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The effects of selective inhibitors of matrix metalloproteinases (MMPs) on bone resorption and the identification of MMPs and TIMP-1 in isolated osteoclasts

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SUMMARY

We have compared the effects of a general matrix metalloproteinase (MMP) inhibitor (CT435) with those of a concentration-dependent specific gelatinase inhibitor (CT543; K_i<20 nM) on bone resorption in vitro. The test systems consisted of measuring: (i) the release of $^{45}Ca^{2+}$ from prelabelled mouse calvarial explants; (ii) the release of $^{45}Ca^{2+}$ from prelabelled osteoid-free calvarial explants co-cultured with purified chicken osteoclasts; and (iii) lacunar resorption by isolated rat osteoclasts cultured on ivory slices.

Both CT435 and CT543 dose-dependently inhibited the release of $^{45}Ca^{2+}$ from neonatal calvarial bones stimulated by either parathyroid hormone or 1,25-dihydroxyvitamin D_3. Moreover, CT543 produced a 40% inhibition at a concentration (10^{-8} M) selective for the inhibition of human gelatinases A and B. CT435 (10^{-5} M) and CT543 (10^{-5} M) partially inhibited the release of $^{45}Ca^{2+}$ from osteoid-free calvarial explants by chicken osteoclasts with a maximum of approximately 25% for unstimulated cultures, and approximately 36% for cultures stimulated by interleukin-1α (IL-1α; 10^{-10} M). Neither inhibitor prevented lacunar resorption on ivory by unstimulated rat osteoclasts, but the compounds produced a partial reduction in both the number and total surface area of lacunae in IL-1α-stimulated cultures, with maximal action at 10^{-5} M. Neither of the inhibitors affected protein or DNA synthesis, nor the IL-1α-stimulated secretion of the lysosomal enzyme β-glucuronidase.

Immunocytochemistry demonstrated that isolated rabbit osteoclasts constitutively expressed gelatinase A and synthesized gelatinase B, collagenase and stromelysin, as well as the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) following IL-1α stimulation.

These experiments have shown that in addition to collagenase, gelatinases A and B are likely to play a significant role in bone resorption. They further suggest that MMPs produced by osteoclasts are released into the sub-osteoclastic resorption zone where they participate in bone collagen degradation.

Key words: MMP, inhibitor, osteoclast

INTRODUCTION

Osteoclasts are the principal resorptive cells in the skeleton, degrading both the mineral and organic constituents of bone in a specialized extracellular compartment, the subosteoclastic resorption zone (Baron et al., 1985; Blair et al., 1986). Solubilization of the mineralized matrix is due to secretion of acid into this region (Silver et al., 1988), whilst degradation of the organic matrix (mainly type I collagen) is due primarily to the activities of proteolytic enzymes that are considered to belong to two major classes, the cysteine proteinases (CPs; reviewed by Delaissé and Vaes, 1992) and matrix metalloproteinases (MMPs; Everts et al., 1992). The MMP family consists of three major subgroups, the interstitial collagenases, the gelatinases and the stromelysins; these are zinc-dependent endopeptidases with the combined ability to degrade the organic components of connective tissue matrices at physiological pH (Murphy and Reynolds, 1993). MMPs are secreted as proenzyme forms, require extracellular activation and are regulated by secreted inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), a multigene family of which TIMP-1 (Docherty et al., 1985) and TIMP-2 (Stetler-Stevenson et al., 1990) are well characterized. An association between collagenase (MMP-1) and bone resorption was initially suggested on the basis of its specificity for type I collagen and the release of the enzyme from bone explants during the resorption process (Sellers et al., 1978; Lenaers-Claeys and Vaes, 1979; Francois-Gillet et al., 1981). This was supported by the demonstration that hormones

and cytokines that stimulate bone resorption induce collagenase synthesis by cultured osteoblast-like cells (Heath et al., 1984; Sakamoto and Sakamoto, 1984; Otsuka et al., 1984), and promote accumulation of the enzyme within bone matrix (Eeckhout et al., 1986; Delaissé et al., 1988). Further evidence was provided when it was shown that both synthetic MMP inhibitors (CI-1; Delaissé et al., 1985; Everts et al., 1992) and natural inhibitors of MMPs (TIMPs -1 and -2; Hill et al., 1993) prevent bone resorption in vitro. Osteoblasts also produce gelatinases A (MMP-2) and B (MMP-9; Rifas et al., 1989; Meikle et al., 1992), enzymes that are capable of degrading denatured type I collagen (Murphy et al., 1985; Wilhelm et al., 1989) and could also participate in the resorption process.

Osteoblast-derived collagenase is thought to be primarily involved in degradation of the unmineralized surface osteoid layer of bone, thereby exposing the underlying mineralized matrix to osteoclastic action (Chambers et al., 1985; Delaissé et al., 1988). Recent morphological studies have suggested that MMPs may also contribute to osteoclast-mediated lacunar resorption (Shimizu et al., 1990; Everts et al., 1992). However, since bone matrix was used as a substrate in these studies, it remains uncertain whether the MMPs were secreted by osteoclasts or released from the bone during osteoclastic dissolution of the mineralized matrix as suggested previously (Eeckhout et al., 1986; Delaissé et al., 1988). Collagenase has recently been detected within rodent osteoclasts (Delaissé et al., 1993) but it has not been established whether this osteoclast-derived enzyme participates in the subosteoclastic degradation of collagen.

The aim of the present investigation was to determine the roles and sites of action of gelatinase and collagenase in bone resorption using a general MMP inhibitor (CT435) and a concentration-dependent specific gelatinase inhibitor (CT543). The culture systems consisted of: (1) neonatal calvarial explants; (2) highly enriched populations of chicken osteoclasts cultured on osteoid-free calvarial explants; and (3) isolated rat osteoclasts cultured on ivory slices. Finally, by means of indirect immunofluorescence, we investigated whether isolated rabbit osteoclasts produce interstitial collagenase, gelatinases A and B, stromelysin (MMP-3) and TIMP-1.

We provide direct evidence for a role of gelatinases A and B in bone resorption and show that MMPs are produced by isolated osteoclasts and participate in osteoclast lacunar resorption.

