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Bisphosphonates Induce Osteoblasts to Secrete an Inhibitor of Osteoclast-Mediated Resorption*

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ABSTRACT

Current knowledge indicates that osteoblasts play an integral role in osteoclastic bone resorption through an osteoclast-stimulating activity produced by osteoblasts in response to resorption-promoting osteotropic factors. Previously, we have shown that the inhibitory action of the bisphosphonates on bone resorption in part is mediated by osteoblasts. The aim of the present study was to investigate whether the bisphosphonate-generated inhibition is due to these compounds decreasing the synthesis of the osteoclast-stimulating activity or is the result of osteoblasts synthesizing an osteoclast resorption inhibitor.

Using the osteoblastic cell line CRP 10/30, which produces osteoclast-stimulating activity, constitutively and employing isolated rat osteoclasts cultured on ivory, evidence was obtained indicating that the bisphosphonates ibandronate and alendronate at a concentration of 10^{-7} M induce osteoblasts to synthesize an osteoclast inhibitor that reduces pit formation by more than 50%. The inhibitor is heat and proteinase labile and has a molecular mass between 1–10 kDa. The reduction of resorption pits is paralleled by a decrease in tartrate-resistant acid phosphatase-positive mono- and multinucleated cells, whereas the mean area resorbed per pit was not changed, suggesting that the inhibitor affects osteoclast formation and/or survival and probably not the osteoclast resorption activity. Rat preosteoblastic cells and rat dermal fibroblasts were found not to produce the inhibitor.

In conclusion, osteoblasts aside from their role of mediating osteoclastic resorption promoters are also involved in inhibiting bone resorption through the synthesis of an osteoclast resorption inhibitor. (*Endocrinology* **137:** 2324–2333, 1996)

BISPHOSPHONATES are analogs of pyrophosphate with a potent inhibitory effect on bone resorption (1). They represent a class of compounds that has been developed for diagnostic and therapeutic use in various disorders of bone and calcium metabolism (see review in Refs. 2 and 3). Their main therapeutic use has been in Paget's disease (see review in Ref. 4), tumoral bone disease (see review in Ref. 5), and, recently, osteoporosis (6–9).

The mechanism by which bisphosphonates inhibit osteoclast-mediated bone resorption, however, is not yet completely clear. Because of their strong chemical affinity with the bone mineral surface (10–12), the inhibiting action of bisphosphonates has primarily been attributed to metabolic damage of actively resorbing osteoclasts after ingestion of these compounds bound to bone (5). This mode of action was supported by data showing that osteoclasts acquire a degenerative appearance and change in function not only *in vivo* (13) but also *in vitro* after culture of cells on bisphosphonate-coated bone particles (14–16). Additional evidence in favor of the above mechanism was provided by the demonstration that alendronate accumulates *in vivo* densely under the osteoclast (17). Interestingly, however, this study and others have shown that the effectiveness on resorption of structurally different bisphosphonates when added to bone mineral *in vitro* does not reflect their relative potencies obtained *in vivo* (18, 19).

Recently, we were able to show using the *in vitro* resorption pit assay that a coculture of isolated osteoclasts with osteoblasts pretreated with bisphosphonate results in an inhibition of osteoclastic resorption (20). The potency corresponded well with the activity observed *in vivo* (19) when five compounds with an activity range of 1–1000 were used. The finding that bisphosphonates act via the osteoblast has recently been confirmed by two other laboratories (21, 22). Additional evidence showing that the bisphosphonate inhibition of resorption may require the presence of osteoblasts was demonstrated with the recent finding that bisphosphonates inhibit interleukin-6 production, a cytokine implicated in osteoclastogenesis (23). These studies and the data on the elaboration of an inhibitor of osteoclast resorption by estrogen-treated osteoblasts (24) strongly suggest that osteoblasts, apart from acting as mediator for resorption-promoting osteotropic factors such as PTH (25), 1,25-dihydroxyvitamin D_3 (26), interleukin-1 (27), or tumor necrosis factor-α and -β (28), evidently also act as intermediaries for substances that reduce osteoclastic bone resorption. The mechanism by which the resorption-promoting substance may operate is thought to involve the synthesis of a soluble factor(s) in response to above osteotropic factors (29, 30).

The objective of the present study was to study by which mechanism osteoblasts mediate the inhibitory effect of bisphosphonate on bone resorption. There may be two possible operational modes: bisphosphonates may inhibit the

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synthesis of an osteoclast-stimulating factor(s), or the drug may induce osteoblasts to synthesize an inhibitor(s) that act(s) either by reducing osteoclast resorption activity or by interfering with the development of the osteoclasts.

The results of the present study show that bisphosphonates induce osteoblasts to secrete an inhibitor that acts on osteoclast formation and/or survival, rather than on osteoclast resorption activity.

Materials and Methods

Materials

Medium 199 with Hanks' salts and 20 mM HEPES (M199), MEM with Earle's salts (MEM; 2.2 g $NaHCO_3$/liter), and gentamicin were purchased from Life Technologies (Basel Switzerland). The bisphosphonate ibandronate [1-hydroxy-3-(methylpentylamino)-propylidene-bisphosphonic acid] was a gift from Boehringer Mannheim (Mannheim, Germany), whereas alendronate (4-amino-1-hydroxy-butylidenebisphosphonic acid) was a gift from Merck Research Laboratories (Rahway, NJ). Stock solutions of bisphosphonates were prepared by dissolving them in 0.9% NaCl at a concentration of 10^{-3} M. The pH of the individual solutions was adjusted to 7.4 with NaOH. Plastic tissue culture plates were purchased from Falcon, Becton Dickinson (Plymouth, UK). Trypsin and bovine PTH (synthetic fragment 1–34) were obtained from Sigma Chemical Co. (Buchs, Switzerland). Radiolabeled AMP and [2,3-^{3}H]proline (20–44/nmol) were purchased from New England Nuclear Corp. (Boston, MA).

Isolation and culture of osteoclasts

Osteoclasts were isolated from femurs of 1-day-old rats (Wistar) as outlined previously (31). Briefly, after killing the animals, femurs were dissected and freed of adherent soft tissue. Thereafter, the bones were cut across the epiphysis, and the marrow was removed using a size 10 dental needle (100 μm in diameter). The femurs were then placed in a dish containing 1 ml M199 supplemented with 0.5% gentamicin. Osteoclasts were gently released from the femurs, using in succession calibrated dental needles of sizes 20 and 30. With four femurs, this procedure took no longer than 10 min. The resulting osteoclast suspension from 4 femurs was brought to a volume of 2 ml with M199. Then, 500 μl of the cell preparation were added to 8 ivory slices kept individually in plastic wells (2.0 × 1.0 cm). After 25-min incubation at 37 C and 5% CO_2-air, nonadherent cells were removed by lateral agitation. Then, 8 slices for the control group and 8 for each treated group were individually transferred into single wells of a 24-well plastic tissue culture plate to which 500 μl of either control or test medium were added. The cultures were carried out in MEM for 24 h at 37 C and 5% CO_2-air.

