Inhibition of bone resorption *in vitro* by selective inhibitors of gelatinase and collagenase

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Two low-molecular-mass inhibitors of matrix metalloproteinases (MMPs), CT1166, a concentration-dependent selective inhibitor of gelatinases A and B, and Ro 31-7467, a concentration-dependent selective inhibitor of collagenase, were examined for their effects on bone resorption and type-I collagenolysis. The test systems consisted of measuring (1) the release of [^{3}H]proline from prelabelled mouse calvarial explants; (2) the release of ^{14}C from prelabelled type-I collagen films by mouse calvarial osteoblasts; and (3) lacunar resorption by isolated rat osteoclasts cultured on ivory slices. In 24 h cultures, CT1166 and Ro 31-7467 inhibited both interleukin-1α- (IL-1α; 10^{-10} M) and 1,25-dihydroxyvitamin D_3 (10^{-8} M)-stimulated bone resorption in cultured neonatal mouse calvariae at concentrations selective for the inhibition of gelatinase (10^{-9} M for CT1166) and collagenase (10^{-8} M for Ro 31-7467) respectively. For each compound the inhibition was dose-dependent, reversible, and complete at a 10^{-7} M concentration. However, CT1166 (10^{-9} M) and Ro 31-7467 (10^{-8} M) in combination were required to completely abolish IL-1α-stimulated bone resorption in mouse calvariae throughout a 96 h culture period. Neither of the inhibitors affected protein synthesis, DNA synthesis nor the IL-1α-stimulated secretion of the lysosomal enzyme, β-glucuronidase. Both CT1166 and Ro 31-7467 partially inhibited IL-1α-stimulated lacunar resorption by isolated osteoclasts, but were without effect on unstimulated lacunar resorption. Rodent osteoclasts produced collagenase and gelatinases-A and -B activity. In contrast the substrate used to assess osteoclast lacunar resorption contained no detectable collagenase or gelatinase activity. Both compounds dose-dependently inhibited 1,25-dihydroxyvitamin D_3 (10^{-8} M)-stimulated degradation of type-I collagen by mouse calvarial osteoblasts; however, complete inhibition of collagenolysis was only achieved at concentrations at which CT1166 and Ro 31-7467 act as general MMP inhibitors. This study demonstrates that collagenase and gelatinases A and/or B participate in bone resorption. While these MMPs may be primarily involved in osteoid removal, we conclude that they may also be released by osteoclasts, where they participate in bone collagen degradation within the resorption lacunae.

INTRODUCTION

Bone resorption involves the removal of both the mineral and organic constituents of bone matrix. Osteoclasts are the cells principally responsible for this process which occurs in the sub-osteoclastic resorption zone, a specialized extracellular compartment bounded by the ruffled border of the cell and the mineralized bone matrix (Baron, 1989). Osteoclasts acidify the sub-osteoclastic resorption zone leading to dissolution of mineral (Blair et al., 1989) while the organic matrix (mainly type-I collagen) is degraded by proteolytic enzymes, especially the matrix metalloproteinases (MMPs) and cysteine proteinases (CPs). Osteoblastic cells play an accessory role in bone resorption by releasing MMPs that degrade the surface osteoid layer (principally type-I collagen), facilitating access of osteoclasts to the mineralized bone (Chambers et al., 1985). The gene family of MMPs includes the collagenases, gelatinases and stromelysins, which are zinc-dependent endopeptidases with the combined ability to degrade the organic components of connective-tissue matrices at physiological pH (Murphy and Reynolds, 1993). The MMPs are specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs), members of a multigene family of which TIMP-1 (Docherty et al., 1985) and TIMP-2 (Stetler-Stevenson et al., 1990) are well characterized.

Several lines of evidence strongly suggest the involvement of MMPs and TIMPs in bone resorption. First, the MMPs and TIMP-1 are produced by osteoblast-like cells and their activities are regulated by factors that influence bone resorption (Heath et al., 1984; Otsuka et al., 1984; Sakamoto and Sakamoto, 1984; Murphy et al., 1985; Meikle et al., 1992). Secondly, both synthetic (CI-1; Delaisse et al., 1985) and natural (TIMPs-1 and -2; Hill et al., 1993) inhibitors of MMPs prevent resorption in bone explants. We have also shown that TIMP-1 inhibits type-I collagenolysis by mouse calvarial osteoblasts (Thomson et al., 1987). Since interstitial collagenase is the only enzyme capable of cleaving native type-I collagen (Murphy and Reynolds, 1985), it is proposed that osteoblast-derived collagenase is responsible for degrading the non-mineralized osteoid layer covering bone surfaces, thereby exposing the underlying mineralized matrix to osteoclastic action (for review see Sakamoto and Sakamoto,