MATERIALS AND METHODS

Materials

The MMP inhibitors CT435 (N_1-[*N*-(2-phenylethyl)-3-cyclohexyl-2-(*S*)-propanamidyl]-N_4-hydroxy-2-(*R*)-(2-methylpropyl) succinamide) and CT543 (N_1-[*N*-(2-morpholinoethyl)-3-cyclohexyl-2-(*S*)-propanamidyl]-N_4-hydroxy-2-(*R*)-(3-phenylpropyl) succinamide) were synthesized by Celltech, Slough, UK. Synthetic human parathyroid hormone (PTH-(1-84)) was purchased from the Division of Biological Standards, National Institute of Medical Research, Mill Hill, UK. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and recombinant human interleukin-1α (IL-1α) were generous gifts from Dr Ian Dickson, Brunel University, Uxbridge, UK and Dr J. Saklatvala, The Babraham Institute, Babraham, Cambridge, UK. Actinomycin D, L-proline, thymidine, indomethacin, alpha-minimal essential medium (α-MEM) and 4-methylumbelliferone were purchased from Sigma Chemical Co., Poole, Dorset, UK. $^{45}CaCl_2$, [*methyl*-^{3}H]thymidine and L-[5-^{3}H]proline were purchased from Amersham International plc, Buckinghamshire, UK. Modified BGJ medium was obtained from Flow Laboratories, Irvine, UK.

MMP inhibitors and their characterization

The inhibitory effects of CT543 and CT435 against the respective human recombinant matrix metalloproteinases were assessed using the synthetic peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 as described (Knight et al., 1992). CT543 has the following K_i values against the MMPs: gelatinase A, 0.14 nM; gelatinase B, 1.9 nM; stromelysin, 24.8 nM; collagenase, 210 nM. CT435 is a general MMP inhibitor with the following K_i values against the MMPs: gelatinase A, 0.33 nM; gelatinase B, 1.47 nM; collagenase, 7.8 nM; stromelysin, 25.3 nM. The conclusions from our experiments using the MMP inhibitors are based on the extrapolation of the K_i values from human enzymes to those of rodent and chicken, as it has not been possible to obtain non-human recombinant enzymes.

Murine calvarial explants

Bone resorption was assessed by analyzing either $^{45}Ca^{2+}$ or [^{3}H]proline release from cultured neonatal mouse calvarial bones as described (Reynolds and Dingle, 1970). Briefly, 1-day-old mice were injected subcutaneously with either 0.074 MBq of $^{45}CaCl_2$ or 0.37 MBq of [^{3}H]proline. After 6 days the calvariae were excised and the posterior two-thirds of the parietal bones were dissected into four pieces and precultured in modified BGJ medium (1 ml) containing 26 mM $NaHCO_3$, 0.85 mM ascorbic acid, 1.4 mM L-glutamine, 5% acid-treated rabbit serum (this contains no detectable proteinase inhibitors or α_2-macroglobulin) and indomethacin (1 μM) for 24 hours (Lerner, 1987; Ljunggren et al., 1991). Bones that were stimulated with $1,25(OH)_2D_3$ or PTH were also treated with the hormones during this initial period. Paired explants were subsequently cultured in fresh modified BGJ medium (1 ml) containing the previous additions without indomethacin. The bones were stimulated with one of the osteotropic hormones in the presence and absence of the MMP inhibitors. Mobilization of radioactivity was expressed as the percentage release of total isotope (calculated as the sum of radioactivity in medium and bone after culture). To determine radioisotope release due to passive exchange of isotope with either cold Ca^{2+} or proline in the culture medium, four parietal bone quarters from each litter were devitalized by 3 cycles of freeze-thawing. The mean percentage release from the devitalized bone was subtracted from each living bone to give the amount of cell-mediated resorption (CMR).

Protein and DNA synthesis were assessed by incubating calvarial halves for 24 hours in BGJ medium (1.5 ml) with and without test substances, and labelling the bones during the last 6 hours with either 0.1 MBq [^{3}H]proline (protein synthesis) or 0.18 MBq [^{3}H]thymidine (DNA synthesis). Actinomycin D was used as a control for protein synthesis and hydroxyurea for DNA synthesis. The lysosomal enzyme, β-glucuronidase, that was released into the medium was determined fluorimetrically using 4-methylumbelliferyl-β-D-glucuronide as the substrate (Achord et al., 1978; NBS Biologicals, Hatfield, Herts). One unit of activity represents the amount of enzyme catalyzing the release of 1 nmole of 4-methylumbelliferone per hour.

Preparation of radiolabelled osteoid-free calvarial bone

Calvariae were dissected from 8-day-old mice that had been injected subcutaneously 6 days previously with 0.18 MBq of $^{45}CaCl_2$. The periosteum, endosteum and lining cells were removed exposing the osteoid layer. The parietal bone was cut into two equal halves and the explants were incubated in α-MEM containing collagenase (1 mg/ml) at 37°C in a humidified atmosphere of 5% CO_2/95% air for 30 minutes to remove the osteoid layer as described (Chambers et al., 1985). The bones were then washed three times in α-MEM to remove exogenous collagenase.

Isolation and culture of chicken osteoclasts on osteoid-free bone

Chick long bones are rich in osteoclasts and frequently used as a source of these cells (Zambonin-Zallone et al., 1982; Osdoby et al., 1982). Enriched cultures of chicken osteoclasts were established using modifications of the method of Blair et al. (1986). The long bones from 17-20 day embryonic chicks were cleaned of adherent soft tissues and placed in ice-cold Ca^{2+}- and Mg^{2+}-free PBS, pH 7.2, containing 0.2% BSA; all subsequent steps using PBS were performed at 4°C. The bones were split to expose the marrow, rinsed three times with PBS, and the endosteum scraped with a scalpel blade into 2.5 ml PBS. The cell suspension was passed in succession through calibrated dental needles of size 20 and 30. The resultant osteoclast suspension was layered onto a seven-step (2.5 ml each, 1.01 to 1.07 g/ml) Percoll gradient (Pharmacia AB, Uppsala, Sweden), centrifuged for 20 minutes at 400 ***g***, and the fractions collected by aspiration. The 1.04 g/ml fraction was washed twice with α-MEM supplemented with 10% fetal calf serum (FCS); up to 60% of the cells were multinucleated osteoclasts. Osteoclasts were plated in 24-well plates (Linbro, Flow Laboratories, 50,000 cells/well), each well containing a piece of $^{45}Ca^{2+}$ osteoid-free parietal bone. The bones were cultured in pairs in 1 ml α-MEM containing 10% acid-treated rabbit serum at 37°C in a humidified atmosphere of 95% air/5% CO_2 for 72 hours with and without either CT435 or CT543. To determine $^{45}Ca^{2+}$ release due to passive exchange of isotope with cold Ca^{2+} in the culture medium, four parietal bone quarters from each litter were cultured without osteoclasts. Mobilization of radioactivity was expressed as before. The percentage release from the osteoclast-free bones was subtracted from each bone substrate with osteoclasts to give the amount of CMR.

