Journal of Cellular Biochemistry 54:145–153 (1994)

Tartrate Resistant Acid Phosphatase Activity in Rat Cultured Osteoclasts Is Inhibited by a Carboxyl Terminal Peptide (Osteostatin) From Parathyroid Hormone-Related Protein

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Abstract A carboxyl-terminal peptide sequence ("osteostatin") from parathyroid hormone related protein has been shown to have an inhibitory effect on osteoclastic bone resorption—an action opposite to its amino-terminal sequence. In this study, we proposed that inhibition of osteoclastic bone resorption by osteostatin was associated with reduction of tartrate resistant acid phosphatase (TRAcP) activity in osteoclasts. Our results have indicated that osteostatin reduced TRAcP activity in a dose dependent manner. This effect of osteostatin was both sensitive (half maximal effect approximately 5×10^{-13} M) and potent (maximum inhibition approximately 50% of control). In the first 90 min of treatment, however, reduction of TRAcP activity was erratic but became persistent and progressive when the time course was extended. Moreover, throughout the experimental period the levels of TRAcP activity in the culture medium had fallen significantly. It appears that osteostatin has a biphasic effect on TRAcP activity, inhibiting its secretion and either suppressing its synthesis or increasing its degradation. In addition, osteostatin induced rapid cellular retraction of both human and rat cultured osteoclasts, which was morphologically distinct from that produced by calcitonin.

Key words: osteoclast, tartrate resistant acid phosphatase, parathyroid hormone-related protein, osteocalcin

Tartrate resistant acid phosphatase (TRAcP) is one of the key lysosomal enzymes in osteoclastic bone resorption [1–4]. It has been detected in the lysosomes of osteoclasts, at their ruffled border as well as in the immediate microenvironment where bone resorption is occurring [2,5,6,7]. Modulation of osteoclast activity by certain hormones is reflected in the level of TRAcP activity. For example, inhibition of osteoclast activity by calcitonin resulted in reduced TRAcP activity [1,7,8], while direct inhibition of TRAcP activity in vitro, by an anti-TRAcP antibody or molybdate, reduced osteoclast bone resorption by more than 90% [4].

Parathyroid hormone-related protein (PTHrP) is a single chain peptide of 139, 141, or 173 amino acids with a pre-pro sequence of 36 amino acids [9,10]. All forms of human PTHrP are identical up to position 139, but each has a unique carboxyl terminal sequence [11–13]. The structural similarity between parathyroid hormone (PTH) and PTHrP is confined to the amino terminal portion of the molecule with 8 of the first 13 amino acids being identical. This region contains the site of biological activity of PTH and the similarity is sufficient to allow PTHrP to exert PTH-like effects by interacting with PTH receptors on osteoblasts to stimulate osteoclastic bone resorption [11–13]. Beyond this there is no significant homology between the two molecules [14–17]. Recent work has demonstrated that a carboxyl terminal sequence (PTHrP [107–139] osteostatin) inhibited bone resorption in the isolated neonatal rat osteoclast resorbing pit assay [18,19].

In this study, we proposed that inhibition of osteoclastic bone resorption by osteostatin may be associated with inhibition of synthesis and/or

Received April 12, 1993; revised August 27, 1993; accepted September 1, 1993.

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secretion of TRAcP in osteoclasts. Osteoclasts were isolated from both rat long bones and human giant cell tumour of bone. Time lapse micrography was used to examine osteoclast retraction induced by osteostatin. Biochemical and quantitative cytochemical assays were used to detect the extracellular and intracellular TRAcP activity in rat cultured osteoclasts. Our results indicate that osteostatin reduces TRAcP activity in rat osteoclasts. Furthermore, osteostatin induced cytoplasmic retraction of both human and rat cultured osteoclasts.

