supernatants to minimal levels (less than 50 pg/ml (limit of assay detection, 7.8 pg/ml) mean percent reduction in M-CSF-treated cultures, 82.25 ± 10%) (Fig. 2), these data suggest that the indomethacin-induced inhibition of osteoclast parameters was the result of reduced levels of PGE₂, thereby demonstrating that PGE₂ is essential for human osteoclast formation.

PGE₂ reversed the inhibitory effect of indomethacin and enhanced M-CSF-induced osteoclast formation (Fig. 2). At 10⁻⁹ M, PGE₂ resulted in osteoclasts being formed in very small numbers, less than that seen in the control (M-CSF) cultures. At 10⁻⁸ M, there was a dramatic 3-fold increase in osteoclast numbers above that observed in M-CSF-treated cultures. The 23c6-positive cells in the cultures treated with M-CSF alone or with M-CSF with PGE₂ (10⁻⁸ M) contained the same number of nuclei, with a range from 1–6 (M-CSF, 1.45 ± 0.76; n = 97 cells vs. M-CSF + PGE₂, 1.48 ± 0.85; n = 128 cells). The increase in 23c6-positive cells was paralleled by a greater area of bone being resorbed in these cultures. Higher concentrations of PGE₂ (10⁻⁷ and 10⁻⁶ M) did not further induce osteoclast formation (Fig. 2).

Because M-CSF has previously been shown to be critical for osteoclast formation, we wished to determine whether this effect was mediated via PGE₂. To achieve this, we added increasing concentrations of M-CSF (0.5–50 ng/ml) to phase II of our cultures, and we measured the PGE₂ concentration by EIA in the culture supernatants. As expected, M-CSF stimulated osteoclast formation and bone resorption in a dose-responsive manner (17), but there was no increase in the level of PGE₂ in these cultures (Fig. 3), indicating that these two local hormones mediate their effect on osteoclast formation independently.

To test the hypothesis that PGE₂ results in the preferential formation of osteoclasts over macrophages, we assessed the numbers of osteoclasts and macrophages that were formed...
in the presence of M-CSF with or without 10^{-6} \text{M} PGE_2 in the same human BM cultures. We found that M-CSF induced both osteoclast and macrophage formation, as well as bone resorption, above that seen in vehicle-treated cultures, whereas there was a significant reduction in the number of CD14-labeled macrophages in the presence of indomethacin and PGE_2. In contrast, the addition of PGE_2 caused a significant increase in the number of 23c6-positive cells, which was associated with the expected increase in bone resorption (Fig. 4).

Both IL 1\alpha and TNF\alpha increased the number of 23c6-positive cells in a dose-responsive manner in the presence of M-CSF. This was associated with an increase in the area of bone resorption (Figs. 5 and 6). A maximal response occurred in response to both cytokines at 1 ng/ml, with IL 1\alpha inducing a 5- to 10-fold increase in bone resorption and 23c6-positive cell numbers. TNF\alpha induced a 3- to 7-fold increase in bone resorption and 23c6-positive cells. The maximum level of
stimulation by the cytokines depended on the basal level of bone resorption in the experiment. BM experiments with high levels of basal osteoclast activity generally gave lower levels of stimulation, probably reflecting the variability in endogenous PGE2 between BM donors. Above this concentration, the parameters plateaued in the presence of IL-1α and showed a reduction in the presence of TNF-α. In the same experiments, we found that the increase in osteoclast parameters, in response to 0.1–10 ng/ml of IL-1α and 0.1 and 1 ng/ml of TNF-α, was associated with an increase in the levels of PGE2 in the culture supernatants at the end of the experiments (5, 6). The stimulation of bone resorption and PGE2 concentration, in response to 1 ng/ml of both cytokines, was confirmed by further experiments (Figs. 7 and 8).

As further confirmation that PGE2 is involved in the osteoclast-inductive effect of IL-1α and TNF-α, we added indomethacin to the IL-1α- and TNF-α-treated cultures. The increase in bone resorption in response to these cytokines (as originally demonstrated in Figs. 5 and 6) was virtually abrogated in the presence of indomethacin. The number of 23c6-positive cells followed the same pattern as bone resorption (data not shown). Addition of 10^{-7} M PGE2 to the TNF-α- or IL-1α indomethacin-treated cultures restored bone resorp-
tion levels to those in TNFα- or IL 1α-treated cultures (Figs. 7 and 8).