Preparation of ivory slices

Ivory slices (200 μm) were cut with a low-speed water-cooled diamond saw (Isomet, Buehler UK Ltd, Coventry, Warwickshire, UK) from a 1 cm^2 rod. Slices were cleaned by ultrasonication for 20 minutes in distilled water, sterilized in 70% ethanol for 1 hour and stored dessicated at room temperature until use.

Isolation and culture of rat osteoclasts on ivory slices

Osteoclasts were prepared from 2- to 3-day-old rats (Wistar). After killing the animals, femora and tibiae were removed and freed of adherent soft tissues, cut across their epiphyses and sectioned longitudinally. Osteoclasts were mechanically disaggregated by curetting the bones into 4 ml phosphate buffered saline and agitating the cell suspension with a pipette. Larger fragments were allowed to settle for 10 seconds before 500 μl aliquots of the supernatant cell suspension were immediately transferred to 6 wells of 24-well culture dishes (Costar), each well containing a single ivory slice. Cells were allowed to settle for 20 minutes at 37°C. The slices were then washed free of non-adherent cells and incubated for 24 hours in a humidified atmosphere of 5% CO_2/95% air at 37°C in 500 μl α-MEM supplemented with 10% acid-treated rabbit serum, 2.0 g/l $NaHCO_3$, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. Vehicle or the MMP inhibitors, CT435 and CT543, were added to the cultures at this time. Due to variability in the number of osteoclasts isolated from each rat, a single experiment consisted of 6 ivory slices bearing the cells from one rat, with 3 slices for each control and test variable. Each experimental variable was repeated 4 times and the results were expressed as the percentage inhibition of the control, which was set at 100%.

At the completion of the culture period the lactate concentrations in the culture media were determined by the lactate oxidase/peroxidase method (Barhan and Trinder, 1972; Sigma). The ivory slices were either stained unfixed with Neutral Red so that live osteoclasts could be counted, or fixed with warm (37°C) formaldehyde-acetone-citrate (1:6.5:2.5) solution. Following fixation the specimens were stained for 30 minutes at 37°C in darkness for tartrate-resistant acid phosphatase (TRAP) activity; osteoclasts were identified as large multinucleated (3 or more nuclei) strongly TRAP-positive cells. In those experiments where the highest concentration of inhibitor was used, osteoclast counts were made over the entire surface of each slice. Cells were then dislodged from the ivory slices and the substrate restained with Toluidine Blue in order to count the resorption lacunae by reflected light microscopy (Boyde et al., 1984). The method used for the precise quantitation of the resorptive capacity of the osteoclasts involved estimating the surface area of each lacuna by image analysis (TC Image, Foster Finlay Ass., UK).

Isolation of osteoclasts from rabbit bone

Isolated rabbit osteoclasts were prepared as previously described (Chambers et al., 1984). Femora and tibiae from 1-week-old rabbits were cleaned of adherent soft tissues and cut across their epiphyses. Each bone was curetted with a scalpel blade into 2 ml of α-MEM in a 35 mm tissue culture dish (Sterilin, UK). The curetted fragments were vigorously agitated with a glass pipette. Larger fragments were allowed to sediment for 10 seconds and 200 μl of the osteoclast suspension was added to each well of an 8-well Lab-tek slide (Nunc, Inc., Naperville, USA), then removed and replaced with 200 μl of α-MEM supplemented with 10% FCS.

Immunolocalization of matrix metalloproteinases and TIMP in rabbit osteoblasts

MMPs and TIMP were immunolocalized in osteoclasts by indirect immunofluorescence following two different culture procedures. Rabbit osteoclasts were used as we previously developed specific sheep antisera to the rabbit MMPs and TIMP-1.

(i) Rabbit bone marrow cells containing osteoclasts were plated onto 8-well Labtek slides containing α-MEM plus 10% FCS as described above. After 2 hours the medium was removed from the cells and replaced with fresh medium; the sodium ionophore monensin (5 μM; Sigma), which inhibits translocation and secretion of newly synthesized proteins, was added for the final 3 hours of culture. This results in intracellular accumulation of antigen in the Golgi apparatus and secretory vesicles of cells.

(ii) Rabbit bone marrow cells containing osteoclasts were cultured as above but in the presence of IL-1α (10^{-10} M) for 24 hours. Monensin was added for the final 3 hours of culture. After removal of culture medium the cell layer was fixed for 5 minutes in 4% paraformaldehyde at room temperature. Cells were permeabilized (0.1% Triton X-100, 5 minutes) to enable IgG penetration, washed with PBS and incubated with specific polyclonal antibodies to either collagenase (Hembry et al., 1986), gelatinase A and B (Murphy et al., 1989a), pig gelatinase B, which cross reacts with rabbit gelatinase B (Murphy et al., 1989b), stromelysin (Murphy et al., 1986), TIMP-1 (Gavrilovic et al., 1987), or normal sheep serum IgGs (50 μg/ml in PBS for 30 minutes at room temperature). The characterization of these antisera, including species specificity, western blots, inhibition curves and immunoabsorption experiments with purified antigen, are detailed in the above references. All the antisera have been used in previous immunolocalization studies in the rabbit (Brown et al., 1989; Green et al., 1990; Hembry et al., 1993). The cells were washed (PBS, 3× 5 minutes) and the second antibody (a pig Fab′ preparation labelled with FITC; Hembry et al., 1985) was applied for 30 minutes. After exhaustive washing they were mounted with Citifluor (University of Kent, Canterbury) and observed by fluorescence microscopy on a Zeiss photomicroscope III with epifluorescence and standard FITC filters or a Bio-Rad MRC 600 confocal microscope with a krypton/argon laser. Photographs were taken on Agfachrome RS 1000 film uprated to 2000 ASA, or from the colour monitor on Ektachrome 100 film.

