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Inhibition of Osteoclastic Acid Phosphatase Abolishes Bone Resorption

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Osteoclastic acid phosphatase is a member of a widely-distributed class of iron-containing proteins with acid phosphatase activity. Antibodies raised against one member of this class cross-react with other members from the same or different species, but not with acid phosphatase isoenzymes of different types. When antibodies to one such protein, porcine uteroferrin, are added to medium in which rat osteoclasts are incubated on devitalised cortical bone, both bone resorption and acid phosphatase activity are markedly inhibited. Furthermore, addition of molybdate (an inhibitor of this class of acid phosphatases) also inhibits both bone resorption and enzyme activity. These observations strongly suggest a functional role for osteoclastic acid phosphatase in bone resorption.

Osteoclasts are known to secrete an isoenzyme of acid phosphatase (orthophosphoric monoester phosphohydrolase, acid optimum; E.C. 3.1.3.2) that is also present normally in other differentiated cells of the monohistocytic series (1, 2) and pathologically in Gaucher cells (3) and hairy cells of leukaemic reticulo-endotheliosis (4). The osteoclastic acid phosphatase isoenzyme is a member of a widely-distributed class of iron-containing proteins with acid phosphatase activity, the structures of which are highly conserved (5). Molybdate inhibits these acid phosphatases (6), but catalytic activity is not inhibited by dextro-rotatory tartrate, a competitive inhibitor of prostatic and lysosomal acid phosphatases; this property of tartrate-resistance is shared by erythrocyte acid phosphatase (7). Further, antisera raised against individual iron-containing acid phosphatases, cross-react with other members of this class of proteins, but not with acid phosphatases of other types (5, 8). An association between osteoclastic acid phosphatase and bone resorption has been suspected from observations that elevated plasma isoenzyme levels are associated with increased bone turnover in metabolic bone disease. Nevertheless, there is no evidence to date for a direct role of acid phosphatase in bone resorption.

Recent advances have allowed the isolation and short-term culture of osteoclasts (9). The cells are freshly disaggregated from neonatal rat long bones, dispersed on devitalised cortical bone substrate (10), and the area of bone resorbed precisely quantitated by scanning electron microscopy (10, 11). We demonstrate that the inhibition of acid phosphatase activity in such bone-osteoclast preparations by a specific antibody or a specific phosphatase inhibitor molybdate is accompanied by a

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marked inhibition of osteoclastic bone resorption. These results strongly suggest a functional role for osteoclastic acid phosphatase in the bone resorptive process.

MATERIALS AND METHODS

Bone-osteoclast cultures and quantitation of resorption: Osteoclasts were mechanically disaggregated by curetting long bones of neonatal rats into HEPES-buffered Medium199 (Flow Laboratories, UK, Ltd.). The cell suspension was agitated with a pipette and larger bone fragments allowed to settle for 10 seconds, before the cells were dropped onto slices of devitalised human cortical bone placed in a well of a Sterilin multiwell dish. Osteoclasts were then allowed to sediment and attach to the bone substrate for 30 minutes (37 °C). The slices were then washed in Minimal Essential Medium containing heat inactivated foetal calf serum (Gibco, UK; 10% v/v) and placed in separate wells containing 2 ml of the same medium. Following incubation for 18 hours (37 °C; 10% humidified CO_2) with or without sodium molybdate, anti-porcine uteroferrin antiserum or purified antibody, the culture medium was sampled and the slices were fixed with glutaraldehyde (10% v/v; BDH, UK), bleached by immersion in sodium hypochlorite solution (10% v/v) and dehydrated in ethanol (80% v/v). The bone slices were then sputter-coated with gold and examined by scanning electron microscopy (Cambridge 360, Cambridge Instruments, Bar Hill, Cambridgeshire, UK) (9-11). The area of bone resorbed and the 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, Berks) (12). It has previously been demonstrated that the area and volume of bone resorbed are closely correlated, and that the area of resorption is a reliable measure of total resorption (10). The absence of significant contamination of bone- osteoclast preparations was demonstrated by a lack of the effect of parathyroid hormone (0.1 U/ml) on bone resorption in these cultures (11).

Measurement of acid phosphatase activity: Total activity was measured by incubating samples (150 µl) of osteoclast-exposed medium in 4-nitrophenyl phosphate (final concentration 10 mM in a total volume of 450 µl in 100 mM citrate buffer; pH 5.0). The reaction was stopped and the coloured quinonoid form developed by adding NaOH (200 µl) before absorptiometry (405 nm). Catalytic activity concentration (µmol substrate hydrolysed per minute (U) per litre sample was calculated from the absorbance coefficient of coloured 4-nitrophenate, $\varepsilon = 1.85\ l \times mmol^{-1} \times mm^{-1}$.

Anti-uteroferrin antiserum: An antiserum against purified porcine uteroferrin (an iron containing protein with acid phosphatase activity) was raised in rabbits and purified by specific immunoaffinity chromatography as described previously (8). The antiserum had been shown to cross-react completely with human tartrate-resistant acid phosphatase from Gaucher's disease spleen, with alveloar macrophages and with increased circulating acid phosphatase activity in pateints with osteoclastic bone disease. SDS-polyacrylamide gel electrophoresis of an extract of Gaucher's disease spleen confirmed that the antibody reacted only with the 37 kDa fraction corresponding to acid phosphatase (8). The antibody-isoenzyme complex has been shown to have reduced catalytic activity. The purified antibody or antiserum was specific for the osteoclastic isoenzyme and did not effect the catalytic activity of other acid phosphatase isoenzymes, including the tartrate-resistant erythrocyte form (8): this specificity has allowed us to differentiate between osteoclastic and erythrocyte isoenzymes found in culture medium.