Because indomethacin is a relatively nonspecific inhibitor of both the constitutive and the inducible forms of cyclooxygenase, PGHS-1 and -2, respectively (29), we added NS-398 as a specific inhibitor of the inducible form. We found that NS-398 exerted an effect similar to that of indomethacin (Fig. 7), except that the observed reduction in PGE2 concentration was not as great, reducing PGE2 levels by 66 ± 4%, as opposed to indomethacin reducing PGE2 by 82.25 ± 10% in M-CSF-treated cultures (a difference of 16.25%, P = 0.036). In cytokine-treated cultures, the effects of indomethacin and NS-398 were inhibitory PGE2 concentration by 99.45 ± 0.15% and 97.73 ± 0.14%, respectively (a difference of 1.72%, P = 0.016). Dose response experiments for indomethacin and NS-398 showed that they have almost identical inhibitory effects on bone resorption in our cultures (data not shown). The increase in the supernatant concentration of PGE2 (in response to TNFα) and its suppression (in the presence of indomethacin and NS-398) provides additional evidence that increasing levels of cytokine are associated with elevated PGE2 production. These results further indicate that PGE2 is critical for osteoclast formation and bone resorption. Similar results were observed when IL 1α was substituted for TNFα (Fig. 8).

**Discussion**

We have found that PGE2 enhances osteoclast formation and bone resorption in M-CSF-treated human BM cultures. This provides strong evidence that this local hormone exerts a similar effect on osteoclast formation in both the human and murine species. Our finding is accounted for by an increase in osteoclast numbers, which are identified as cells expressing the vitronectin receptor (using monoclonal anti-
compared with M-CSF control. B, Effect of IL 1α on human osteoclast development and that they mediate their effects non-existent (9, 11). Further studies, however, are required to see if osteoclast formation and bone resorption are virtually nonexistent (9, 11). Our findings corroborate those of others, in that not only does PGE2 increase osteoclast formation but, in its absence of a rise in the levels of PGE2 largely excludes the possibility, as suggested by others (11), that the defect in the op/op osteopetrotic mouse mutant is caused by a failure to produce sufficient PGE2 from BM macrophages.

PGE2 has previously been found to inhibit bone resorption by the mature neonatal rat osteoclast (6, 8). It may therefore have been predicted that there would be less bone resorption per 23c6-positive cell in the presence of PGE2 compared with control cultures, as shown by Collins and Chambers (10) in murine osteoclast cultures. However, this was not the case in our experiments. It may be that PGs do not exert an inhibitory effect on human osteoclastic resorption activity. The alternative is that an effect was not detected because of the difficulty in assessing the quantity of bone resorbed per 23c6-positive cell in our cultures, because bone resorption may be the cumulative result of the activity of cells, which numbers vary during the culture period. To investigate this issue, it would be more appropriate to use the isolated mature human fetal osteoclast bone slice assay, just as we have done when we investigated the effect of M-CSF on mature human osteoclasts (30).

The osteoclastic response to PGE2 was not gradual; instead there was a very dramatic increase in osteoclast parameters, which occurred at 10^-5 M PGE2. This suggests that PGE2 concentration is a crucial means by which the effect of the hormone is controlled and, in particular, the means by which its action is contained locally. This provides insight into how PGE2 could exert different effects on the same cells in different microenvironments [it inhibits mature rat osteoclasts from resorbing bone (6, 8) and stimulates bone formation (31, 32)].

Figs. 8A and 8B. A, Effect of IL 1α (1 ng/ml) ± indomethacin (10^-6 M) ± PGE2 (10^-7 M) on bone resorption (n = 3) and PGE2 concentration in culture supernatants (n = 1) in phase II human BM cultures. P < 0.0001, compared with M-CSF control. B. Effect of IL 1α (1 ng/ml) ± NS-398 (4 x 10^-6 M) ± PGE2 (10^-7 M) on bone resorption in phase II human BM cultures (n = 3). Each experiment contains five or six bone slices per variable. P < 0.0001, compared with M-CSF control.

It has previously been suggested that PGs inhibit human osteoclast-like cell formation in vitro (13). One possible explanation for this data being apparently contradictory to ours is that the osteoclast-like cells in that report may have been macrophage polykaryons and not bone resorptive osteoclasts. We propose this because we have demonstrated, for the first time, that PGE2 reduces macrophage CD 14-positive macrophages in cells in culture where human osteoclast numbers and bone resorption are enhanced. Our finding implies that osteoclasts and macrophages share a common precursor, and this is consistent with reports showing that in c-fos knock-out mice (33) and NF-kB knock-mice (34), osteoclasts are absent but macrophage numbers are increased.