RESULTS

Effect of the MMP inhibitors on calvarial bone resorption

We initially examined the effects of a range of doses of the MMP inhibitors on 1,25$(OH)_2D_3$-stimulated release of $^{45}Ca^{2+}$ from prelabelled mouse calvarial bones during a 48 hour culture period (Fig. 1). Both compounds inhibited the release of $^{45}Ca^{2+}$ dose-dependently, CT435 in the range 10^{-10} to 10^{-8} M and CT543 in the range 10^{-9} to 10^{-6} M. During the 48 hours of culture we found that CT543 produced about a 40% inhibition in stimulated resorption at a concentration (10^{-8} M) selective for gelatinase inhibition, whilst CT435, a general MMP inhibitor, produced 100% inhibition at the same concentration. CT543 only abolished stimulated bone resorption completely at a concentration (10^{-6} M) that would inhibit all MMPs.

We then tested the dose of each MMP inhibitor that was 100% effective, on both the basal and PTH-stimulated release of $^{45}Ca^{2+}$ and [^{3}H]proline from prelabelled mouse calvarial bones during a 48 hour culture period (Fig. 2). Whilst the inhibitory effects of CT435 (10^{-8} M) and CT543 (10^{-6} M) on PTH-stimulated bone resorption were complete, higher concentrations of the inhibitors failed to prevent the basal (unstimulated) release of $^{45}Ca^{2+}$ (Fig. 2A). In contrast, CT435 (10^{-7} M) and CT543 (10^{-5} M) produced a statistically significant reduction in the release of [^{3}H]proline from control bones (Fig. 2B). The MMP inhibitors were as effective as calcitonin (10^{-8} M) at inhibiting bone resorption.

To exclude the possibility that cell death was contributing to the inhibition a recovery experiment was performed. Calvarial bones were treated with PTH and one of the inhibitors for the first 48 hours, and then cultured with PTH alone for a further 4 days. The inhibitory effects of CT435 and CT543 seen during the initial culture period were gradually lost, the amount of PTH-stimulated $^{45}Ca^{2+}$ release returning to levels without the inhibitors by 96-144 hours (Table 1). Further evidence that inhibition was not due to toxic effects included the findings that neither of the MMP inhibitors at a concentration of 10^{-6} M affected either protein or DNA synthesis, as assessed by the incorporation of [^{3}H]proline and [^{3}H]thymidine into explants (Table 2). As expected, actinomycin D (10^{-4} M) and hydroxyurea (10^{-4} M) completely blocked the uptake of [^{3}H]thymidine and [^{3}H]proline, respectively. Finally, neither of the compounds at 10^{-6} M affected the PTH-stimulated release of the lysosomal enzyme, β-glucuronidase (PTH stimulated, $2.6\pm0.3\times10^{-3}$ units/ml; PTH + CT435, $2.9\pm0.4\times10^{-3}$ units/ml; PTH + CT543, $2.5\pm0.3\times10^{-3}$ units/ml).

These experiments illustrate that gelatinases A and/or B play a role in the degradation of the organic matrix during bone resorption. Furthermore, as CT543, like CT435, only com-

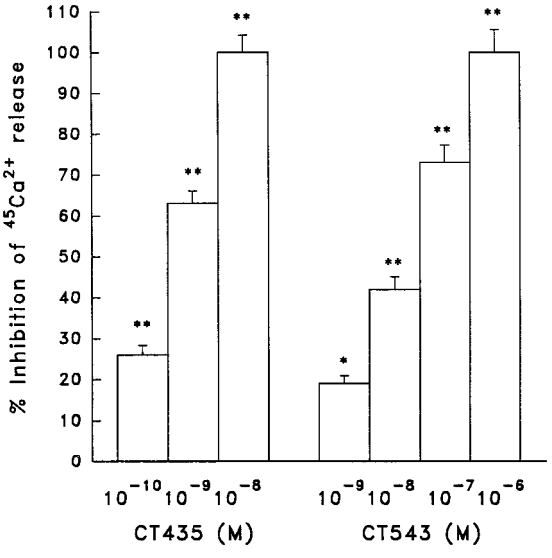

Fig. 1. Effects of the MMP inhibitors at different concentrations on the 1,25$(OH)_2D_3$ (10^{-8} M)-stimulated release of $^{45}Ca^{2+}$ from calvarial bones after a 48 hour incubation period. The results are expressed as percentage inhibition of 1,25$(OH)_2D_3$-stimulated $^{45}Ca^{2+}$ release, which was arbitrarily set to 100%. Each value is the mean ± s.e.m. of 6 pairs of bones. The inhibitory effects of CT435 (10^{-10} to 10^{-8} M), and CT543 (10^{-9} to 10^{-6} M) were statistically significant (*P<0.05, **P<0.01). The percentage release of $^{45}Ca^{2+}$ from 1,25$(OH)_2D_3$-stimulated bones was 16.9±1.7 (CT435) and 17.4±1.6 (CT543).

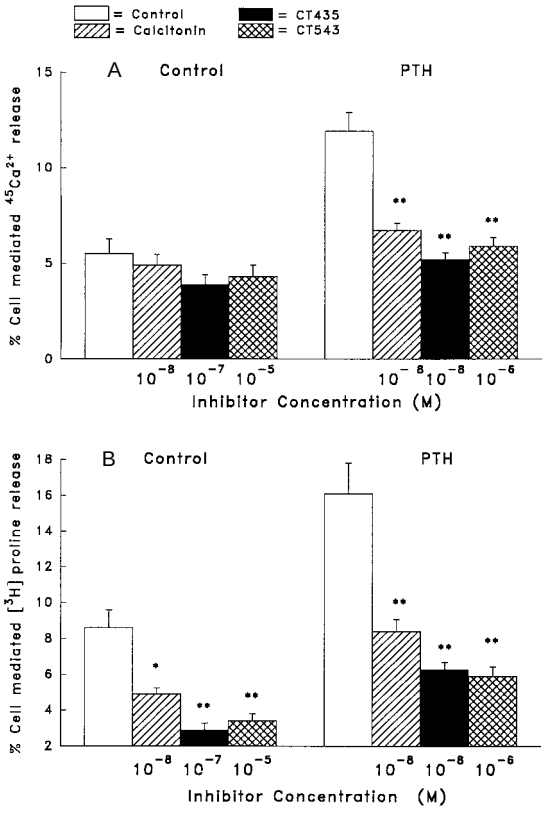

Fig. 2. Effects of the MMP inhibitors on basal (control) and PTH (2×10^{-8} M)-stimulated release of $^{45}Ca^{2+}$ (A) and [^{3}H]proline (B) from calvarial bones. Values are expressed as the mean percentage (± s.e.m.) of radioisotope released from five pairs of cultured bones after a 48 hour incubation. The stimulatory effect of PTH was significantly different from the untreated controls (**P<0.01). The inhibitory effects of CT435, CT543 and calcitonin on $^{45}Ca^{2+}$ and [^{3}H]proline release were significantly different from those of PTH alone and on the control release of [^{3}H]proline (*P<0.05; **P<0.01).