MATERIALS AND METHODS

Chemicals

Osteostatin was obtained from Professor B.E. Kemp, University of Melbourne. The sequence is from position 107 to 139 of human PTHrP and has the following composition: Thr Arg Ser Ala Trp Leu Asp Ser Gly Val Thr Gly Ser Gly Leu Glu Gly Asp His Leu Ser Asp Thr Ser Thr Thr Ser Leu Glu Leu Asp Ser Arg-COOH. The molecular weight of osteostatin is approximately 3,700 Da [18]. Nitrophenyl phosphate (disodium salt, anhydrous) was obtained from ICN Biomedicals. Naphthol-ASBI-phosphate and dimethyl formamide were from Sigma Chemical Co., St. Louis, MO. Tissue culture chamber slides were obtained from Lab-tek (Australia). Minimum essential medium (MEM), medium 199 with Earle's salts (M199), and fetal bovine serum (FBS) were obtained from ICN, Australia. All other chemicals used were of the highest biochemical grade available and were purchased from standard suppliers.

Osteoclast Preparations

Rat osteoclasts were isolated from 1-day-old Wistar rats as previously described [20]. In brief, the long bones were removed then placed in M199 (6 bones per ml, at 4°C) in a 30 mm culture dish. The bones were cut across at the diaphysis, split longitudinally, and then finely chopped. The resulting suspension was gently triturated with a wide bore pipette to encourage osteoclasts to dissociate from the bone surface. The cells were incubated at 37°C in humidified air for 30 min to allow osteoclasts to settle and adhere to the slide. Cultures were then rinsed with MEM (37°C) to remove non-adherent cells. Osteoclast cultures were then incubated for a further 30 min in 95% air /5% CO_2 with MEM before experimentation.

Human osteoclast-like cells were obtained from an osteoclastoma removed from a 25-year-old woman. Tumour tissue containing both osteoclast-like cells and tumour cells was chopped in M199 as previously described [21]. The cell suspensions were pipetted into the wells of chamber slides. After incubation at 37°C for 60 min, the cultures were washed to remove non-osteoclast-like cells, including tumour cells. They were then incubated at 37°C in MEM for a further 30 min before experimentation.

Time-Lapse Micrography

An inverted microscope and camera with variotimer (Nikon, Japan) was used to examine osteoclast retraction induced by osteostatin. Fields were chosen near the middle of the well to minimise edge effects and filmed on Kodak T64 film with 10 min lapse between exposures.

Biochemical Assay of Extracellular TRAcP Activity

Total activity of TRAcP in the culture media was measured by incubating 150 μl of osteoclast conditioned medium with 450 μl of 10 mM 4-nitrophenyl phosphate (40 mg of 4-nitrophenyl phosphate dissolved in 10 ml of citrate/tartrate buffer; 100 mM/100 mM, pH 5.0) at 37°C for 1 h [4]. The reaction was stopped and the coloured quinonoid form of the reaction product developed by the addition of 200 μl 0.2 M sodium hydroxide. The absorbance of the reaction product of TRAcP activity was measured with a Pharacia Ultrospec III UV/Vis spectrophotometer. Three osteoclasts cultures (approximately 100 osteoclasts each culture) in each experimental group were assessed.

Quantitative Cytochemical Assay of Intracellular TRAcP Activity

Quantitative cytochemical assay was used to assess TRAcP activity within osteoclasts. Four miligram Naphthol-ASB1 phosphate was solublized in 0.3 ml dimethyl formamide and then made up to 20 ml using 100 mM/100 mM acetate/tartrate buffer. Fast Garnet GBC (0.5 mg/ml) was added as a colour conjugate [22]. Osteoclasts adherant to chamber slides were incubated in the medium for 20 min at 37°C. The enzyme reaction was stopped by fixation of slides with 1% paraformaldehyde. The reaction product which is directly related to the activity of TRAcP within the cell was measured using a cytoscan microscope interfaced to a chromatic image