Cultivation of osteoblasts and preparation of conditioned medium

Normal clonal osteoblast-like cells CRP 10/30 established from 1-day-old rat calvariae (Wistar) (32) were plated at a density of 10^4 cells/cm^2 in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA) and grown to confluence in MEM (Earle's) supplemented with 10% FBS and 1% penicillin/streptomycin. Media were changed every third day. At confluence, media were removed, and cells were washed twice with 10 ml PBS before being incubated in serum-free MEM containing the indicated bisphosphonate or vehicle solution. Unless stated otherwise, the bisphosphonate ibandronate at a concentration of 10^{-7} M was used in most of the experiments. The reason for using ibandronate at this dose is based on a previous study, in which it was determined that, maximal inhibition by ibandronate of osteoclastic resorption was achieved at a concentration of 10^{-7} M (20). After 5-min incubation at 37 C in the presence of 5% CO_2/air, media were removed, and cells were washed twice with 15 ml MEM. Then, fresh serum-free MEM was added, and cells were cultured for an additional 24 h. Resulting conditioned media were collected, centrifuged at 2000 × g, and kept at −15 C until use. Before being tested, each conditioned medium was concentrated to one tenth its volume by ultrafiltration using an Amicon device (Amicon Corp., Lexington, MA) equipped with a YM1 membrane (M_r cut-off, 1 kDa). The filtration was carried out with a pressure of 4 psi using a gas mixture of 1% CO_2-air. Concentrated samples were then reconstituted with fresh MEM to its initial volume. This procedure was used to prevent conditioned medium that might have become deficient in nutrients and other cell maintenance substances to affect the viability of the osteoclasts in the pit assay.

Isolation and cultivation of fibroblasts

Skin from 1-day-old rats (Wistar) was dissected; freed of facia, fat, and blood vessels; and subsequently minced and placed into 15 ml DMEM containing 3 mg collagenase and incubated for 1 h at 37 C under 5% CO_2-air. Cells released during this period were passed through a Nitex filter, and the filtrate was centrifuged for 7 min at 400 × g. The resulting cell pellet was resuspended in 5 ml DMEM and plated for immediate use or kept in liquid nitrogen for future operations.

Preparation of ivory slices

Elephant ivory (kindly obtained from Dr. B. Irrall, Bundesamt für Veterinärwesen, Berne, Switzerland) was used as mineral substrate to assess osteoclast resorption activity. The ivory was cut into 4 × 4 × 0.1-mm slices with a Isomet low speed saw (Buehler Instrument, Evanston, IL). Resulting slices were cleaned by ultrasound for 30 sec in deionized water. Thereafter, the pieces were air dried, gas sterilized, and subsequently degassed under vacuum for 24 h.

Measurement of osteoclast resorption activity

After a culture period of 24 h, the ivory slices were freed of adherent cells by ultrasound in 70% propanol. Thereafter, the slices were washed, air-dried, and subsequently sputter-coated with gold (SCD 004 Coater, Balzers, Lichtenstein). The number of resorption pits on each ivory slice was counted using a light microscope equipped with a tangential light at a magnification of ×200. A pit was defined as a depression in the ivory surface with a continuous rim and an area of at least 250 μm^2. Osteoclast resorption activity in most cases is expressed as the number of excavation per eight slices, which corresponds to a surface of 128 mm^2. To ensure that the pit analysis was performed unbiased, the reader was blinded. In the majority of cases, pits were counted independently by two people.

Pit areas were calculated from pit images that were captured by a camera attached to the reflected light microscope with the aid of image analysis software (NIH Image).

Determination of tartrate-resistant acid phosphatase (TRAP)-positive (TRAP$^+$) cells

The determinations of TRAP$^+$ mononuclear (MoNC) and multinucleated cells (MNC) were carried out as previously reported (33).

Heat and proteinase K treatment of conditioned medium

To examine whether the inhibiting activity is heat resistant, a 10-fold concentrated conditioned media of untreated and bisphosphonate-treated CRP 10/30 cells was subjected to boiling for 10 min. After heating, the samples were reconstituted to the original volume with fresh MEM and subsequently tested in the resorption pit assay against the osteoclast-stimulating activity of 10% FBS.

To determine whether the inhibiting activity resists proteolytic degradation, inhibitor containing conditioned medium was subjected to proteinase K treatment. Briefly, one part each of conditioned medium of bisphosphonate-treated and untreated (control) CRP 10/30 cells after concentrating to one tenth of their original volume and subsequent reconstitution with fresh MEM was used to determine whether conditioned medium of bisphosphonate-treated cells contains inhibitory activity on TRAP$^+$ MNC formation. Another part of the conditioned medium was used to separate the osteoclast-stimulating activity from the osteoclast inhibitor, which was achieved with a PM 10 filter (M_r cut-off,

10 kDa). Thereafter, the ultrafiltrates containing the resorption inhibitor and the retentate containing the osteoclast-stimulating activity were examined for $TRAP^+$ MNC formation. Finally, one part of the inhibitor containing the ultrafiltrate fraction was incubated with 1 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) for 2 h at 37 C. After the incubation, the medium was reconstituted to the original volume with fresh MEM. Before assaying for inhibitory activity, the proteinase K was removed from the test samples by subjecting the samples once again to ultrafiltration using a PM 10 filter with a 10 kDa molecular mass cut-off.

Cell proliferation

Cell proliferation was determined by cell count, employing a Coulter counter (Coulter Electronics, Dunstable, UK).

Alkaline phosphatase and cellular response to PTH

Alkaline phosphatase activity was measured according to a previously described method (34). To examine the effect of PTH on control and bisphosphonate-treated CRP 10/30 cells, they were challenged with bovine PTH (10^{-8} M). The effect of the hormone on adenylate cyclase activity was determined according to a method detailed previously (35).