Table 1. Recovery from the inhibitory effects of MMP inhibitors on PTH-stimulated release of $^{45}Ca^{2+}$ from mouse calvarial bones

Treatment		% Cell mediated $^{45}Ca^{2+}$ release		
0-48 h	48-144 h	0-48 h	49-96 h	96-144 h
PTH	PTH	16.7±2.9	15.4±1.8	13.9±0.6
PTH+CT435	PTH	5.6±0.8	9.7±1.2	18.7±2.0
PTH+CT543	PTH	6.1±0.7	8.9±0.9	17.9±1.6

Values are means ± s.e.m. for five calvarial bones prelabelled with 0.15 MBq $^{45}Ca^{2+}$. PTH, CT435 and CT543 were added at final concentrations of 10^{-9} M, 10^{-7} M and 10^{-6} M.

pletely abolished stimulated bone resorption at a concentration at which it acts as a general MMP inhibitor this indicates that other MMPs, presumably collagenase, contribute to the process. Due to the complexity of the calvarial assay we were unable to conclude whether the MMPs were active in osteoclast lacunar resorption or were only inhibiting osteoid degradation. Hence we investigated the effects of the MMP inhibitors on chicken osteoclasts cultured on radiolabelled osteoid-free calvarial explants. Chickens were chosen as the source of osteoclasts due to their rich supply of these cells and because the cells can be partially purified from contaminating cells. This enabled large numbers of osteoclasts to be obtained so that accurate determinations could be made of the effects of the inhibitors on the release of radioisotopes from prelabelled bone explants.

Effect of the MMP inhibitors on chick osteoclasts cultured on osteoid-free mouse calvarial explants

In unstimulated cultures, the resorptive activity of chicken osteoclasts was dose-dependently inhibited by both CT435 and CT543 (Table 3). The inhibition was only partial with a maximum of 29.2±4.9% for CT435 and 20.5±2.4% for CT543 both at a 10^{-5} M concentration. In cultures stimulated by IL-1 (10^{-10} M), the inhibitory effects of both at 10^{-5} M were greater, with CT435 producing a 41.7±4.2% inhibition and CT543 a 35.9±4.6% inhibition (Table 3).

These data indicate that the MMPs participate in the osteoclastic degradation of the collagenous bone matrix but provide no conclusive information as to whether the enzymes are produced by osteoclasts or are stored and released by bone matrix during the resorptive process. Consequently we examined the effects of the two MMP inhibitors on the resorptive activity of isolated rat osteoclasts cultured on ivory slices. Ivory was chosen as the biological substrate as it is a mineralized bone-like material; in contrast to bone it presents a homogeneous surface allowing accurate determinations of lacunar resorption to be made. In addition, unlike bone, ivory contains no detectable collagenase activity and only trace amounts of progelatinase A (data not shown) making it a suitable substrate for assessing the contribution of osteoclast-derived MMPs to collagen degradation.

Table 2. Effect of MMP inhibitors on [^{3}H]thymidine uptake into DNA and [^{3}H]proline incorporation into proteins in murine calvarial bones

Addition	Amount (M)	[^{3}H]Thymidine (dpm/half calvaria) DNA fraction	[^{3}H]Proline (dpm/half calvaria)
Control	–	12394±1564	24643±2101
CT435	10^{-6}	13465±2183	25457±2350
CT543	10^{-6}	10786±1436	23630±2510
Actinomycin D	10^{-4}	–	3401±298
Hydroxyurea	10^{-4}	906±184	–
Devitalized bone	–	798±163	2577±219

Values are means ± s.e.m. for five calvarial halves, labelled with either 0.18 MBq [^{3}H]-thymidine (DNA synthesis) or 0.1 MBq [^{3}H]proline (protein synthesis) for the last 4 hours of a 24 hours culture period.

Table 3. Effect of CT435 and CT543 on the release of $^{45}Ca^{2+}$ from osteoid-free mouse calvarial bones by chicken osteoclasts

Inhibitor	Inhibitor concentration (M)	Stimulation of cultures	% Inhibition of $^{45}Ca^{2+}$ release
CT435	10^{-8}	–	3.9±1.6
	10^{-7}	–	15.6±1.7*
	10^{-6}	–	25.6±2.5*
	10^{-5}	–	29.2±4.9*
CT435	10^{-8}	IL-1 (10^{-10} M)	8.6±1.1
	10^{-7}	IL-1 (10^{-10} M)	19.4±2.7*
	10^{-6}	IL-1 (10^{-10} M)	37.2±3.6*
	10^{-5}	IL-1 (10^{-10} M)	41.7±4.2*
CT543	10^{-7}	–	2.7±0.9
	10^{-6}	–	14.9±1.5*
	10^{-5}	–	20.5±2.4*
CT543	10^{-7}	IL-1 (10^{-10} M)	8.9±2.3*
	10^{-6}	IL-1 (10^{-10} M)	24.1±2.9*
	10^{-5}	IL-1 (10^{-10} M)	35.9±4.6*

Prelabelled osteoid-free mouse calvarial bones were cultured with chicken osteoclasts for 72 hours in the absence or presence of varying concentrations of either CT435 or CT543. The results are expressed as the percentage inhibition of either the unstimulated or IL-1-stimulated $^{45}Ca^{2+}$ release that was arbitrarily set to 100%. Each value is the mean±s.e.m. of 3 pairs of bones.

*Significantly different from the control cultures at $P<0.05$. The percentage release of $^{45}Ca^{2+}$ from unstimulated explants was 8.6±1.9 (CT435) and 7.5±1.6 (CT543) and the percentage release from IL-1α-stimulated explants was 15.1±2.2 (CT435) and 16.2±2.4 (CT543).