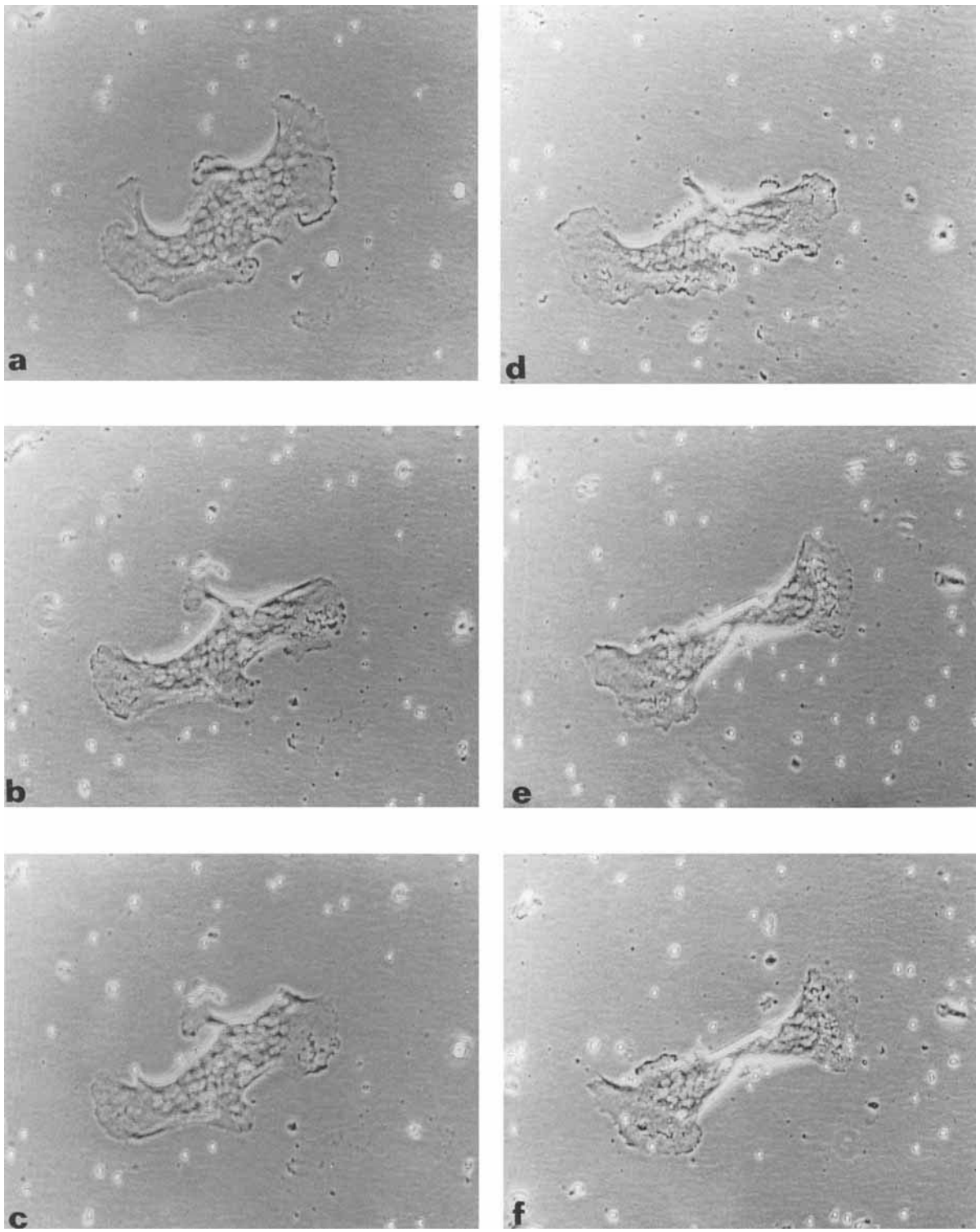

Fig. 1. Time-lapse cinematography of human osteoclast retraction induced by osteostatin at a concentration of 5×10^{-13} M. (**a**) Osteoclast before addition of osteostatin; (**b**) 20 min after adding osteostatin; (**c**) 30 min after adding osteostatin; (**d**) 50 min after adding osteostatin; (**e**) 90 min after adding osteostatin; (**f**) 180 min after adding osteostatin. ×400. Micrography were obtained from one of duplicate experiments.

analysis system (Leica, Australia) at a wavelength of 560 nm. Results for each osteoclast were expressed as the integrated optical density (IOD) divided by the number of nuclei with it [21,22]. Fifty osteoclasts in each experimental group were assessed.

Statistical Analyses

Normality of the data was confirmed by calculation of skewness and kurtosis before the use of standard test. Student's t test and regression analysis of the data were performed using a computer statistical package.