Abbreviations used: CPs, cysteine proteinases; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; FITC, fluorescein isothiocyanate; IL-1α, interleukin-1α; rhIL-1α, recombinant human IL-1α; FCS, fetal calf serum; DMEM, Dulbecco's modification of Eagle's medium; BGJ, Biggers, Gwatkin and Judah; αMEM, alpha minimal essential medium; MMPs, matrix metalloproteinases; MNP, mononuclear phagocyte; TIMP, tissue inhibitor of metalloproteinases; TRAP, tartrate-resistant acid phosphatase; Ro 31-7467, 2(*R* or *S*)-[(5-bromo-2,3-dihydro-6-hydroxy-1,3-dioxo-1*H*-benz[*de*]isoquinolin-2-yl)methyl](hydroxy)-phosphinyl]-*N*(*R* or *S*)-(2-oxo-3-azacyclotridecanyl)-4-methylvaleramide; CT1166, *N*1-{*N*-[2-(morpholinosulphonylamino)-ethyl]-3-cyclohexyl-2-(*S*)-propanamidyl}-*N*4-hydroxy-2-(*R*)-[3-(4-methylphenyl)propyl]-succinamide.

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1986). Mouse and human osteoblasts also produce gelatinases A and B (Rifas et al., 1989; Lorenzo et al., 1992; Meikle et al., 1992), enzymes that can degrade denatured type-I collagen (Murphy et al., 1985; Wilhelm et al., 1989), but their contribution to bone collagen degradation has not been investigated.

Osteoclasts can degrade bone collagen independently of MMP secretion (Blair et al., 1986; Delaisse et al., 1987), but recent morphological evidence using mouse calvariae has suggested that MMPs participate in bone collagen degradation within the sub-osteoclastic resorption zone (Everts et al., 1992). The cellular origin of the enzymes, however, was unclear reflecting the complexity of organ culture models. The presence of procollagenase within the bone matrix of such models (Eeckhout et al., 1986; Delaisse et al., 1988) has further added to the uncertainty. In this study we initially investigated the effects of concentration-dependent collagenase (Ro 31-7467) and gelatinase (CT1166) inhibitors on bone resorption *in vitro* using a neonatal mouse calvarial assay. We then extended the study to determine the contribution of the enzymes to the two separate stages of bone resorption. First, osteoid degradation was examined by culturing primary mouse osteoblasts on prelabelled type-I collagen films in the presence and absence of the inhibitors. Secondly, the effects of the compounds on osteoclast lacunar resorption were investigated by culturing rat osteoclasts on ivory slices. Finally, to assess whether the MMPs were being released by osteoclasts rather than from the substrate during the resorption process, we determined that ivory contained no collagenase activity and a very low content of progelatinase.

MATERIALS AND METHODS

The gelatinase inhibitor, CT1166, was synthesized by Celltech (Slough, U.K.) and the collagenase inhibitor, Ro 31-7467 by Roche (Welwyn Garden City, Herts., U.K.). 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and recombinant human interleukin-1α (rhIL-1α) were generous gifts from Roche Products Ltd and Dr. Jeremy Saklatvala, Strangeways Research Laboratory, Cambridge, U.K., respectively. Actinomycin, D,L-proline, thymidine, indomethacin and 4-methylumbelliferone were purchased from Sigma Chemical Co., U.K. Methyl-[^{3}H]thymidine, [^{14}C]acetic anhydride and L-[5-^{3}H]proline were purchased from Amersham International, Amersham. Bucks., U.K. Modified Biggers, Gwatkin and Judah (BGJ) medium was from Flow Laboratories. Trypsin, dispase and bacterial collagenase were from Boehringer Mannheim, Germany.

MMP inhibitors and their characterization

(1) CT1166

Recombinant human progelatinase A, progelatinase B, interstitial procollagenase and prostromelysin were purified from NSO-cell-conditioned culture medium (Murphy et al., 1992a,b; O'Connell et al., 1994). Enzyme assays to determine IC_{50} values for CT1166 against enzymes other than gelatinase A were performed at 37 °C using the fluorescent peptide substrate dinitrophenyl-Pro-Leu-Gly-Leu-Trp-Ala-Arg-NH_2 (DnpPLGLWAR; Stack and Gray, 1989). Assays to determine the IC_{50} value against gelatinase A were performed at 23 °C using the fluorescent peptide substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl-Ala-Arg-NH_2 (McaPLGLDpaAR; Knight et al., 1992). Essentially, enzyme (0.05–2.5 nM) and a range of inhibitor concentrations (0.1–50 × IC_{50}) were incubated for 2 h in 0.1 M Tris/HCl buffer, pH 7.5, containing 0.1 M NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35. The reaction was started by the addition of substrate. DnpPLGLWAR assays were performed for 16 h and were terminated with 0.1 M sodium acetate buffer, pH 4. McaPLGLDpaAR assays were performed for 2 min with hydrolysis rates being determined over the first minute of the reaction. Apparent K_i values (IC_{50}) were established using the equation for tight-binding inhibition as described (Morrison and Walsh, 1988):

$$V_i = V_o/2E\ (\{K_{i,app.} + [I]^2 + 2(K_{i,app.} - [I])[E] + [E]^2\}^{\frac{1}{2}} - K_{i,app.} + [I] - [E])$$

where V_o is the initial rate in the absence of inhibitor, V_i is the initial rate in the presence of inhibitor, [E] is the total enzyme concentration and [I] the total inhibitor concentration in the reaction mixture. $K_{i,app.}$ (IC_{50}) was assumed to approximate to the true K_i as in both assays $[S] \ll K_m$ for the substrate hydrolysis.