Effect of MMP inhibitors on isolated rat osteoclast bone resorption

Image analysis of ivory slices on which osteoclasts had been cultured without any addition revealed typical resorption pits. In unstimulated cultures, the resorptive activity of rat osteoclasts was unaffected by either inhibitor up to a 10^{-5} M concentration. However, when the osteoclast cultures were stimulated by IL-1α (10^{-10} M), both CT435 and CT543 dose-dependently inhibited the number of resorption lacunae (Fig. 3A). The inhibition was only partial at 10^{-5} M, with a maximum of 30.2±3.6% for CT435 and 26.2±3.1% for CT543.

When the extent of bone resorption was assessed by measuring the total surface area of the lacunae (Fig. 3B), it was found that the degree of inhibition was similar to that for the percentage reduction in the number of resorption lacunae. The reason for this was that the mean surface area of each lacunae in the inhibitor-treated cultures (469±78 μm^2 for CT435;

535±65 μm^2 for CT543) was similar to that in the control cultures (546±89 μm^2).

To determine whether the inhibitors were exerting a non-specific cytotoxic effect on the rat osteoclasts, the cultures were inspected both after Neutral Red staining and following fixation and staining for TRAP. In the presence of MMP inhibitors multinucleated cells took up Neutral Red just as well as controls, suggesting that the viability of the cells was not affected. Morphological examination also showed that the inhibitors did not appear to alter the peripheral ruffles of the cells, indicating that the compounds did not interfere with the mobility of the osteoclasts. Furthermore, at the highest concentration used (10^{-5} M), neither CT435 nor CT543 had an inhibitory effect on glycolysis as assessed by measuring the amount of lactate produced by the osteoclast cultures (control, 17.1±1.9 μmol/l; CT435, 18.4±1.2 μmol/l; and CT543, 16.2±1.3 μmol/l). Finally, at the highest concentration used neither of the inhibitors altered the number of osteoclasts, as compared to controls. The mean number of TRAP-positive multinucleated cells per 1 cm^2 slice (15 slices per treatment) was 76.3±5.3 for the control, 69.4±5.9 for CT435 (10^{-5} M) and 81.6±7.1 for CT543 (10^{-5} M).

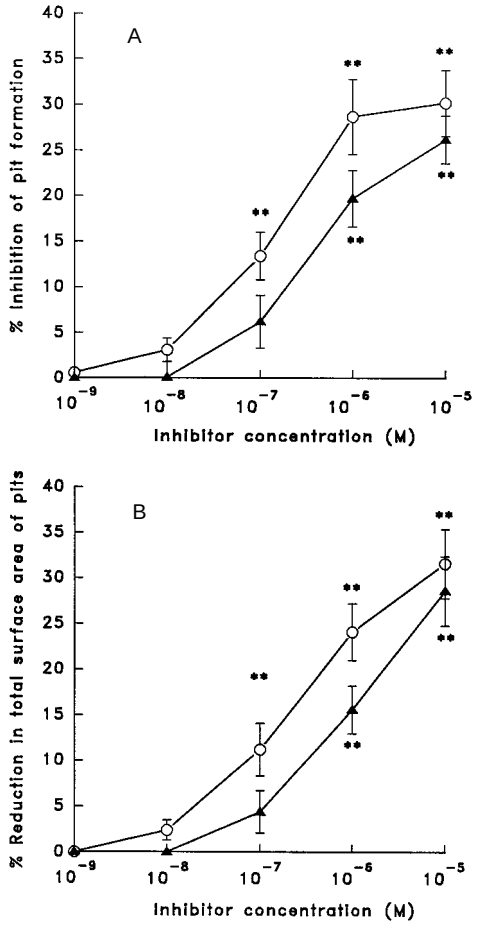

Fig. 3. Effects of the CT435 and CT543 on (A) the number and (B) the total surface area of rat osteoclast lacunae. Each value is the percentage inhibition in IL-1α-stimulated osteoclast lacunar resorption arbitrarily set to 100%. The values represent the mean ± s.e.m. from five individual experiments. The number (A) and surface area (B) of pits on the inhibitor-treated slices were significantly different from control (*P<0.05 and **P<0.01). The number of lacunae in the control cultures was 1432 (CT435) and 1267 (CT543). The mean surface area of the lacunae in the control cultures was 532±79 μm^2 (CT435) and 559±97 μm^2 (CT543). ○—○, CT435; ▲—▲, CT543.

These experiments indicate that the MMPs are produced by osteoclasts at least under certain states of activation, in this case induced by IL-1α. As the selective gelatinase inhibitor, CT543, only inhibited resorption at a concentration at which it acts as a general inhibitor, the data provided no insight into which particular members of the MMP family are produced by osteoclasts. We addressed this issue by undertaking immunofluorescence experiments with specific antisera to MMPs and TIMP-1 on rabbit osteoclasts.

Immunolocalization of MMPs and TIMP-1 in rabbit osteoclasts

In none of the unstimulated osteoclast cultures could collagenase, gelatinase B, stromelysin or TIMP-1 be immunolocalized: only gelatinase A could be detected within the perinuclear Golgi apparatus and secretory vesicles in unstimulated cells (Fig. 4A). Since gelatinase B could not be detected in unstimulated cells using the specific antibody for this enzyme, we conclude that the positive staining observed with the gelatinase A/B antibody is due to the presence of gelatinase A. Treatment with IL-1α (10^{-10} M) for 24 hours had a dramatic effect, stimulating gelatinases A/B, gelatinase B, collagenase, stromelysin and TIMP-1 production in osteoclasts (Fig. 4B-F). Cells stained with normal sheep serum were negative in both stimulated (Fig. 4G) and unstimulated cultures (data not shown). The MMPs and TIMP-1 were distributed throughout the prominent Golgi complexes surrounding each nucleus and the many Golgi vesicles within osteoclasts. Hence MMPs and TIMP-1 could be observed along the entire secretory pathway. In IL-1α-stimulated cultures, more osteoclasts were positive for gelatinases A and B than the other enzymes and the intensity of the perinuclear staining was brighter. These data show that cultured rabbit osteoclasts can produce the full spectrum of MMPs and TIMP-1, and gelatinases A and B appear to be the predominant enzymes. However, we observed that expression of MMPs was more prevalent in the smaller/less multinucleated osteoclasts and were seen infrequently in the larger cells.