Determination of collagen synthesis

After having reached confluence, media of control as well as bisphosphonate (10^{-7} M ibandronate)-treated CRP 10/30 cells were changed and replaced with medium containing 1% FBS, 50 μg/ml ascorbic acid, 100 μg/ml β-aminopropionitril fumarate, and 10^{-7} M ibandronate. After 18-h incubation, 10 μCi/ml [^{3}H]proline were added to the cultures. Incorporation of radiolabeled proline into newly synthesized collagen was allowed to occur for the last 6 h. Preparation of samples and calculations to quantify the synthesis of collagen were conducted as detailed previously (36).

Statistical analysis

In each experiment, a single osteoclast suspension from the same animal pool was used for treatment and control groups. Thus, one value represents the total number of pits found on eight slices (128 mm^2). In experiments for which results are given as a percentage of the control value, the difference between control and treated groups is given by a one-column test, assuming a sampling from a Gaussian population, with the hypothetical value set at 100%.

Results

Effect of medium conditioned by untreated and bisphosphonate-treated osteoblastic cells on pit formation by isolated osteoclasts

Treatments of isolated osteoclasts with medium conditioned by the osteoblastic cell line CRP 10/30 resulted in an average nearly 5-fold stimulation of osteoclast-mediated pit formation compared to that in cells treated with plain MEM (Fig. 1a), thus confirming an earlier study, which demonstrated that CRP 10/30 cells produce constitutively a factor with osteoclast-stimulating activity (33). When the osteoblastic cells were pretreated for 5 min with 10^{-7} M bisphosphonate, the resulting conditioned medium from cells cultured for 24 h in absence of the bisphosphonate produced a more than 50% ($P < 0.05$) reduction of pits compared to medium conditioned by control (untreated) cells. Experiments undertaken to evaluate whether a longer than 5-min exposure of osteoblasts to bisphosphonates would increase the osteoclast-inhibiting activity revealed that although a slight increase in activity was measured after a 10-min treatment, a 30- or 60-min exposure of CRP 10/30 cells to bisphosphonate did not result in a further increase in activity (data not shown).

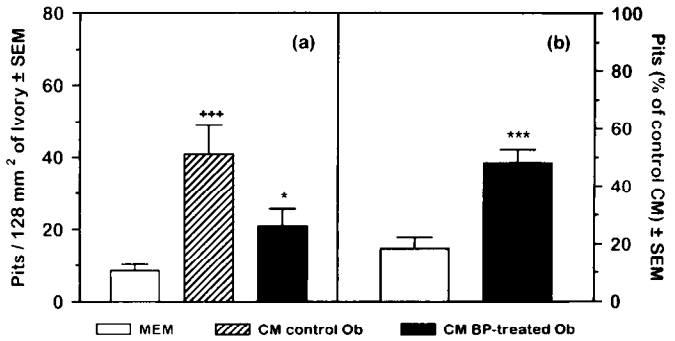

FIG. 1. Effects of MEM and conditioned media of untreated (control) and bisphosphonate (ibandronate)-treated osteoblastic CRP 10/30 cells on pit formation of isolated rat osteoclasts. (a), Average number of pits found on 128 mm^2 of ivory surface. (b), Data are presented as the number of pits expressed as a percentage of the number of pits formed by conditioned medium of control cells. Each *bar* represents the mean ± SEM of 12 individual experiments. Each experiment was performed with different conditioned media (CM) and osteoclast preparations of different animal pools. *, $P < 0.05$; ***, $P < 0.001$ (*vs.* control CM). +++, $P < 0.001$ (*vs.* MEM).

Because of the large interexperiment variations in the resorption pit assay, the SEM was considerable, even for a large number of experiments (n = 12). To lessen the large variability in the experimental data, the results were, therefore, expressed as a percentage of the control value (conditioned medium obtained with untreated cells; Fig. 1b).

Appearance of inhibitory activity: time-course study

To determine the time course during which the osteoclast-inhibiting activity in medium conditioned by bisphosphonate-treated CRP 10/30 cells appears, CRP 10/30 cells were pretreated for 5 min with bisphosphonate or vehicle. After the treatment, cells were washed and continued to be incubated in fresh MEM without bisphosphonate. After the indicated times of culture, media were collected and tested for $TRAP^+$ MNC and pit formation. The results presented (Fig. 2a) show that up to 6 h after bisphosphonate treatment, MNC formation increased about equally regardless of whether the osteoclast preparation was cultured in medium derived from control or treated cells. However, thereafter, conditioned medium from control cells caused a further increase in MNC formation, whereas medium conditioned by bisphosphonate-treated cells inhibited it in a time-dependent manner. Similar results were obtained when the effects of both media were tested on pit formation (Fig. 2b). During the initial 6 h after treatment, pit formation was increased by conditioned media of both control and bisphosphonate-treated osteoblastic CRP 10/30 cells. Thereafter, the number of pits decreased in cultures with medium conditioned by bisphosphonate-treated cells, whereas the number of pits produced by osteoclasts cultured in control medium kept increasing. After 24 h of incubation, both media reached plateau levels, at which no further stimulation or inhibition was noticed.

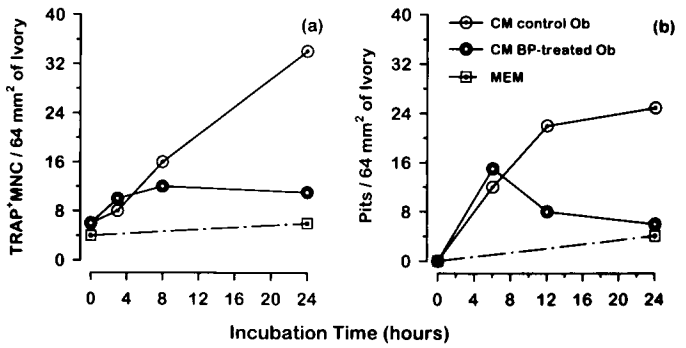

FIG. 2. Time course for the effect of conditioned media (CM) produced by control and bisphosphonate-treated CRP 10/30 cells on TRAP⁺ MNC (a) and pit formation (b) of osteoclasts cultured on ivory slices. At the indicated times, cultures were terminated, and the conditioned media were examined for TRAP⁺ MNC and pit formation. Results similar to those shown were obtained with two additional time-course studies.

Indication for an osteoblast-derived osteoclast inhibitor

The results of the time-course study described above suggest that the inhibitory effect of medium conditioned by bisphosphonate-treated osteoblastic CRP 10/30 cells might be due to the synthesis of an osteoclast inhibitor rather than the result of inhibition of osteoclast-stimulating activity. To resolve this question, the effect of conditioned medium of bisphosphonate-treated cells on medium conditioned by control cells was investigated. This was achieved by mixing both media at equal volumes. To prevent dilution of the respective test media through mixing, both were concentrated to one tenth of their original volumes by ultrafiltration using a membrane with a molecular mass cut-off of 1 kDa. Thereafter, the condensed media were reconstituted to half their original volumes with fresh MEM and then mixed in equal parts. The results, expressed as a percentage of the number of pits obtained with control cells, revealed that not only conditioned media of bisphosphonate-treated cells (Fig. 3, *solid bar*), but also a mixture of both control and treated cells (Fig. 3, *cross-hatched bar*), caused a diminution in pit number that is highly statistically significant.