(2) Ro 31-7467

Gelatinase A was purified from CCD43 human dermal fibroblast serum-free conditioned medium in the presence of recombinant human IL-1α (rhIL-1α) (25 ng/ml). The concentrated culture medium was buffered with 25 mM sodium cacodylate, pH 7.2, 10 mM $CaCl_2$, 200 mM NaCl, 0.05% Brij (buffer A), applied to a gelatin–agarose column (3.4 cm × 19 cm) and bound gelatinase A was eluted with 10% DMSO in buffer A.

Collagenase was purified from the unbound fraction from the gelatin–agarose column. This fraction was applied to a column of dextran sulphate (2.4 cm × 10 cm), and collagenase was eluted with 2 M NaCl in 0.05 M Tris/HCl, pH 7.4, 10 mM $CaCl_2$ and 0.05% Brij. The fractions containing collagenase were dialysed against 0.05 M Tris/HCl, pH 7.4, 10 mM $CaCl_2$, 0.5 M NaCl, 0.05% Brij (buffer B) and further purified on an hydroxamic acid inhibitor affinity column (2 cm × 8 cm) prepared by coupling L-prolyl-L-leucylglycine–hydroxamic acid to activated CH-Sepharose 4B as described (Moore et al., 1986). Bound collagenase was eluted with buffer B at pH 9.0 and adjusted to pH 7.6 immediately.

Stromelysin was purified from GM625B human fibroblast serum-free conditioned medium in the presence of rhIL-1α (25 ng/ml). Following centrifugation at 48000 ***g*** for 30 min the culture medium was applied to an anti-prostromelysin affinity column (1.8 cm × 6 cm) and stromelysin was eluted with 50 mM Tris/HCl, pH 7.5, 5 mM $CaCl_2$, 0.02% NaN_3 (buffer C) and 6 M urea. Urea was removed by dialysis against buffer C.

Enzyme assays to determine the IC_{50} of Ro 31-7467 against gelatinase A, collagenase and stromelysin were performed at 37 °C by cleavage of the synthetic hexapeptide, acetyl-Pro-Gln-Gly-Leu-Leu-Gly-OEt (0.25 mM for gelatinase A; 1 mM for collagenase; and 2 mM for stromelysin) to give the tripeptide product Leu-Leu-Gly-OEt which was derivatized by reaction with pirylsulphonic acid and as described previously (Johnson et al., 1987). Enzyme concentrations were 1–2 nM. In addition, anti-collagenase activity was determined by degradation of ^{14}C-acetylated type-I collagen as the substrate according to Johnson-Wint (1980) and stromelysin by degradation of ^{14}C-acetylated β-casein.

Neonatal mouse calvarial assay

Bone resorption was assessed by analysing [^{3}H]proline release from cultured neonatal mouse calvarial bones (Reynolds and Dingle, 1970). Briefly, 1-day-old mice were injected subcutaneously with 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% (v/v) acid-treated rabbit serum and 1 μM indomethacin for 24 h (Lerner, 1987; Ljunggren et al., 1991). Bones that were stimulated with either 1,25-$(OH)_2D_3$ or IL-1α were also treated with the hormones during this initial period. Paired bones were subsequently cultured in fresh modified BGJ medium (1 ml) containing the previous additions excluding indomethacin. The bones were stimulated with either 1,25-$(OH)_2D_3$ or IL-1 with and without the MMP inhibitors. Mobilization of radioactivity was expressed as the percentage release of initial isotope (calculated as the sum of radioactivity in medium and bone after culture). To determine [^{3}H]proline release due to passive exchange of isotope with unlabelled proline in the culture medium, four parietal bone quarters from each litter were devitalized by three cycles of freeze–thawing. The percentage release from the devitalized bones 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 96 h in modified BGJ medium (3.0 ml) containing the previous additions with and without test substances, and labelling the bones during the last 6 h with either 0.037 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, which was released into the medium, was determined fluorimetrically using 4-methylumbelliferyl-β-D-glucuronide as the substrate (Achord et al., 1978; NBS Biologicals, Hatfield, Hertfordshire, U.K.). One unit of activity represents the amount of enzyme catalysing the release of 1 nmol of 4-methylumbelliferone per h.