DISCUSSION

Using a concentration-dependent specific gelatinase inhibitor we have shown that gelatinases A and/or B participate in bone resorption. We have also demonstrated, using sensitive immunocytochemical techniques that rabbit osteoclasts constitutively express gelatinase A (MMP-2) and can synthesize gelatinase B (MMP-9), stromelysin (MMP-3) and TIMP-1 in response to IL-1α stimulation and confirm that they also synthesize collagenase (MMP-1; Delaissé et al., 1993). Our data further indicate that osteoclast-derived MMPs participate in osteoclast lacunar resorption.

Because cleavage of the triple helices of the collagen molecule is the rate-limiting step in type I collagen degradation (Murphy and Reynolds, 1985), it has been assumed that collagenase is the principal MMP involved in bone resorption

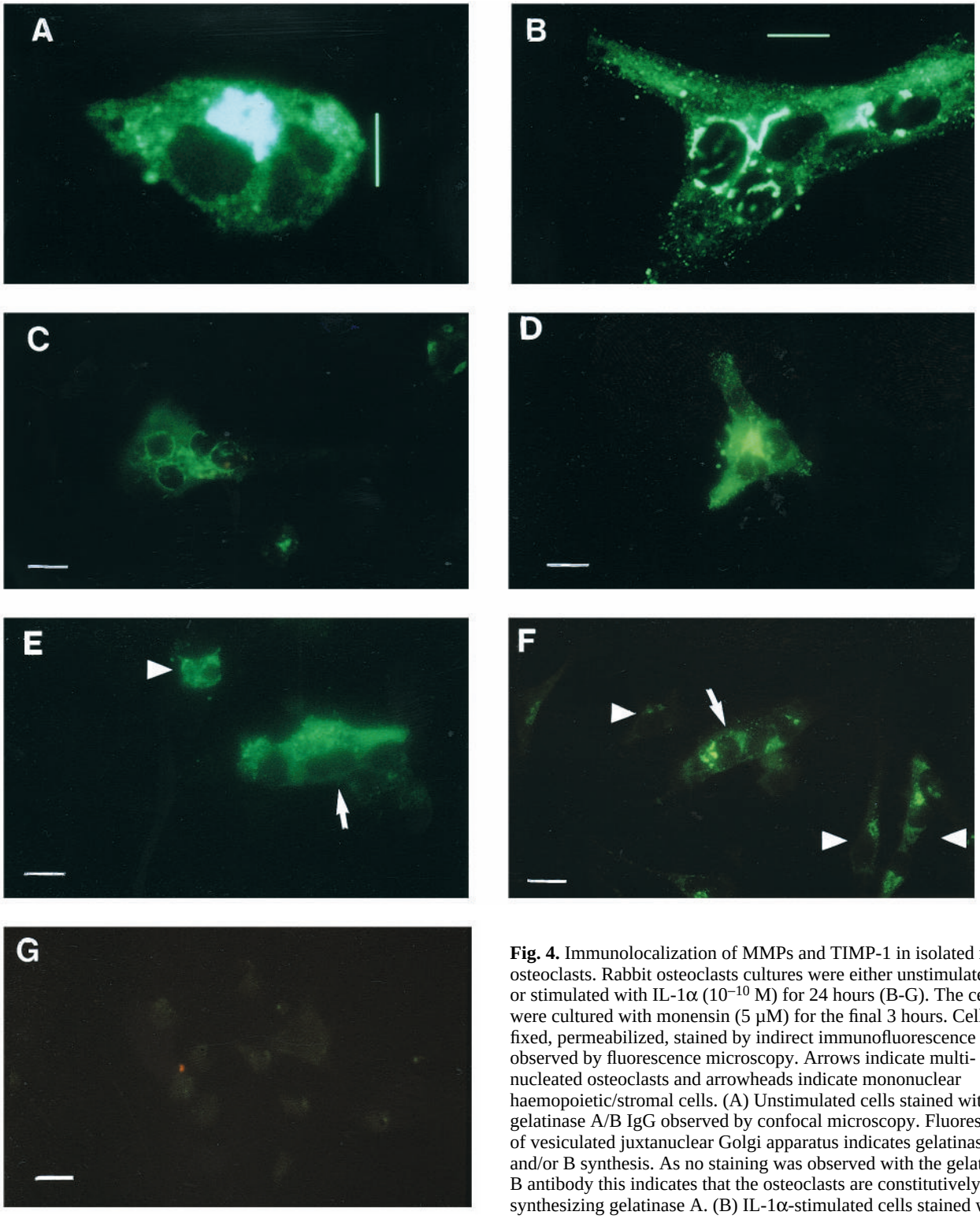

Fig. 4. Immunolocalization of MMPs and TIMP-1 in isolated rabbit osteoclasts. Rabbit osteoclasts cultures were either unstimulated (A) or stimulated with IL-1α (10^{-10} M) for 24 hours (B-G). The cells were cultured with monensin (5 μM) for the final 3 hours. Cells were fixed, permeabilized, stained by indirect immunofluorescence and observed by fluorescence microscopy. Arrows indicate multinucleated osteoclasts and arrowheads indicate mononuclear haemopoietic/stromal cells. (A) Unstimulated cells stained with anti-gelatinase A/B IgG observed by confocal microscopy. Fluorescence of vesiculated juxtanuclear Golgi apparatus indicates gelatinase A and/or B synthesis. As no staining was observed with the gelatinase B antibody this indicates that the osteoclasts are constitutively synthesizing gelatinase A. (B) IL-1α-stimulated cells stained with anti-gelatinase A/B IgG observed using the confocal microscope; optical sectioning clearly demonstrates the perinuclear distribution of gelatinase A/B associated with each nucleus. (C-G) IL-1α-stimulated cells stained with anti-gelatinase B IgG, anti-collagenase IgG, anti-stromelysin IgG, anti-TIMP-1 IgG and normal sheep serum IgG, respectively. Intracellular fluorescence of the vesiculated Golgi apparatus and the secretory vesicles indicates the synthesis of: (C) gelatinase B; (D) collagenase; (E) stromelysin, with trinucleated osteoclast (arrow) and mononuclear cell (arrowhead); and (F) TIMP-1, with trinucleated osteoclast (arrow) and mononuclear cells (arrowhead). Cells were stained with normal sheep serum IgG (G) where no intracellular fluorescence is visible. Bars, 10 μm.