Effect of dilution

The medium of bisphosphonate-treated cells was subjected to serial dilutions. The data in Fig. 4 show that with increasing dilutions, an initial rise in pit formation was followed by a subsequent decline. Knowing that conditioned medium of bisphosphonate-treated cells contain both a stimulator and an inhibitor of osteoclastic resorption, the above-mentioned rise in pit formation is presumably due to a "dilution out" of the inhibitor, allowing the stimulator to take action. With increasing dilutions, the stimulator likewise loses activity, which is documented by a decline in pit number.

Separation of the osteoclast inhibitor from the osteoclast-stimulating activity

Based on the fact that the mol wt of the osteoclast-stimulating activity was determined to be greater than 10 kDa, both conditioned media were subjected to ultrafiltration using a membrane with a molecular mass cut-off of 10 kDa. The retentates were reconstituted to the original volume, whereas the filtrates were first condensed to one tenth of their volumes, employing a membrane with a molecular mass cutoff of 1 kDa. The individual ultrafiltration fractions were then brought to the original volume with fresh MEM and subsequently tested in the resorption pit assay. The results obtained, which are summarized in Fig. 5, show that osteoclasts cultured in the presence of the filtration retentates (>10 kDa) of conditioned medium derived from both control (*diagonally hatched bar*) and treated (*filled bars*) osteoblastic cells had an enhanced number of pits about equal to that in osteoclasts maintained in plain MEM (*open bar*). These results indicate that the osteoclast-stimulating activity was retained in both conditioned medium fractions containing molecules with molecular mass greater than 10 kDa, indicating the removal

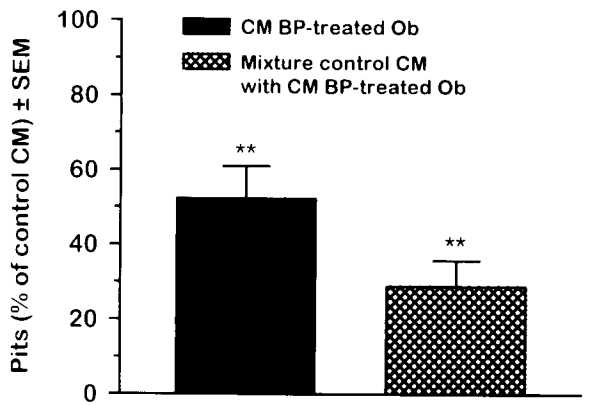

FIG. 3. Pit formation of osteoclasts, cultured in the presence of conditioned media (CM) of bisphosphonate-treated and control osteoblastic cells. In addition, the effect of a mixture of both conditioned media on pit formation is shown (*cross-hatched bar*). Results are expressed as a percentage of the pits excavated by osteoclasts in the presence of control CM. Each *bar* represents the mean ± SEM of four independent experiments, using different CMs and osteoclasts of different animal pools for each experiment. **, $P < 0.01$ *vs.* control CM.

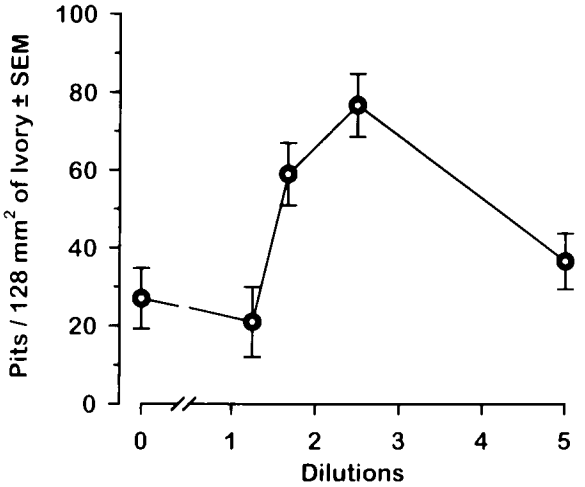

FIG. 4. Effects of different dilutions of medium conditioned by bisphosphonate-treated CRP 10/30 cells on pit formation by osteoclasts. Each *data point* represents the mean ± SEM of four separate experiments using conditioned media prepared from different cell cultures and osteoclasts of different animal pools.

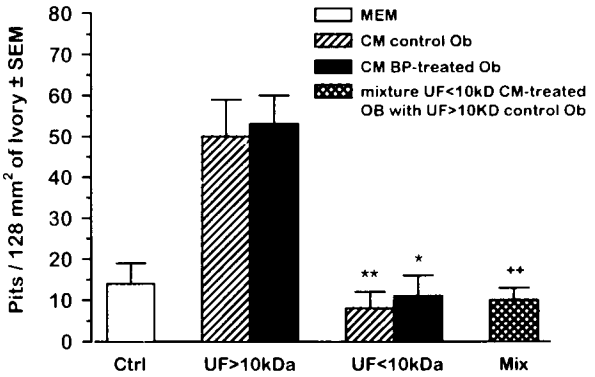

FIG. 5. Pit formation by osteoclasts cultured in the presence of conditioned media (CM) that were separated into fractions containing substances with a molecular mass either larger or smaller than 10 kDa, but larger than 1 kDa. The fractionation of CM was carried out using an ultrafiltration device. The experimental data shown represent the mean ± SEM of three individual experiments using same conditioned media, but osteoclasts from different animal pools. *, $P < 0.05$; **, $P < 0.01$ (*vs.* ultrafiltrate >10 kDa). ++, $P < 0.01$ (*vs.* ultrafiltrate >10 kDa; control Ob).

of the inhibitor from medium of bisphosphonate-treated cells. The notably fewer pits excavated by osteoclasts cultured in ultrafiltrates containing molecules with a molecular mass less than 10 kDa renders additional evidence that osteoclast-stimulating activity in conditioned media of control and treated cells has a molecular mass larger than 10 kDa. Whether the ultrafiltrate (<10 kDa) of medium conditioned by bisphosphonate-treated CRP 10/30 cells contained the osteoclast inhibitor was determined by testing the effect of this fraction on the pit-stimulating activity contained in the control medium fraction (>10 kDa; presented by the *cross-hatched bar*; Fig. 5); the results show that the inhibitor was present in the ultrafiltrate fraction (<10 kDa) of conditioned medium derived from bisphosphonate-treated cells.