RESULTS

Osteoclast Retraction

The morphological changes induced by osteostatin were similar in rat and human osteoclasts. Figure 1 shows that a human osteoclast treated with osteostatin at concentration of 10^{-13} M losses its intense lamellipodal ruffling activity within 10 min. After 20 min treatment, reduction of spreading area was observed while the central cell body had gradually retracted (Fig. 1). Measurement of spreading area by a computer microreading program indicated that the osteoclast reduced about 50% of spreading area at 180 min after adding osteostatin (Fig. 2). The morphological characteristics of osteoclast retraction induced by osteostatin differed from those induced by calcitonin (Fig. 3). Compared to controls, over 70% (51/70) of rat osteoclasts treated with osteostatin displayed rounded cell bodies and relatively smooth peripheries, while their nuclei were tightly clumped (Fig. 3b). On the other hand, although osteoclasts treated with calcitonin also retracted, they demonstrated characteristically long thin filopodia which radiated to the substratum (Fig. 3c).

Dose-Response of Osteostatin Effect on TRAcP Activity in Rat Osteoclasts

Statistical analyses indicated that the distribution of TRAcP activity in both the control and test groups was approximately normal (skewness, −0.79; kurtosis, −0.16). Moreover, under basal conditions, TRAcP activity did not vary significantly in osteoclasts within the experimental period. Quantitative cytochemical assay showed that the activity of intracellular TRAcP responds to osteostatin in a biphasic fashion (Fig. 4A). Low doses of osteostatin (between 1×10^{-15} M and 1×10^{-13} M) resulted in a

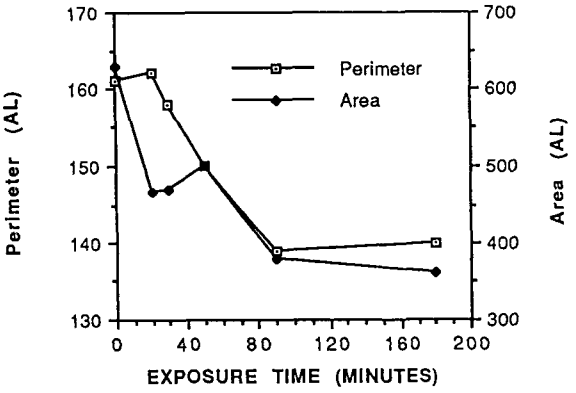

Fig. 2. Change of area and perimeter of the osteoclast showed in Figure 1. AL is arbitrary length of microreading program.

reduction in TRAcP activity in the order of 20%, but this did not vary significantly with increasing concentrations of osteostatin up to 1×10^{-13} M. Higher doses between 1×10^{-12} M and 1×10^{-10} M resulted in approximately 50% of maximum reduction in TRAcP activity. Biochemical assays showed that treatment with osteostatin at concentrations between 10^{-15} M and 10^{-12} M resulted in a decrease in extracellular TRAcP activity in a dose dependant manner, with a maximum decrease of around 40% at concentration of 10^{-12} M compared to controls (Fig. 4B). It appears that the ED_{50} is approximately 5×10^{-13} M.

Time Course of Osteostatin Effect on TRAcP Activity in Rat Osteoclasts

Short-time course studies of osteoclasts exposed to osteostatin for periods between 10 to 90 min showed erratic variations in intracellular TRAcP activity detected by quantitative cytochemical assay (Fig. 5A). The mean values of IOD/No. of nuclei showed a sharp decrease in intracellular TRAcP activity followed by an increase in activity over exposure periods between 40 and 90 min (Fig. 5A). However, continued exposure to osteostatin for 120 min resulted in a reduction in TRAcP activity of 17% when compared to the controls, and after 3 h exposure the level of activity had dropped to 20% below control values. The biochemical assays of extracellular TRAcP activity in the media collected from the cultures which had been exposed to osteostatin between 10 and 180 min showed a reduction in activity when compared to the controls, but the levels were variable during the experimental period (Fig. 5B). Regression analysis demonstrates a trend towards a reduction in

