The Effect of Extracellular Calcium Elevation on Morphology and Function of Isolated Rat Osteoclasts

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Osteoclasts are large multinucleate cells unique in their capacity to resorb bone. These cells are exposed locally to high levels of ionised calcium during the process of resorption. We have therefore examined the effect of elevated extracellular calcium on the morphology and function of freshly disaggregated rat osteoclasts. Cell size and motility were quantitated by time-lapse video recording together with digitisation and computer-centred image analysis. In order to assess the resorptive capacity of isolated osteoclasts, we measured the total area of resorption of devitalised cortical bone by means of scanning electron microscopy and computer-based morphometry. The results show that elevation of the extracellular calcium concentration causes a dramatic reduction of cell size, accompanied by a marked diminution of enzyme release and abolition of bone resorption. We propose that ionised calcium might play an important role in the local regulation of osteoclastic bone resorption.

KEY WORDS: osteoclast; bone resorption; extracellular calcium; cell motility; acid phosphatase.

INTRODUCTION

The overactivity of osteoclasts leads to progressive loss of bone eventually causing osteoporosis and fracture. Nevertheless, our knowledge of how osteoclast activity is regulated in health and disease is incomplete. The recent development of elegant methods for osteoclast culture have made possible the study of osteoclasts in isolation from other bone cells (1). Using these techniques, it has been shown that the cells are exquisitely sensitive to calcitonin, the only known physiological inhibitor of osteoclast function (2, for review 3). However, as the osteoclast is exposed to high levels of ionised calcium during bone resorption, it is imperative that we understand any effects of calcium on osteoclast behaviour. We show here that an increase in the ambient calcium concentration is followed by dramatic...
shape change, diminished enzyme release and abolition of resorptive activity. This, we believe, represents a fundamental regulatory mechanism for the feedback control of osteoclast function. Thus, excess osteoclast activity will generate concentrations of calcium that would limit further bone resorption. A preliminary report of this work has been presented previously (4).

MATERIALS AND METHODS

Osteoclast Isolation and Culture

Osteoclasts were mechanically disaggregated by curetting long bones of neonatal rats into HEPES-buffered Medium 199 (Flow Laboratories, UK) (1). The cell suspension was agitated with a pipette and the larger bone fragments allowed to settle for 10 seconds. The cells were then dropped onto slices of devitalised human cortical bone placed in a well of a Sterilin multiwell dish or onto plastic petri dishes (Cell Cult, Sterilin). Osteoclasts were allowed to sediment and attach to the substrate for 30 minutes (37°C). The substrate was then washed in Minimal Essential Medium containing heat-inactivated foetal calf serum (Gibco, UK; 10% v/v) and placed in separate wells containing 2 mls of the same medium.

Bone-Osteoclast Resorption Assay

The osteoclast-bone preparations were incubated for 18 hours (37°C; 10% humidified CO₂) with or without added calcium chloride (final concentration 20 mM), magnesium chloride (20 mM), potassium chloride (20 mM) or human calcitonin (hCT, 100 pmol/l) the slices were fixed with glutaraldehyde (10% v/v), bleached by immersion in sodium hypochlorite solution (10% v/v; BDH, UK) and dehydrated in ethanol (80% v/v). The bone slices were then sputter-coated with gold and examined under a scanning electron microscope (Cambridge 360, Cambridge Instruments, Bar Hill, Cambridgeshire). The size and number of osteoclastic excavations was assessed by tracing the outline of each excavation into a digitiser linked to an IBM-AT-controlled image processor (Sight Systems, Newbury, Berkshire).

Analysis of Cell Size and Motility

Petri dishes with adherent osteoclasts were placed in a humidified incubation chamber (37°C) of an inverted phase-contrast microscope (Diaphot, Nikon UK, Ltd.) linked via CCD camera to a time-lapse video recorder (Mitsubishi, Japan). The signal was fed into a 256-gray level imaging system (Sight Systems, Newbury, Berks) centered on a 60-megabyte dual hard disc IBM microcomputer. The capture of digitised gray images into the computer memory was followed by redigitisation of the cell boundary using a microsoft mouse (Microsoft), an edge detection facility and a binary overlay. A tracing of each cell was retrieved
sequentially, overlaid on the previous outline and analysed using a software package programmed to measure the area within each tracing together with area change due to cell movement (shape area or ΔA) (Datta, Zaidi & MacIntyre, unpublished observations). Motility has been computed as the rate of change ΔA per unit absolute cell area and expressed as a percentage of control cell motility.

**Acid Phosphatase Assay**

For acid phosphatase measurements supernatant from bone-osteoclast cultures was sampled 60 minutes following incubation. Tartrate-resistant acid phosphatase activity was measured by incubating samples (usually 150 μl) with 4-nitrophenyl phosphate (final concentration 10 mM) in citrate buffer (100 mM, pH 5.0) with dextrorotatory tartrate (150 mM) at 37°C for 30 minutes in a total volume of 450 μl. The reaction was stopped and the quinonoid form of 4-nitrophenate ion was developed by the addition of 200 μl NaOH (0.5 mM) before absorbometry. Catalytic activity (μmol substrate hydrolysed per minute (U) per 1 sample) was calculated from the absorbance coefficient of 4-nitrophenate, e = 1.851 × mmol⁻¹ × mm⁻¹.