Cell specificity of the inhibitor

To determine whether the production of osteoclast inhibitor is confined to bone cells, conditioned media of rat dermal fibroblasts were treated according to the same protocol as that used for osteoblastic CRP 10/30 cells. Recently, we showed that that rat dermal fibroblasts do not express osteoclast-stimulating activity (31), for this reason, 10% FBS, which was previously found to support osteoclast-mediated pit formation (31), was added to the pit assay. Figure 6 illustrates that there was no difference between the number of pits produced by osteoclasts cultured in the presence of control medium or in medium of bisphosphonate-treated fibroblasts. In contrast, using conditioned media of control and bisphosphonate-treated osteoblastic CRP 10/30 cells as a positive control concurrently in the same experiment, a clear inhibition of pit formation was obtained by conditioned medium of bisphosphonate-treated CRP 10/30 cells.

Effect of the osteoclast inhibitor on TRAP⁺ MNC formation

To assess whether the reduction in pit formation by the resorption inhibitor is paralleled by a diminution of TRAP⁺ MNC, ivory slices were subjected to staining for TRAP⁺

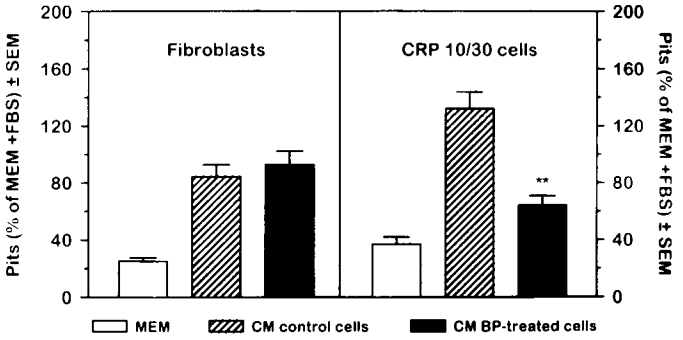

FIG. 6. Effect of medium conditioned by bisphosphonate-treated rat cells on pit formation of rat skin fibroblasts and osteoblastic CRP 10/30. The results obtained are expressed as a percentage of the pits produced by osteoclasts cultured in the presence of MEM supplemented with 10% FBS. The data represent the mean ± SEM of five experiments using different conditioned media (CM) and osteoclast preparations from different animal pools. **, $P < 0.01$ *vs.* CM of CRP 10/30 cells.

MNC before processing them for detection of pits. The data (Fig. 7a) show that conditioned media of control osteoblastic cells caused an approximately 2-fold increase in MNC compared to that in plain MEM-containing cultures, whereas conditioned medium of bisphosphonate-treated cells and a mixture of media of treated and untreated cells produced a reduction in MNC of about 50%. The ratio of MNC to the number of pits formed showed that there was one pit formed per approximately two TRAP⁺ MNC. The ratio of both parameters was the same regardless of whether the osteoclasts were cultured in the presence of MEM or conditioned medium of control or bisphosphonate-treated cells (Fig. 7b).

Mode of action of the osteoclast resorption inhibitor

The data obtained show that the reduction of pit number was accompanied by a reduction of TRAP⁺ MNC. To determine whether the formation of TRAP⁺ MoNC, the immediate precursors of MNC, or the mean area resorbed per pit were altered by the inhibitor, experimental procedures were applied that allowed all of the above-mentioned parameters to be determined within the same experiment. The

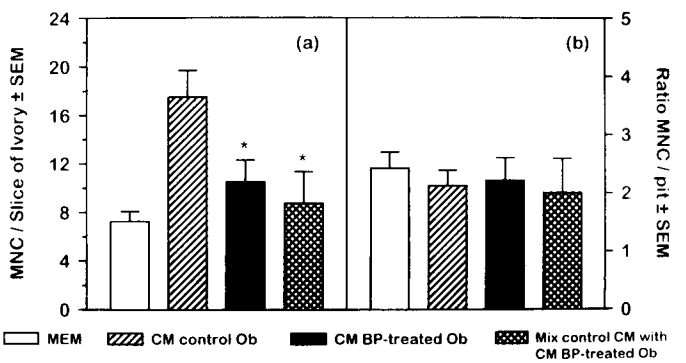

FIG. 7. Effect of conditioned media (CM) of bisphosphonate-treated CRP 10/30 cells on TRAP⁺ MNC formation (a) and on the ratio of MNC per resorption pit (b). The results shown are representative of one experiment of four other experiments that produced similar results. Each *bar* represents the mean number of pits ± SEM produced by the same pool of osteoclasts on eight different ivory slices cultured individually in separate wells. *, $P < 0.05$ *vs.* CM of control cells.

results (Fig. 8) illustrate that the decrease in pit number by medium conditioned with bisphosphonate-treated osteoblastic cells was paralleled by a decline in both $TRAP^+$ MNC and MoNC formation. In contrast, the mean area per pit resorbed was not altered.

Effect of alendronate on the synthesize the osteoclast resorption inhibitor

All of the preceding experiments were carried out with the bisphosphonate ibandronate at 10^{-7} M. To determine whether the results achieved were due to the structural properties of this particular bisphosphonate, we examined alendronate, another widely studied bisphosphonate, together with ibandronate. Scoring both the number of $TRAP^+$ MNC and pits to monitor the effects of the two bisphosphonates, the results revealed that medium conditioned by alendronate- and ibandronate-treated CRP 10/30 cells inhibited the number of MNC (Fig. 9a) and pits (Fig. 9b), to approximately the same extent at a concentration of 10^{-7} M. The IC_{50} values for ibandronate and alendronate were 3.5×10^{-10} and 2×10^{-9} M, respectively (20).

Do other osteoblastic cells produce osteoclast resorption inhibitor in response to bisphosphonate treatment?