This is the first report demonstrating that IL 1α and TNFα enhance human osteoclastic bone resorption in vitro. There are several findings to indicate that the osteoclast-inductive effect of IL 1α and TNFα was mediated by PGE2, which itself induces osteoclast formation. First, the increase in osteoclast numbers and bone resorption that occurred in response to these cytokines correlated with increasing levels of PGE2. Second, PGE2 induced a dramatic increase in osteoclast parameters at 10^-8 M, which was approximately the same PGE2 concentration found in the cultures stimulated with 1 ng/ml of IL 1α or TNFα (10^-8 M) is approximately equal to 3.6 ng/ml; TNFα 1 ng/ml gives rise to a PGE2 concentration of 2.3 ng/ml, or 7 x 10^-9 M; IL 1α 1 ng/ml gives rise to a PGE2 concentration of 14.6 ng/ml, or 4 x 10^-8 M). Finally, the
indomethacin and NS-398 inhibition of osteoclast numbers and bone resorption in cultures treated with TNFα or IL1α was reversed by the addition of PGE2.

The elevation in osteoclast parameters in response to PGE2, whether this be in response to the addition of PGE2 or the cytokines IL 1α or TNFα, occurred dramatically at a concentration of approximately 10^{-8} M PGE2. In the presence of this concentration of PGE2, osteoclast parameters were increased up to 10-fold. Because PGE2 was only added at the beginning of phase II, and because we have previously shown that osteoclasts only start to develop on day 3 of this phase of the cultures, this suggests that PGE2 exerts its effect on osteoclast formation by exerting an effect on a late precursor, possibly inducing differentiation rather than proliferation. This proposal is consistent with the report from Marshall et al. (35), who showed, using bromo-deoxyuridine, that osteoclast formation stimulated by PGE2 was the result of differentiation and not proliferation.

PGE2 is synthesized by the constitutive and inducible cyclooxygenase enzymes PGHS-1 and -2, respectively. The former is expressed in many mammalian cells, whereas the latter is generally undetectable under physiological conditions but is induced by inflammatory cytokines. Therefore, it is consistent that the addition of indomethacin, a relatively nonselective inhibitor of cyclooxygenase activity, should reduce PGE2 concentration by 82.5% ± 10% in M-CSF-treated cultures. In contrast, NS-398, a specific PGHS-2 inhibitor that is 1,000 times more active on PGHS-2 than PGHS-1 (36), is consistent that the addition of indomethacin, a relatively nonselective inhibitor of cyclooxygenase activity, should reduce PGE2 concentration by only 66% in our cultures, implying that PGHS-2 was predominantly responsible for the PGE2 production in the M-CSF-treated cultures. The greater inhibition of PGE2 in the presence of cytokines, compared with cultures treated with only M-CSF, is caused by the increase in absolute PGE2 concentration by IL 1α and TNFα. The differential inhibition of PGE2 concentration by NS-398 and indomethacin probably reflects the inhibition of only the inducible form of the enzyme by NS-398. Suppression of PGE2 levels by NS-398 in cultures that have not been treated and indomethacin probably reflects the inhibition of only the inducible form of the enzyme.

It is interesting to speculate that the increased level of PGE2 in the culture supernatant, in response to IL 1α and TNFα in our cultures, is largely the result of up-regulation of PGH-2, just as it is in the neonatal mouse calvarial system (25). However, an alternative explanation is that the cytokines increase release of arachidonic acid that can subsequently be converted into PGE2.

Our in vitro results are consistent with the observations made in vivo that PGs are involved in the development of increased bone resorption in disuse osteopenia (37), and in some animal models of hypercalcemia of malignancy (38), and are associated with a small proportion of solid malignant tumors (39). However, apart from the work of Seyberth et al. (39), there is no convincing evidence that PGs reach levels in vivo that are capable of inducing hypercalcemia by inducing bone resorption. This suggests that there are PG-independent mechanisms by which bone resorption can be increased, and there is now evidence that tumors producing PTH-related peptides account for many cases of hypercalcemia of malignancy (40). Nevertheless, this does not exclude the importance of PGs in local osteoclast formation and bone resorption at tumor sites.

This is the first report to establish unequivocally that PGE2, IL 1α, and TNFα increase bone resorption in human BM cultures, and it provides a sound platform on which to base further experiments. Continued exploitation of this assay, including analysis of the expression of PGHS-1 and -2 mRNA and protein in human BM before culture and the effect of proinflammatory cytokines on their expression in vitro, will allow elucidation of the mechanisms by which local hormones interact to regulate human osteoclastic bone resorption in health and disease.

References

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