**RESULTS**

The elevation of extracellular calcium ([Ca²⁺]ₑ) to 20 mM led to a marked reduction of the total area of bone resorbed per bone slice (R) (Fig. 1a) and the total number of excavations (per bone slice, ±SEM; control −9.0 ± 2.5 vs [Ca²⁺]ₑ − 0.8 ± 0.1; n = 8; p < 0.01, Students t-test). The magnitude of reduction was equivalent to an effect produced by 100 pmol/l hCT (Fig. 1a) (number of excavations per bone slice ±SEM; control 13 ± 3.0 vs hCT − 1.0 ± 0.3; n = 8; p < 0.01). Prolonged exposure of osteoclasts to calcium had no effect on cell number or viability, ruling out possible toxic effects. This was accompanied by a significant reduction of enzyme (acid phosphatase) release (E) (p < 0.01), similar to that seen with calcitonin (p < 0.05, Student’s t-test) (Fig. 1b). The acute effect of extracellular calcium elevation was characterised by a marked 65% reduction of osteoclast size (p, 0.01) (A) within minutes of exposure to elevated ionised calcium (half time = 7.8 minutes) (Fig. 1c). In contrast, calcitonin (half time = 23 minutes) produced a response (significant p < 0.05) which was less in magnitude than that due to calcium. These responses were followed by complete recovery. Interestingly, while calcitonin caused a complete inhibition of osteoclast motility (p < 0.01) (M) within 15 minutes of exposure, extracellular calcium elevation had no effects on membrane ruffling, pseudopodial motility, cell migration or granule movement (Fig. 1d). Because the exposure to elevated extracellular calcium was followed by marked cell retraction, motility has been calculated as the change in shape area (ΔA) per unit absolute cell area (A). In addition, we found that magnesium (20 mM) and potassium (20 mM) did not inhibit resorption, enzyme release, osteoclast size or cell motility (data not shown), suggesting that the response was specifically mediated by ionised calcium.
Fig. 1. The effect of elevated extracellular calcium (20 mM) (filled bars) and human calcitonin (100 pmol/l) (hatched bars) on the total area of bone resorption (R, n = 8 bone slices per group) (a), enzyme release (E, n = 4 experiments) (b), spread area (A, n = 8 individual cells) (c) and motility (M, n = 8 individual cells) (d) of isolated rat osteoclasts. The results have been expressed as percentages of the mean control values ± standard error of mean. The control responses are shown as plain bars.

DISCUSSION

A complex of interacting factors are now known to stimulate osteoclastic bone resorption: such factors include parathyroid hormone, 1,25-dihydroxycholecalciferol, parathyroid hormone-related peptide, prostaglandins, cytokines and transforming growth factors (for review see 4). However, the only established physiological inhibitor of osteoclastic function is the thirty-two aminoacid polypeptide, calcitonin (2, 5, 6). Here we suggest that the elevated ambient levels of ionic calcium generated as a consequence of resorption can inhibit further osteoclastic activity. Although the inhibitory effects of calcitonin and extracellular calcium elevation on bone resorption and enzyme release were remarkably similar, there were some notable differences between the effects of the two compounds on osteoclast shape and motility. The exposure of isolated osteoclasts to levels of extracellular calcium likely to be generated at the resorptive sites led to dramatic cell retraction; the magnitude of retraction with calcium was markedly greater than that produced by calcitonin (65% and 34% respectively). Furthermore, whereas calcitonin caused complete cessation of osteoclast motility, exposure to elevated ionised calcium did not.

Normally, during the process of bone resorption the ruffled border of the osteoclast lies in apposition with the bone and seals off the surface surrounding the resorption site. Into this microcompartment the osteoclast secretes protons
and hydrolytic enzymes for the dissolution of mineral and digestion of matrix (7). It is therefore likely that factors disrupting the osteoclast membrane-bone seal will abolish the process of resorption. We have also demonstrated that osteoclast adherence to substratum is dependent crucially on appropriate concentrations of calcium and magnesium: osteoclast adhesion is markedly reduced in the presence of high ambient calcium concentrations (Zaidi, Datta, Makgoba, and MacIntyre unpublished observations). The observed effects on osteoclast adhesion and spread area when taken together strongly suggest that elevated calcium levels prevent the creation and maintenance of the microenvironment essential for the resorptive process. A marked reduction of acid phosphatase release by elevated extracellular calcium also points to the induction of intracellular pathways. Acid phosphatase has recently been implicated in the removal of pyrophosphate, a natural inhibitor of bone resorption; the diminution of its release or activity is thus associated with inhibition of resorption (8). The exact mechanism of calcium-induced inhibition of osteoclastic bone resorption is still incompletely understood and requires urgent investigations.

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REFERENCES