The osteoblastic cell line CRP 10/30 is a bone cell type that, contrary to other cloned osteoblasts, produces an osteoclast-stimulating activity without the addition of an osteotropic factor. To determine whether the synthesis of the osteoclast resorption inhibitor by way of treatment with bisphosphonates is unique to CRP 10/30 cells, we used the osteoblastic cell line UMR 106, which also synthesizes an osteoclast-stimulating activity, but only after being challenged with calciotropic hormones such as PTH (20). Pretreatment of

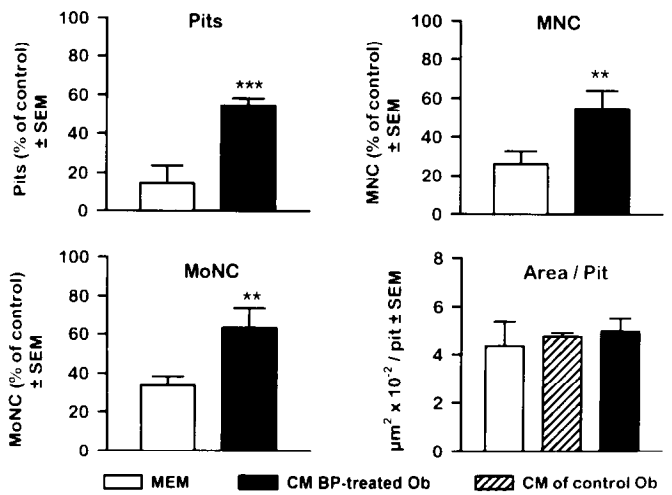

FIG. 8. Effect of media conditioned by bisphosphonate-treated CRP 10/30 cells on the formation of resorption pits, $TRAP^+$ MNC, and MoNC and on the mean area resorbed per pit. All parameters were determined concurrently with the same osteoclast preparation. The numbers of pits, MNC, and MoNC are expressed as a percentage of the results obtained with control conditioned medium (CM). Each data set represents the mean ± SEM of four individual experiments, using different CM and osteoclast preparations from different animal pools. **, $P < 0.01$; ***, $P < 0.001$ (*vs.* control CM).

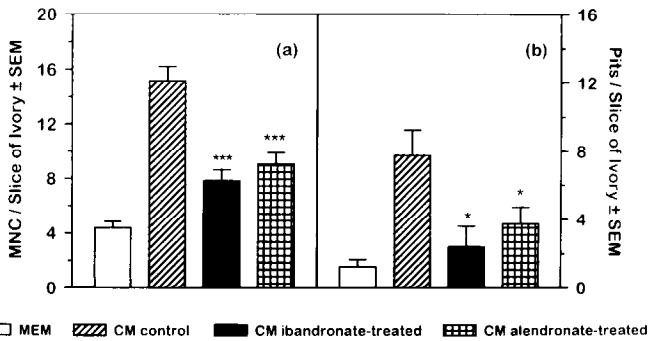

FIG. 9. Comparative study of the effects of two bisphosphonates, ibandronate and alendronate, at 10^{-7} M on the formation of $TRAP^+$ MNC (a) and resorption pits (b). MNC and pits were determined in the same osteoclast preparation. The graph shows the results of a representative experiment of four additional experiments that produced similar results. The data shown are the mean ± SEM of eight separately cultured ivory slices incubated in the presence of the same conditioned medium. *, $P < 0.05$; ***, $P < 0.001$ [*vs.* control conditioned medium (CM)].

UMR-106 cells for 5 min with or without bisphosphonate followed by cultivating the cells for 24 h in the presence of PTH (10^{-8} M) but in the absence of the drug revealed that conditioned medium of control cells treated with PTH produced about 3 times as many $TRAP^+$ MNC as conditioned medium of UMR-106 cells pretreated with the bisphosphonate and subsequently cultured in the presence of PTH (Fig. 10). The lower number of MNC per slice obtained with PTH containing plain MEM (4.13 ± 0.55) compared to the number of pits obtained with conditioned medium of hormone-treated cells (9.56 ± 1.25; Fig. 10) indicates that the increase in MNC formation was due to a soluble factor released by UMR-106 cells in response to PTH and was not the result of the hormone acting on cells that may contaminate the isolated osteoclast preparation.

Additional osteoblastic cell lines that were tested for their ability to produce the inhibitor are listed in Table 1. The results show that, apart from CRP 10/30 and UMR-106 cells,

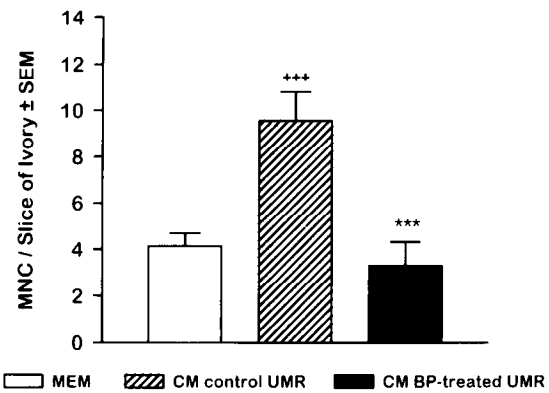

FIG. 10. Effect of bisphosphonate on the synthesis of resorption-inhibiting activity by the osteoblastic UMR-106 cell line. After treatment of UMR-106 cells with the bisphosphonate, control and treated cells were cultivated for an additional 24 h in the presence of PTH but in the absence of bisphosphonate. The data shown represent the mean ± SEM of three different experiments using the same conditioned medium but osteoclasts from different animal pools. ***, $P < 0.005$ *vs.* conditioned medium (CM) of bisphosphonate untreated UMR; +++, $P < 0.005$ *vs.* MEM.

TABLE 1. Synthesis of osteoclast resorption inhibitor and resorption stimulator by various cell types

Cell name	Cell phenotype	Osteoclast inhibitor	Osteoclast stimulator
CRP 10/30	Osteoblast	Yes	Yes
UMR-106	Osteoblast	Yes	Yes[a]
IRC 10/30mycl	Immortalized CRP 10/30	Yes	No
CRP 10/3	Osteoblast-late stage	Yes	No
CRP 5/4	Preosteoblast	No	No
Fibroblast	Skin fibroblasts	No	No

[a] Only after treatment with PTH.

IRC 10/30 mycl cells, the immortalized derivative of CRP 10/30 cells, and CRP 10/3 cells, a late stage (osteocytic) osteoblastic cell line, produce the inhibitor, whereas preosteoblastic clonal CRP 5/4 cells do not.

Physical properties of the osteoclast resorption inhibitor

To investigate whether the inhibitor is heat stable, conditioned media of untreated and bisphosphonate-treated CRP 10/30 cells were subjected to heat treatment (10-min boiling water bath). As the heat treatment destroyed the osteoclast-stimulating activity, the samples were tested for osteoclast-stimulating activity against 10% FBS. The results obtained show that the heat treatment destroyed the osteoclast-inhibiting activity (Fig. 11), indicating that the inhibitor is heat labile.