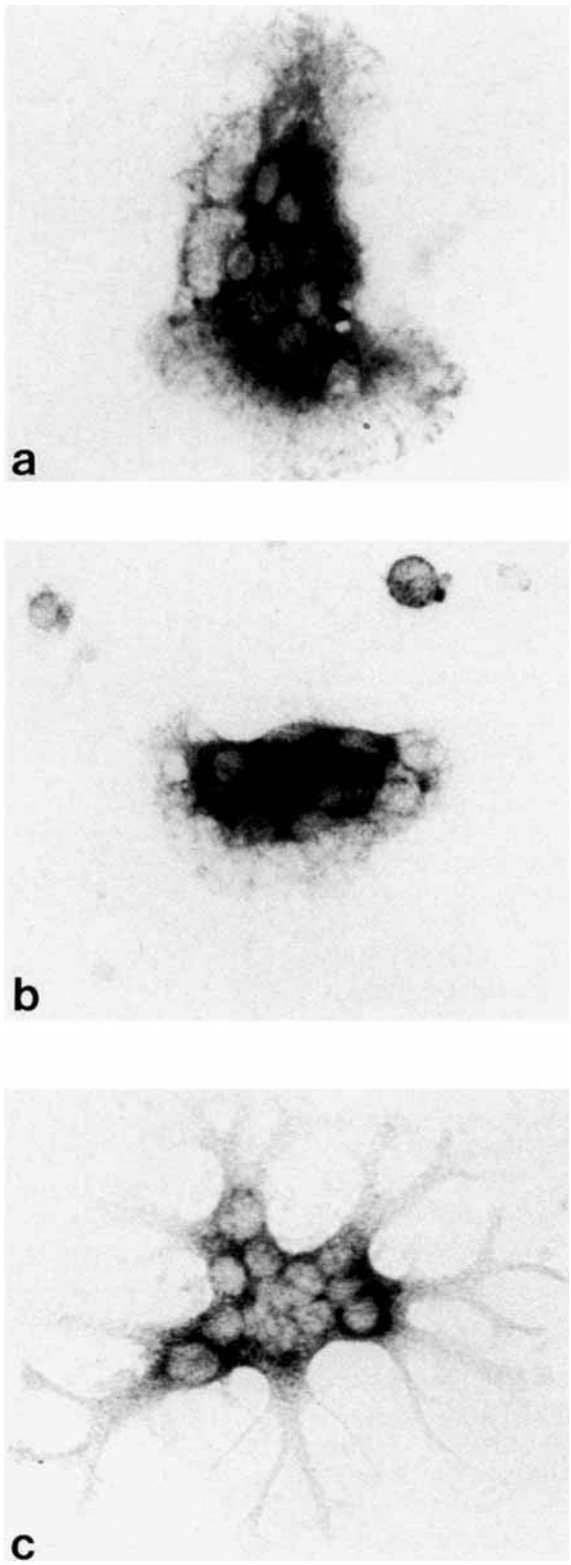

Fig. 3. Comparison of osteoclast retraction induced by osteostatin and calcitonin. (**a**) Rat osteoclasts with vehicle; (**b**) rat osteoclasts treated with osteostatin at a concentration of 10^{-10} M for 4 h showed reduction of spread area while the nuclei became tightly clumped; (**c**) rat osteoclasts treated with salmon calcitonin at a concentration of 10^{-10} M for 4 h notes the long thin filopodia. ×400.

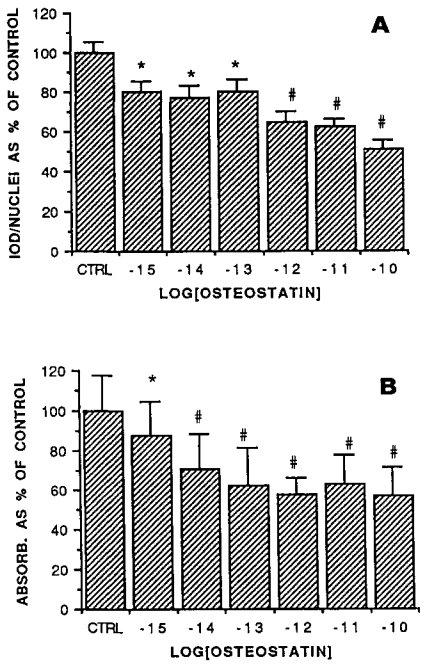

Fig. 4. Dose-response of osteostatin effect on both intracellular (A) and extracellular (B) TRAcP activities. Rat osteoclasts were incubated on chamber slides for 24 h with osteostatin before assays. Points represents mean ± SEM. *, $P < 0.05$ (vs. control), #, $P < 0.01$ (vs. control). The data were obtained from one of two triplicated experiments.