Collagenase and gelatinases A and B produced by calvarial halves cultured for 48 h in the presence of IL-1α (10^{-10} M) were assessed as described below.

Preparation of osteoblasts from neonatal mouse calvariae

Calvarial osteoblasts were prepared and characterized as described previously (Heath et al., 1984). Briefly, neonatal mouse calvariae (40–50) were dissected free from adherent soft tissue, washed in Ca^{2+}- and Mg^{2+}-free Tyrode's solution (10 min) and sequentially digested with 1 mg/ml trypsin (10 min), 2 mg/ml dispase (30 min) and 4 mg/ml collagenase (3 × 30 min). Cells released by the last two collagenase digestions were washed and grown in Dulbecco's modification of Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS; Globepharm, Esher, Surrey) and antibiotics for 2 days prior to use. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2:95% air.

Preparation of collagen films

Radiolabelled collagen films were prepared as described previously (Gavrilovic et al., 1985). Aliquots of ^{14}C-acetylated collagen (rat skin type I; 150 μg in 300 μl of 10 mM phosphate buffer, pH 7.4, containing 300 mM NaCl and 0.02% sodium azide) were dispensed into tissue culture wells (Linbro, 16 mm diam.) and dried at 37 °C. The collagen was then washed twice with sterile distilled water and once with DMEM.

Preparation of acid-treated serum

To destroy serum inhibitors of neutral proteinases, aliquots (20 ml) of heat-inactivated rabbit serum (Globepharm) were acidified to pH 3.2 with 1 M HCl and incubated for 35 min at 37 °C. The pH was then returned to 7.4 with 1 M NaOH.

Culture of osteoblasts on collagen films

Osteoblasts (1×10^5/well) were settled on to collagen films in 1 ml of DMEM plus 10% (v/v) FCS, incubated for 16 h at 37 °C and washed with serum-free DMEM. Cells were then cultured in DMEM (1 ml) supplemented with 5% (v/v) acid-treated rabbit serum as described above. Either 1,25-$(OH)_2D_3$ alone (final concentration 10^{-8} M, added in 5 μl of ethanol) or 1,25-$(OH)_2D_3$ plus either CT1166 or Ro 31-7467, or 5 μl of ethanol alone, was then added to the wells and the cultures maintained at 37 °C for 48 h. The basal release of ^{14}C by unstimulated osteoblasts was subtracted from the 1,25-$(OH)_2D_3$-stimulated release in the presence and absence of inhibitors to give the corrected values for stimulated lysis. At the end of the culture period the media were centrifuged (15 min, 12000 ***g***) to remove any collagen fibrils and radioactivity released during collagen degradation quantified by liquid scintillation counting. The lactate concentrations in the culture media were determined as before. Residual collagen was digested with bacterial collagenase (50 μg/ml) and assayed for radioactivity. Collagenolysis was expressed as radioactivity released from the films as a percentage of the total ± S.E.M.

Isolated osteoclast bone resorption assay

The osteoclast bone resorption assay is based on the ability of isolated osteoclasts to resorb devitalized cortical bone or ivory slices *in vitro* (Boyde et al., 1984; Chambers et al., 1984). Ivory slices (200 μm thick) were cut with a low-speed, water-cooled diamond saw (Isomet, Buehler UK Ltd., Coventry, Warwickshire, U.K.) from a 1 cm^2 rod. Osteoclasts were prepared from 2–3-day-old Wistar rats. 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 of PBS and agitating the cell suspension with a pipette. Larger fragments were allowed to settle for 10 s before 500 μl aliquots of the supernatant cell suspension were transferred to six wells of 24-well culture dishes (Costar), each containing a single ivory slice. Cells were allowed to settle for 20 min at 37 °C. The substrate was then washed free of non-adherent cells and the slices incubated for 24 h in a humidified atmosphere of 5% CO_2/95% air at 37 °C in 500 μl of alpha minimal essential medium (α-MEM) supplemented with 5% (v/v) acid-treated rabbit serum, 2.0 g/l $NaHCO_3$, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The osteoclast cultures were either unstimulated or stimulated with IL-1 (10^{-10} M). Due to the variability in the number of osteoclasts isolated from each rat, a single experiment consisted of six bone slices bearing the cells from one rat with three slices for each control and test variable. Each experimental variable was repeated five 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 Co., U.K.). The collagenase and gelatinases A and B activities in the IL-1α-stimulated rat osteoclast cultures were determined as described below. The release of the lysosomal enzyme, β-glucuronidase, was determined as previously described (Achord et al., 1978). 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, by

vol.) solution. Following fixation the specimens were stained for 30 min at 37 °C in darkness for tartrate-resistant acid phosphatase (TRAP) activity; osteoclasts were identified as large multinucleated (three 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 (Boyde et al., 1984). The method used for the precise quantification of the resorptive capacity of the osteoclasts involved estimating the surface area of each lacuna using an image analyser (T.C. Image, Foster Finlay Ass., U.K.).