To investigate whether the inhibitor is a protein, conditioned media (containing the inhibitor) of bisphosphonate-treated CRP 10/30 cells were exposed to proteinase K, a nonspecific protease. The results presented in Fig. 12 show that the proteolytic enzyme eliminates the inhibitory activity, indicating that the inhibitor is a protein.

Effect of bisphosphonate on the osteoblastic phenotype

The synthesis of an osteoclast resorption inhibitor by bisphosphonate-treated CRP 10/30 cells shows that bone cells do respond to nanomolar concentrations of bisphosphonates. It was, therefore, of interest to assess whether at

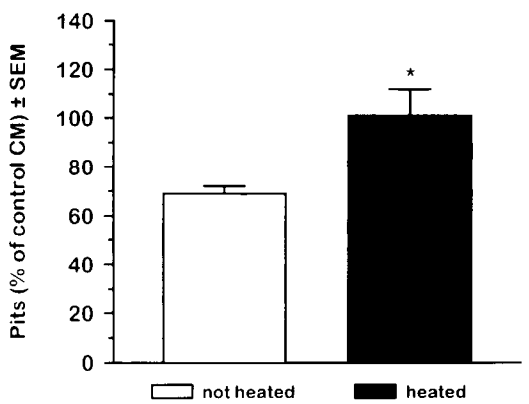

FIG. 11. Heat treatment of the osteoclast inhibitor. The effect of heat treatment on osteoclast-inhibiting activity was measured against the osteoclast stimulatory activity of 10% FBS. The results shown are expressed as the percentage of pits obtained with conditioned medium of control cells and represent the mean ± SEM of three separate experiments using different conditioned media and osteoclasts from different animal pools. *, $P < 0.05$.

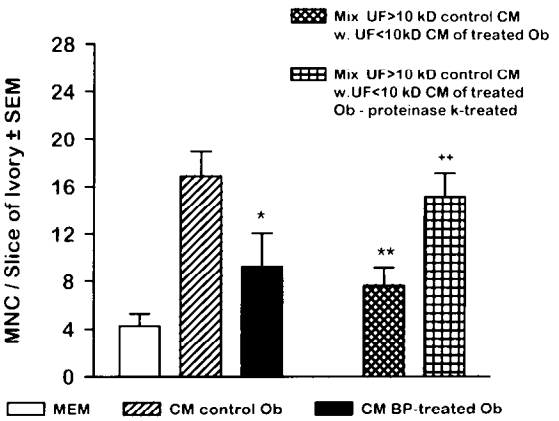

FIG. 12. Effect of proteinase K treatment on the osteoclast inhibitor. The experiment was carried out as detailed in *Materials and Methods*. The results shown are representative of one experiment of three that produced similar results. The data represent the mean ± SEM of eight ivory slices cultured separately in the presence of the same conditioned medium (CM) and osteoclasts from the same animal pool. *, $P < 0.05$; **, $P < 0.01$ (*vs.* CM of control cells). ++, $P < 0.01$ (*vs.* no proteinase treatment).

these concentrations other parameters of the osteoblast became influenced by the bisphosphonate. Cultivation of CRP 10/30 cells in the presence and absence of ibandronate (10^{-7} M) for 72 h showed that neither cell growth, alkaline phosphatase, PTH responsiveness, nor the synthesis of collagen was affected by the bisphosphonate concentration used (Table 2).

Discussion

Bisphosphonates are potent inhibitors of bone resorption when tested in a variety of systems both in culture and *in vivo*. Because bisphosphonates have a strong affinity for hydroxyapatite (10, 11), and hence for bone, it was generally accepted that the mode of action of these compounds is due to the direct inhibitory effect of bisphosphonate-coated bone on the ability of mature osteoclasts to resorb (2, 15, 37). However, previous studies using several assay techniques *in vitro* have failed to reproduce the relative potencies that various bisphosphonates display *in vivo*. Employing the osteoblastic cell line CRP 10/30, we recently presented data indicating that the inhibitory action of bisphosphonates on bone resorption is mediated at least in part by the osteoblast (20). Thus, proof had been obtained showing that bisphosphonates, apart from acting directly on the osteoclast, may also act on bone resorption independently of the need for a contact with bisphosphonate-containing mineral. This finding, which has recently been confirmed by two independent studies (21, 22), infers that the osteoblast, besides being involved in mediating the effects of bone resorption-promoting hormones and cytokines (25–28), also appears to act as a mediator for bone resorption-inhibiting substances. The fact that an inhibiting activity of osteoclastic bone resorption is also elaborated by osteoblasts under the influence of estrogen indicates that physiological resorption-inhibiting substances may also act via the osteoblast-osteoclast axis (24).

Previously, it was demonstrated that CRP 10/30 cells syn-

TABLE 2. Effects of ibandronate on growth and phenotype of CRP 10/30 cells

	Cell no. ($\times 10^{-4}$)	ALP (nmol/min · 10^6 cells)	PTH response[a]		Collagen synthesis (% of total protein)
			No PTH	With PTH	
Control	11.09 ± 1.03	120.45 ± 1.83	2.36 ± 1.38	17.77 ± 2.93	11.12 ± 0.18
Bisphosphonate	9.95 ± 0.23	139.49 ± 1.81	3.33 ± 1.92	21.15 ± 2.71	11.83 ± 0.13

ALP, Alkaline phosphatase.
[a] PTH response is expressed as [^{3}H]cAMP (counts per min $\times 10^{-2}$) $\times 10^{-6}$ cells.

thesize an osteoclast-stimulating activity without the addition of bone resorption-supporting substance (31). This circumstance led us to hypothesize that the inhibitory action of the bisphosphonate on bone resorption may be due to suppression of the synthesis of the osteoclast-stimulating activity mentioned above. With the present study we have presented evidence indicating that the osteoblast-mediated inhibition by bisphosphonates of resorption is not the result of an inhibition of the osteoblast-derived osteoclast-stimulating activity, but is due to the synthesis of an inhibitor that presumably acts directly on the osteoclastic resorption process. Its susceptibility toward heat and protease treatment suggests that the inhibitor may be a polypeptide that, based on ultrafiltration experiments, has a molecular mass ranging between 1–10 kDa. The similar results obtained with two different bisphosphonates, ibandronate and alendronate, show that the induction of the inhibitor may be an occurrence common to other bisphosphonates as well. Furthermore, having employed other osteoblastic cell lines of different developmental stages and fibroblasts, it may be concluded that the inhibitor appears to be synthesized after pretreatment with bisphosphonates only by cells that express the mature osteoblastic phenotype. This information, thus, is consistent with literature reports indicating that it also requires mature osteoblasts for the synthesis of an osteoclast-stimulating activity (24, 26).