(Delaissé et al., 1985, 1988; Eeckhout et al., 1986; Eeckhout, 1990). The ability, however, of CT543 to produce a 40% inhibition in calvarial bone resorption at a concentration at which it acts as a selective inhibitor of gelatinases A and B indicates that these MMPs play a significant role in the degradation of the organic matrix of bone. At present an inhibitor that can distinguish between gelatinases A and B is not available. The finding that the MMP inhibitors prevented the basal release of [^{3}H]proline in contrast to $^{45}Ca^{2+}$ suggests that the demineralization process was allowed to proceed until the collagenous matrix was encountered, the degradation of which was inhibited.

Although we have not demonstrated MMPs within the sub-osteoclastic resorption zone, we propose that osteoclasts under certain states of activation secrete MMPs that then participate in lacunar bone resorption. Firstly, our data shows that CT435 and CT543 partially prevented the resorptive activity of both isolated chicken and rat osteoclasts. Secondly, we have demonstrated that rabbit osteoclasts synthesize gelatinase A constitutively and produce collagenase, gelatinase B, stromelysin and TIMP-1 in response to IL-1α. Thirdly, the intracellular localization of MMPs to the perinuclear Golgi apparatus and cytoplasmic vesicles indicates that the enzymes are within the biosynthetic and secretory pathways of the cell. The fact that the MMPs were observed more frequently in the smaller osteoclasts suggests that expression of MMPs may be related to the life-cycle of the cell.

MMPs that are produced constitutively by both stromal and haemopoietic cells, which cannot be completely eliminated from osteoclast cultures, probably do not participate in osteoclast resorption. This conclusion is supported by the data that the MMP inhibitors had no effect on the resorptive activity of unstimulated cultures. Moreover, access of non-osteoclast derived MMPs to the sealed environment of resorption lacunae will be limited by the large molecular mass of the enzymes.

One problem with trying to implicate a role for MMPs in osteoclast-mediated resorption is explaining how the enzymes retain biological activity at acid pH values encountered within the sub-osteoclastic resorption zone. However, temporospatial variations in pH may occur within the resorption zone depending upon the efficiency of osteoclast acid secretion and the buffering potential of the Ca^{2+} and PO_4^{3-} released during mineral dissolution. It is noteworthy that stromelysin retains significant activity at pH 5 (Galloway et al., 1983).

The hypothesis that the MMPs are synthesized and secreted by osteoclasts is supported by the recent localization of (pro)collagenase within rodent osteoclasts and in the sub-osteoclastic resorption zone (Delaissé et al., 1993). Stromelysin has also been localized in resorbing rat osteoclasts (Case et al., 1989) and gelatinase B mRNA has been detected in osteoclasts during mouse development (Reponen et al., 1994). Finally, mononuclear phagocytes that are ontogenetically related to osteoclasts have been found to synthesize and secrete the MMPs (Cury et al., 1988; Shapiro et al., 1990; Welgus et al., 1990). However, our data demonstrating that the MMP inhibitors completely prevented stimulated calvarial resorption in contrast to osteoclast lacunar resorption supports the view that in bone, osteoblasts are the major source of MMPs whose principal function is degradation of the osteoid layer, as proposed by Chambers et al. (1985).

The inability of both MMP inhibitors to prevent the resorptive activity of unstimulated rat osteoclasts in contrast to chicken osteoclasts is unlikely to be due to the higher basal level of resorptive activity exhibited by chicken osteoclasts (Arnett and Dempster, 1987), since Delaissé et al. (1987) found that the MMP inhibitor, CI-1 failed to inhibit the action of chicken osteoclasts cultured on dentine, a substrate similar to ivory. Perhaps it may be explained by differences in composition of the two substrates, bone and ivory, that were used in this study to assess osteoclastic resorption. Living bone is known to contain procollagenase (see introductory remarks) and it has been proposed by Delaissé et al. (1988) that the zymogen may be released and activated by osteoclasts during bone resorption to participate in the degradation of collagen within the osteoclast resorption lacuna. We found no detectable (pro)collagenase within ivory, which suggests that ivory is an ideal mineralized substrate for assessing the contribution of osteoclast-derived MMPs to the subosteoclastic degradation of collagen.

Although this study demonstrates a role for MMPs in osteoclast lacunar resorption, substantial evidence indicates that lysosomal cysteine proteinases are also involved in this process (Delaissé et al., 1987; Everts et al., 1988, 1992; Rifkin et al., 1991). Using selective inactivators of cysteine proteinases we have recently shown that cathepsin B (EC 3.4.22.1) plays an intracellular role and cathepsins L (EC 3.4.22.15) and/or S (EC 3.4.22.27) an extracellular role in osteoclast lacunar resorption (Hill et al., 1994). In addition to its collagenolytic activity, cathepsin B in conjunction with stromelysin can activate procollagenase (Murphy et al., 1992), and this might be the primary function of CPs within the osteoclast resorption zone. There are also reasons to believe that the collagenolytic activities of both classes of enzyme are enhanced by elevated Ca^{2+} concentrations that will be encountered within the resorption lacuna consequential to mineral dissolution (Etherington and Birkedal-Hansen, 1987; Eeckhout, 1990). Furthermore, it has been suggested that bone collagen degradation is more complete when the enzymes act in sequence rather than independently (Danielsen, 1990). We therefore conclude that both classes of enzyme co-operate in osteoclast bone collagen degradation.

Since lysosomal CPs are active at acidic pH values within the osteoclast resorption zone (Maciewicz and Etherington, 1988; Delaissé et al., 1991) one could speculate that the osteoclast secretion of these enzymes coincides with release of H^+ ions. The MMPs may be secreted at a later stage in the resorptive process, once the bone matrix has been demineralized and the acid neutralized by the released bone salts. This sequence of events would probably lead to a more complete degradation of type I collagen as demonstrated by Danielsen (1990).

In conclusion, this study has demonstrated that gelatinases A and B contribute to bone resorption in addition to other members of the MMP family. Moreover the full spectrum of MMPs including their tissue inhibitor are synthesized by rabbit osteoclasts and the enzymes participate in osteoclast lacunar resorption.

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