Extraction of collagenase and gelatinase from ivory and bone

Ivory was reduced to a fine powder in a Spex freezer mill (Glen Creston Instruments, Stanmore, Middlesex) at −196 °C and extracted as described previously (Eeckhout et al., 1986). Briefly, 150 mg of ivory powder was suspended at 10 mg/ml in CNTN buffer [10 mM cacodylate/HCl (pH 6.0)/1 M NaCl/Triton X-100 (0.1 mg/ml)/1 μM $ZnCl_2/NaN_3$ (0.1 mg/ml)] and mixed at 4 °C for 24 h. The supernatant was removed and supplemented with 10 μl of 0.2 M $CaCl_2$/ml. The procedure was repeated four times and the supernatant extracts were combined and dialysed against TCB buffer [50 mM Tris/HCl (pH 7.4)/10 mM $CaCl_2$/0.05% Brij] for 12 h. Neonatal mouse calvarial bones (150 mg wet weight) were subjected to a similar extraction protocol as they are known to contain measurable amounts of collagenase (Eeckhout et al., 1986).

Purification of gelatinases A and B

The calvarial bone and ivory extracts were applied separately to gelatin–Sepharose columns (2 cm × 0.5 cm) equilibrated in TCB buffer containing 200 mM NaCl. Gelatinase was eluted by the addition of 10% (v/v) DMSO to TCB buffer.

Zymogram analysis of gelatinases A and B

Gelatin-degrading activity was assessed by electrophoresis on non-reducing SDS/8%-polyacrylamide gels (Laemmli and Favre, 1973) incorporating 0.5 mg/ml of denatured type-I collagen (Heussen and Dowdle, 1980). Staining with Coomassie Brillant Blue revealed zones of lysis, indicating gelatin-degrading activity.

Collagenase assay

Collagenase activity in ivory and bone, as well as samples of culture supernatants from mouse calvariae and rat osteoclasts, was assayed at 35 °C for 18 h using ^{14}C-acetylated type-I collagen isolated from rat skin as described elsewhere (Sellers and Reynolds, 1977). Under these conditions 1 unit of collagenase degrades 1 μg of substrate per min.

Immunolocalization of gelatinases A/B and collagenase in mouse osteoclasts

Gelatinase A/B and collagenase were immunolocalized in mouse osteoclasts by indirect immunofluorescence using specific sheep antisera raised to the rabbit MMPs which cross-react with the mouse enzymes (Hembry et al., 1986; Murphy et al., 1989). Mouse bone-marrow cells containing osteoclasts were prepared from the femora and tibiae of 2–3-day-old mice. The cells were plated on to 8-well Labtek slides containing α-MEM plus 10% (v/v) FCS. After 2 h the medium was removed from the cells and replaced with fresh medium containing IL-1α (10^{-10} M) for 16 h. Monensin (5 μM; Sigma), which inhibits translocation and secretion of newly synthesized proteins, was added for the final 3 h of culture. This results in the intracellular accumulation of antigen in the Golgi apparatus and secretory vesicles of cells. After removal of culture medium the cell layer was fixed for 5 min in 4% paraformaldehyde at room temperature. Cells were permeabilized (0.1% Triton X-100, 5 min) 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., 1989), or normal sheep serum IgGs (50 μg/ml in PBS for 30 min 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. The cells were washed (PBS, 3 × 5 min) and the second antibody (a pig Fab′) preparation labelled with fluorescein isothiocyanate (FITC) (Hembry et al., 1985) was applied for 30 min. 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.

Statistical analysis

Differences between groups were analysed by means of the non-parametric Mann Whitney test, rather than the unpaired Student's *t*-test to avoid assumptions that the data are normally distributed.

RESULTS

Inhibitor characteristics

CT1166 has the following IC_{50} values against the MMPs: gelatinase A, 0.01 nM; gelatinase B, 0.016 nM; stromelysin, 2.75 nM; and collagenase, 385 nM.

Ro 31-7467 has the following IC_{50} values against the MMPs: collagenase, 16.8 nM using the hexapeptide and type-I collagen; gelatinase A, 208.8 nM using the hexapeptide; and stromelysin, 238.8 nM with the hexapeptide.

The structures of CT1166 and Ro 31-7467 are shown in Figure 1.