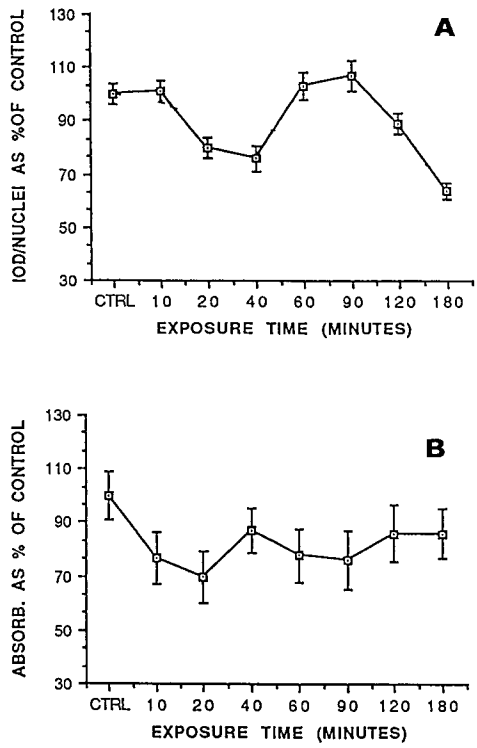

Fig. 5. Short-time course studies of osteostatin effect on both intracellular (A) and extracellular (B) TRAcP activities. Rat osteoclasts were incubated on chamber slides with osteostatin at a concentration of 5×10^{-13} M for period between 10 to 180 min before assay. Points represents mean ± SEM. The data were obtained from one of triplicated experiments (rA = 0.5, rB = 0.45).

TRAcP activity over the total time of the experiment (rA = 0.5; rB = 0.45).

Long-time course studies of intracellular TRAcP activity showed that following extended periods of exposure to osteostatin the activity of TRAcP within the cell fell gradually (Fig. 6A). A maximum reduction in activity was observed after 24 h exposure and a 50% decrease was seen when compared to controls. The biochemical assay of extracellular activity showed a gradual reduction in TRAcP activity over exposure times of 4 to 48 h. A maximum reduction in activity of 50% was observed after 48 h exposure (Fig. 6B).

DISCUSSION

In this study, the kinetics of osteostatin action on both intracellular and extracellular TRAcP activities have been determined by cytochemical and biochemical assays. The results indicated that osteostatin reduces TRAcP activity progressively in a dose-dependent manner. This effect of osteostatin was both sensitive (half maximal effect approximately 5×10^{-13} M) and potent (maximum inhibition approximately 50% of control). In the time-course studies, although erratic variation of TRAcP activity in both biochemical and quantitative cytochemical assays was seen in osteoclasts exposed to osteostatin between 10 to 90 min, regression analysis showed that osteostatin gradually induced a decrease in TRAcP activity. Treatment with osteostatin for longer time periods resulted in TRAcP activity declining in both the culture media and the cells, suggesting that osteostatin persistently inhibits TRAcP activity by suppression of its synthesis or increase in degradation. These findings may reflect a biphasic effect of osteostatin on osteoclast TRAcP activity. Possibly the initial response to osteostatin is to reduce both secretion and synthesis of TRAcP and is then followed by an inhibition of synthesis or increase in its degradation. Investigation of osteostatin effect on TRAcP gene transcript in osteoclasts is in the process in our laboratory in order to determine any inhibitory effects on TRAcP synthesis.