The idea that bisphosphonates may not operate solely through a direct action on osteoclast activity or recruitment, but through mechanisms of other cell systems related to bone resorption as well has been evidenced by several studies. Thus, it was shown that bisphosphonates affect other cells that may play a role in bone turnover, including osteoblasts and macrophages (38–40), of which in the latter case both proliferation and activity are inhibited *in vitro* (41, 42). Additional support for the idea that osteoblasts are target cells for bisphosphonates came from two recent studies in which it was shown that alendronate supports osteoblast-mediated bone formation (43) and that it inhibits the production of interleukin-6 (23). The fact that the osteoblast acts as a mediator for both bone resorption-stimulating hormones or cytokines and resorption-inhibiting substances underscores the importance of the osteoblast in regulating osteoclastic bone resorption.

The isolated osteoclast pit assay used in the present study has proven to be very useful to examine direct and indirect effects of cytokines and hormones on osteoclasts. As the assay is performed over a period of 24 h, it has been thought to reflect mainly activation or inhibition of mature osteoclasts, rather than modulation of osteoclast differentiation. Previously, however, it was demonstrated that isolated osteoclasts can be cultured on mineralized matrexes, such as devitalized bone slices, for up to 144 h. Over this culture period, an increase in TRAP$^+$ multinucleated osteoclast-like cell formation, rather than cell attrition, has been observed (44). Using these findings as a basis, we assessed TRAP$^+$ MNC and MoNC formation of osteoclast preparations that were cultured for 24 h on ivory slices in the presence of medium conditioned by control or bisphosphonate-treated osteoblastic cells. The results revealed that during 24-h incubation, the number of TRAP$^+$ MNC and MoNC increased in the presence of an osteoclast-stimulating activity by approximately 5-fold over that in MEM-containing osteoclast cultures. Therefore, any stimulator or inhibitor can act through the number of osteoclasts and, thus, through their formation and/or survival. In addition, a diminution in pit number observed in the presence of medium conditioned by bisphosphonate-treated CRP 10/30 cells was paralleled by a reduction of TRAP$^+$ MNC as well as MoNC. These data suggest that the inhibiting action of the bisphosphonate-induced osteoclast inhibitor appears to be on the development of TRAP$^+$ cells, presumably osteoclasts, and not on the osteoclast resorption activity. This conclusion gained additional support from observations that the mean area per pit resorbed was not changed by the resorption inhibitor. Whether the decline in the number of TRAP$^+$ cells is due to inhibition of the proliferation of osteoclastic precursor cells or is the result of interference in the differentiation and fusion of postmitotic preosteoclasts to TRAP$^+$ osteoclasts is difficult to determine at the present time. In addition, it can not be ruled out entirely that the decrease in TRAP$^+$ cell was the result of the inhibitor acting on the adherence of these cells to the ivory surface. In a recent communication, it was suggested that the inhibiting action of bisphosphonates on bone resorption may be due to these compounds inducing apoptosis in TRAP$^+$ MNC (45); thus, cell survival may also be involved.

The prospect of bisphosphonates acting on osteoclast recruitment has been reported previously (46). It is not known whether the reported effects were obtained through contaminating osteoblasts in the marrow culture. In a more recent study, however, using an osteoclast recruitment assay to assess the effect of bisphosphonates on osteoclast formation, it was shown that bisphosphonates added directly to the system did not cause inhibition of osteoclastogenesis, but it required the addition of osteoblasts in the assay for bisphosphonates to reduce the number of TRAP$^+$ MNC (22). The possibility of cell detachment of newly formed TRAP$^+$ from the substrate was not discussed in this report.

The nature of the osteoblast-derived inhibitor is not known. The fact that the activity of the inhibitor is destroyed by treatment with proteinase K shows that the factor is a polypeptide that, according to ultrafiltration experiments,

has a molecular mass ranging between 1–10 kDa. Moreover, the activity was found to be heat labile, which makes the inhibitor appear to resemble that found in conditioned medium of ROS 17/2.8 cells (47). There are several reports that describe factors and cytokines that inhibit osteoclastic bone resorption, such as leukemia-inhibiting factor (48) or interferon-γ (49), but each of these cytokines has a molecular mass greater than 10 kDa. Interleukin-4, another inhibitor of bone resorption *in vivo* (50) or *in vitro* (51) and of both osteoclastic activity and formation of osteoclast-like cells in murine bone marrow culture, is in all probability not produced by osteoblasts. A possible candidate that might resemble the osteoclast inhibitor described in the present communication is the carboxyl-terminal cleavage peptide-(107–139) fragment of PTH-related protein (PTHrP). The cleavage peptide was found to exert a potent antiresorptive action on pit formation in isolated disaggregated rat osteoclasts (44) Interestingly, the PTHrP-(107–139) fragment inhibited the total area resorbed per bone slice without reducing the size of the individual pits, which is in line with our results. Recently, we were able to demonstrate that the osteoblastic cell line CRP 10/30 synthesizes PTHrP (52).

As mentioned above, 17β-estradiol appears to stimulate the elaboration of a cell/matrix surface-associated osteoclast-derived inhibitor of osteoclastic bone resorption. However, this inhibitor is solely associated with the osteoblast extracellular matrix, whereas this is not the case with the inhibitor synthesized in response to bisphosphonates, which is found primarily in the culture medium (our unpublished observation).

The question of the passage of the inhibitor from osteoblasts to the osteoclast has not been resolved. Indeed, the osteoclasts can be but are by no means always in close proximity to the osteoblasts. One possibility would be that the inhibitor becomes bound to the mineralized matrix from where it would be released when bone mineral is desolved by osteoclastic resorption. Another prospect is that it is transferred from the osteocytes.

Although it may be unlikely, the bisphosphonates themselves could gain access directly from the general circulation after their administration. Another possibility, consistent with their long duration of action, is that they are released from bone into the microenvironment of osteoblasts, either by passive diffusion or during resorption.

In conclusion, with the present study, evidence has been collected showing that osteoblasts *in vitro* mediate the action of bisphosphonates on bone resorption by synthesizing an inhibitor of osteoclast recruitment and/or survival. This finding not only offers a new additional mechanism of bisphosphonate action on resorption, but it accentuates the important role that osteoblasts play in regulating osteoclastic bone resorption. Thus, osteoblasts may mediate the action not only of stimulating but also of inhibiting substances on bone resorption. These drugs may, therefore, become a valuable tool to elucidate the precise mechanism that governs osteoclast-mediated bone resorption in health and disease.

Acknowledgments

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