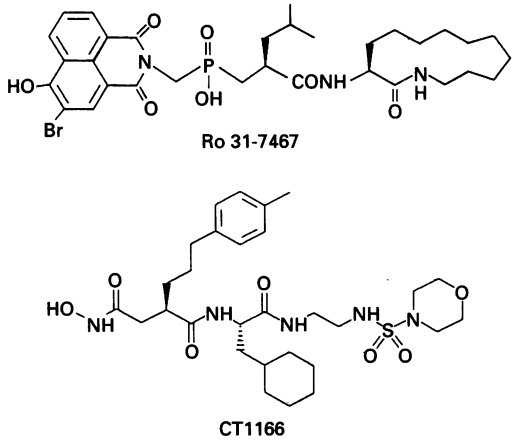

Figure 1 Structures of CT1166 and Ro 31-7467

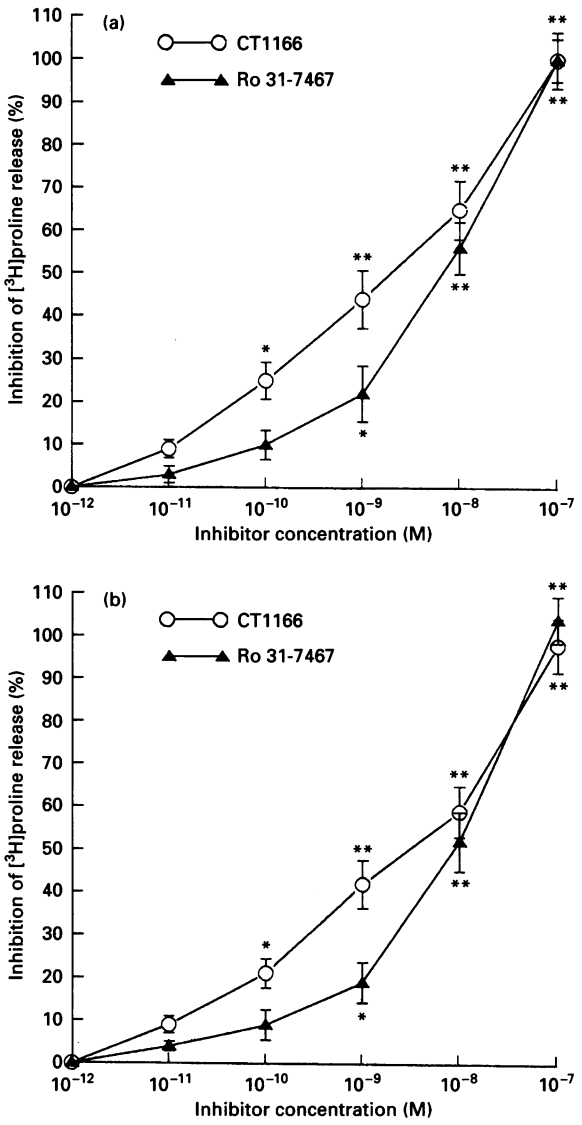

Figure 2 Effect of CT1166 and Ro 31-7467 at different concentrations on the 1,25-$(OH)_2D_3$ (10^{-8} M)-stimulated (a) and IL-1 (10^{-10} M)-stimulated (b) release of [^{3}H]proline from calvarial bones after a 24 h incubation period

The results are expressed as percentage inhibition of stimulated [^{3}H]proline release, which was arbitrarily set to 100%. Each point shows the mean ± S.E.M. of five pairs of bones. The inhibitory effects of CT1166 (10^{-10}–10^{-7} M) and Ro 31-7467 (10^{-9}–10^{-7} M) were statistically significant at all doses. * $P < 0.05$, ** $P < 0.01$ compared with control. The 1,25-$(OH)_2D_3$- and IL-1α-stimulated release of [^{3}H]proline were 7.6 ± 0.9 and 8.2 ± 0.8 respectively. The calvarial halves were prelabelled with 23478 ± 3695 d.p.m. and the percentage release of [^{3}H]proline from devitalized bones was 4.7 ± 0.6%.

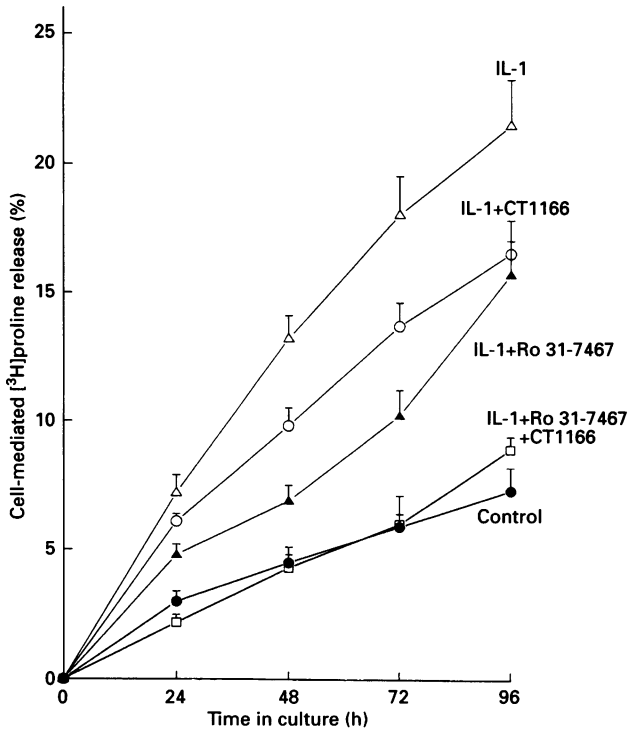

Figure 3 Time course of the effects of CT1166 (10^{-9} M) and Ro 31-7467 (10^{-8} M) on [^{3}H]proline release from labelled mouse calvarial bones stimulated by IL-1 (10^{-10} M)