Our results have also indicated that osteostatin produces cellular retraction in both

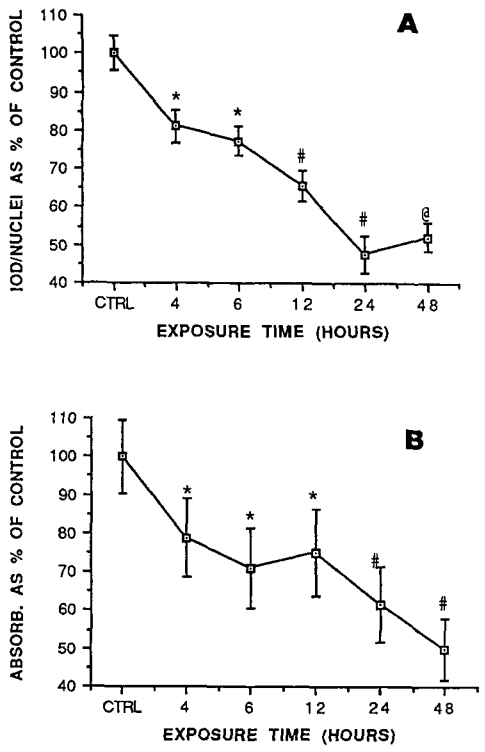

Fig. 6. Long-time course studies of osteostatin effect on both intracellular (A) and extracellular (B) TRAcP activities. Rat osteoclasts were incubated on chamber slides with osteostatin at a concentration of 5×10^{-13} M for period between 4 to 48 h before assay. Points represents mean ± SEM. *, $P < 0.01$ (vs. control). @, $P = 0.72$ (vs. 24 hours). The data were obtained from one of triplicated experiments.

cultured human and rat osteoclasts. Interestingly, the morphology of the retraction is distinct from that seen in osteoclasts treated with calcitonin. The osteostatin treated osteoclasts displayed round, smooth cellular bodies with relatively smooth peripheries, while their nuclei were tightly clumped together. Furthermore, it appears that osteostatin does not affect the motility of osteoclasts (Holloway and Nicholson, unpublished data). Calcitonin treated osteoclasts, on the other hand, demonstrated long thin filopodia which radiated to the substratum. There is no clear explanation for these differences. It appears that the manner in which osteostatin affects the cytoskeletal system differs from that triggered by calcitonin [23]. The signal transduction mechanisms operating for osteostatin in osteoclasts are distinct from those for calcitonin [18]: there is no elevation of cAMP in response to osteostatin; both inorganic and organic calcium channel blockers fail to inhibit the effect of osteostatin; it appears that protein kinase C mediates the action of osteostatin in osteoclasts. By contrast, protein kinase A and calcium calmodulin kinase are involved in the action of calcitonin on osteoclasts [23].

The PTHrP gene is a complex transcriptional unit that by alternative splicing gives rise to messenger RNAs encoding different peptides [24]. Moreover, there are many potential proteolytic sites within the PTHrP molecule and there is evidence for cleavage products in the circulation [12,25–28]. Studies by Fenton et al. [19] have indicated that the osteoclast-inhibitory activity was localised to the [107–111] sequence at the carboxyl-terminal end of PTHrP. Interestingly, this sequence, TRSAW, is a very highly conserved pentapeptide present in human, mouse, rat, and chicken [27]. The native chicken PTHrP [107–111] pentapeptide, ARSAW, has also been shown to inhibit osteoclast bone resorption [18]. It appears that PTHrP contains peptide sequences with opposing actions, the N-terminal portion of PTHrP has a indirect stimulatory effect on osteoclasts via osteoblasts while the C-terminal portion appears to directly inhibit osteoclast activity [19].

Although PTHrP was originally cloned from a human malignant tumour associated with hypercalcemia, it has also been found in numerous normal adult and fetal tissues [29]. Expression of PTHrP mRNA was found in bone cells in areas of periosteum in rat fetal bones [30]. The release of PTHrP by fetal bones in culture was increased by PTH and 1,25-dihydroxyvitamin D3, and decreased by calcitonin [31]. Since PTHrP could be processed to N-terminal and C-terminal fragments with opposing actions, it suggests that PTHrP may play an important role in local bone turnover during bone development.

In summary, we have shown that osteostatin acts on rat osteoclasts to reduce TRAcP activity. Furthermore, osteostatin administration is associated with cellular retraction in both human and rat osteoclasts which is distinct from osteoclast retraction induced by calcitonin.

ACKNOWLEDGMENTS

This work was supported by grants from the National Health and Medical Research Council of Australia the Sir Charles Gairdner Hospital Research foundation and the Medical Research Fund of Western Australia.

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