RESULTS AND DISCUSSION

Anti-porcine uteroferrin antiserum was found to inhibit bone resorption by as much as 98% whereas non-immune rabbit serum had no effect (Figure 1). Similarly the addition of immunopurified anti-uteroferrin antibodies resulted in a marked inhibition of bone resorptive activity ($p \leq 0.001$) (Figure 1). The total acid phosphatase activity in the medium was also reduced from 3.38

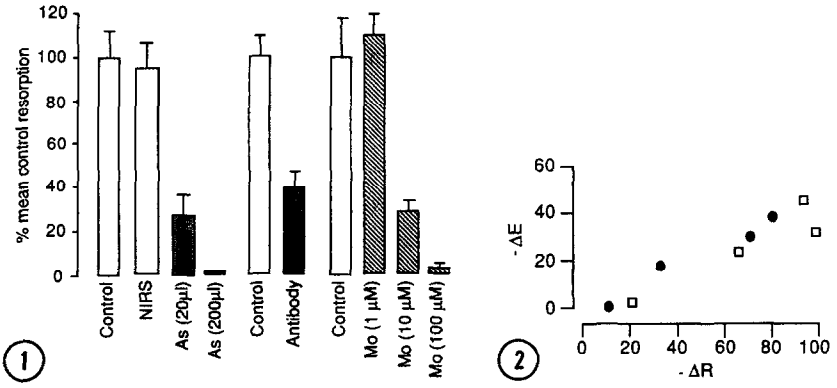

FIGURE 1

Effect of anti-porcine uteroferrin antiserum (As) (20 µl and 200 µl), affinity-purified antibody (Antibody; 60 µg/ml and molybdate ions (Mo; 1, 10 and 100 µM) on the area of bone resorbed by isolated rat osteoclasts, expressed as a percentage of mean control resorption. NIRS: non-immune rabbit serum.

FIGURE 2

Correlation between the percentage fall in measured resorption (per bone slice) (-ΔR) and the percentage fall in supernatant enzyme concentration (-ΔE) using affinity-purified anti-porcine uteroferrin antibody (closed circles) and molybdate ions (open squares).

to 2.14 U/l and the tartrate-resistant activity from 2.93 to 1.95 U/l. Addition of the phosphatase inhibitor molybdate to the bone-osteoclast cultures at a concentration of 100 µM abolished bone resorption (Figure 1) and reduced tartrate-resistant acid phosphatase activity by about 40% (from 3.66 to 2.10 IU/l, maintaining the molybdate concentration at 100 µM). The data from both immunological and chemical inhibition shows a significant correlation ($r = 0.78$; $p \leq 0.01$) between the inhibition of bone resorption and the reduction of supernatant enzyme concentration (Figure 2). The replication of the inhibitory effect of the antibody by the phosphatase inhibitor molybdate is good evidence against a non-specific effect.

We confirmed that the anti-porcine uteroferrin antibody reacted with with rat osteoclastic acid phosphatase in the following way. Osteoclasts were allowed to settle on a polystyrene surface and the supernatant medium was recovered after incubation at 37 °C. Following a 2 hour incubation with Sepharose 4B-bound antibody (8), approximately 93% of the tartrate-resistant acid phosphatase activity was removed from the medium; the unbound fraction is presumed to be to unreactive lysosomal acid phosphatase. We also confirmed that the effects of the antiserum, purified antibody and molybdate were not toxic: on light microscopy the osteoclasts dispersed on bone did not show any signs of cytotoxicity, and the numbers of cells were not significantly different with those found on control (untreated) bone slices [cells per slice ± SEM; control 4.5 ± 0.8, antiserum (200 µl in 2 ml) 5.0 ± 0.6; control 3.6 ± 0.9, purified antibody 2.8± 0.8; control 6.9 ± 1.2, molybdate ions (100 µM) 6.4 ± 0.8].

Osteoclasts resorb bone by settling on bone surfaces and secreting acid, enzymes and free redicals into the space between the bone and cell. The enzymes include proteolytic enzymes in addition to acid phosphatase. Our results clearly demonstrate that the inhibition of osteoclastic acid

phosphatase activity is accompanied by a marked reduction of bone resorption. Admittedly the measured concentration of acid phosphatase in the culture medium might not precisely reflect levels of the enzyme at the bone-cell interface. However the inhibition of resorption by a specific antibody that reduces the number of active molecules present, aswell as by molybdate, an inhibitor of phosphatase activity suggests that the enzyme has a primary role in the process of resorption by osteoclasts.

The nature of the proposed role of acid phosphatase in bone resorption is uncertain. However, the enzyme is also a pyrophosphatase (6) and thus is probably responsible for the removal of pyrophosphate. This compound is a potent inhibitor of apatite solubilisation, the essential first step in bone resorption. It seems possible that the inhibition of this step of pyrophosphate removal by acid phosphatase antiserum and molybdate may explain the inhibition of resorption observed. Free radical scavengers, without a known effect on acid phosphatase, can also inhibit bone resorption (13), implying that acid phosphatase activity, although essential, is not sufficient for resorption. Further it is conceivable that our findings may partly explain the action of diphosphonates: some of these are potent inhibitors of bone resorption and are also potent inhibitors of acid phosphatase activity (14). For example, 3-amino-1-hydroxy-propylidene-1,1-bisphosphonate (APD) at a concentration of 12 mM and a substrate concentration of 1 mM reduces the activity of human iron-containing acid phosphatase by 80% in our assay (unpublished observations). If correct, our hypothesis may lead to the design of novel compounds of improved activity on inhibition of the enhanced osteoclastic activity responsible for bone loss in osteoporosis.

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