The release of [^{3}H]proline was determined after the removal of 100 μl of medium at each time point. Values are expressed as the mean percentage (± S.E.M.) of radioisotope released from five bones. The stimulatory effect of IL-1 was significant at all time points ($P < 0.01$). CT1166 and Ro 31-7467 produced a significant inhibition of the IL-1-stimulated release of [^{3}H]proline throughout the 96 h culture period ($P < 0.05$). The calvarial halves were prelabelled with 26218 ± 4029 d.p.m. and the percentage release of [^{3}H]proline from devitalized bones was 5.1 ± 0.4%.

Table 1 Recovery from the inhibitory effects of CT1166 and Ro 31-7467 on the IL-1-stimulated release of [^{3}H]proline from mouse calvarial bones in culture

Values are means ± S.E.M. for five calvarial bones prelabelled with 0.37 MBq [^{3}H]proline. IL-1, CT1166 and Ro 31-7467 were added at final concentrations of 10^{-10} M, 10^{-7} M and 10^{-7} M respectively. * Significantly different from IL-1 alone at ($P < 0.01$).

Treatment		Cell-mediated [^{3}H]proline release (%)		
0–48 h	48–144 h	0–48 h	48–96 h	96–144 h
IL-1	IL-1	14.2 ± 1.9	12.1 ± 1.3	11.6 ± 1.5
IL-1 + CT1166	IL-1	7.3 ± 0.8*	11.3 ± 1.1	13.6 ± 1.5
IL-1 + Ro 31-7467	IL-1	5.1 ± 0.6*	10.2 ± 0.8	12.4 ± 1.4

Effects of MMP inhibitors on calvarial bone resorption *in vitro*

As shown in Figure 2 CT1166 and Ro 31-7467 dose-dependently inhibited the release of [^{3}H]proline from calvarial explants stimulated by either 1,25-$(OH)_2D_3$ (10^{-8} M) or IL-1α (10^{-10} M) during 24 h. CT1166 produced a statistically significant inhibition in the release of [^{3}H]proline at a 10^{-10} M concentration, and Ro 31-7467 at a 10^{-9} M concentration. However, Ro 31-7467 produced a 50% inhibition in the stimulated release of [^{3}H]proline, and CT1166 a 40% inhibition at concentrations selective for inhibition of collagenase (10^{-8} M for Ro 31-7467) and gelatinase (10^{-9} M for CT1166) respectively. The inhibitory effects of CT1166 on stimulated bone resorption were only complete at a concentration (10^{-7} M) high enough to inhibit gelatinase and stromelysin but not collagenase. The inhibitory effects of Ro 31-7467 were only complete at a concentration (10^{-7} M) at which it acts as a general MMP inhibitor.

In 96 h bone cultures the IL-1α-stimulated release of [^{3}H]-proline was partially prevented by both compounds at concentrations selective for the inhibition of collagenase and gelatinase respectively (Figure 3). Although the inhibitory activity of

Table 2 Effect of CT1166 and Ro 31-7467 on [^{3}H]thymidine uptake into DNA and [^{3}H]proline incorporation into proteins in mouse calvarial bones

Values are means ± S.E.M. for five calvarial halves, labelled with either 0.037 MBq [^{3}H]proline (protein synthesis) or 0.18 MBq [^{3}H]thymidine (DNA synthesis) for the last 6 h of a 96 h culture period.

Addition	Amount	[^{3}H]Thymidine (d.p.m./half calvaria)	[^{3}H]Proline (d.p.m./half calvaria)
Control	–	24201 ± 4219	15631 ± 2317
CT1166	10^{-7} M	25884 ± 3697	12976 ± 2476
Ro 31-7467	10^{-7} M	22306 ± 2967	16019 ± 2169
Devitalized bone	–	4596 ± 419	3654 ± 397
Actinomycin D	10^{-4} M	–	5431 ± 485
Hydroxyurea	10^{-4} M	5875 ± 482	–

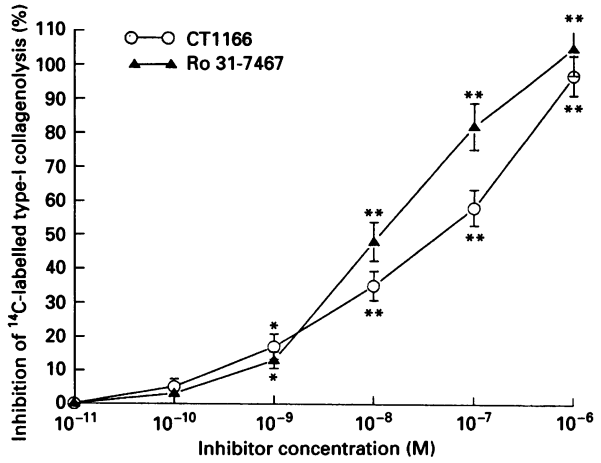

Figure 4 Effects of CT1166 and Ro 31-7467 at different concentrations on the degradation of ^{14}C-labelled type-I collagen films by mouse osteoblasts stimulated by 1,25-$(OH)_2D_3$ (10^{-8} M) after 72 h

The results are expressed as percentage inhibition of 1,25-$(OH)_2D_3$-stimulated ^{14}C release, which was arbitrarily set to 100%. Each point is the mean ± S.E.M. of six wells. The inhibitory effects of CT1166 (10^{-9}–10^{-6} M) and Ro 31-7467 (10^{-9}–10^{-6}M) were statistically significant. * $P < 0.05$, ** $P < 0.01$ compared with control. The percentage release of isotope by 1,25-$(OH)_2D_3$-stimulated mouse osteoblasts was 55.1 ± 3.1 which was obtained after subtracting the unstimulated release of isotope (19.1 ± 2.6).

CT1166 was less than that of Ro 31-7467 for the first 72 h their effects were similar over the last 24 h. Only CT1166 (10^{-9} M) and Ro 31-7467 (10^{-8} M) in combination completely abolished the IL-1α-stimulated release of [^{3}H]proline throughout the 96 h. We then carried out a recovery experiment involving treatment of bones with IL-1α (10^{-10} M) and either CT1166 (10^{-7} M) or Ro 31-7467 (10^{-7} M) for the first 48 h and then cultured in the presence of IL-1α only for a second period, the inhibitory effects of both compounds seen during the initial culture period were subsequently lost (Table 1). Neither CT1166 (10^{-7} M) nor Ro 31-7467 (10^{-7} M) inhibited DNA synthesis or protein synthesis in calvarial bones, whereas actinomycin D (10^{-4} M) blocked the uptake of [^{3}H]proline and hydroxyurea (10^{-4} M) the uptake of [^{3}H]thymidine (Table 2). At the highest doses tested, neither CT1166 (10^{-7} M) nor Ro 31-7467 (10^{-7} M) influenced the stimulated release of the lysosomal enzyme β-glucuronidase (data not shown).

Table 3 Effect of CT1166 and Ro 31-7467 on lactate production by rat osteoclasts and mouse osteoblast cultures

Values are means ± S.E.M. for six osteoclast and five osteoblast cultures.

		Lactate concentration (μmol/l)	
Addition	Amount	Rat osteoclasts	Mouse osteoblasts
Control	–	14.0 ± 0.5	14.6 ± 0.4
CT1166	10^{-5} (M)	13.6 ± 0.9	13.8 ± 1.0
Ro 31-7467	10^{-5} (M)	14.3 ± 0.8	24.8 ± 1.1

Effects of the MMP inhibitors on the degradation of type-I collagen by mouse osteoblasts stimulated with 1,25-$(OH)_2D_3$

CT1166 and Ro 31-7467 dose-dependently inhibited 1,25-$(OH)_2D_3$-stimulated collagen breakdown by mouse osteoblasts (Figure 4). However, the concentrations of the respective MMP inhibitors required to produce a similar degree of inhibition to that demonstrated for bone resorption was about 10-fold greater, for both compounds. Ro 31-7467 and CT1166 produced about a 50% and 20% reduction in type-I collagenolysis respectively, at concentrations selective for the inhibition of collagenase (10^{-8} M for Ro 31-7467) and gelatinase (10^{-9} M for CT1166). However, both compounds completely abolished type-I collagenolysis only at concentrations in which they act as general inhibitors of MMPs. The effects of the inhibitors on glycolysis was tested by measuring the amount of lactate produced by the osteoblast cultures. Neither CT1166 nor Ro 31-7467 reduced lactate levels compared with control cultures (Table 3).

Effects of the MMP inhibitors on isolated osteoclast bone resorption

We examined the direct effect of the MMP inhibitors on osteoclast function in an isolated osteoclast resorption pit assay. In contrast to bone, ivory (a mineralized bone-like material) was chosen as the biological substrate as it presents a homogenous surface so that accurate determinations of lacunar resorption can be made. Image analysis of the 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 concentration of 5×10^{-5} M (data not shown). However, when the osteoclast cultures were stimulated by IL-1α (10^{-10} M), both compounds produced a dose-dependent reduction in the number of resorption lacunae (data not shown). Their inhibitory effects were incomplete, however, with Ro 31-7467 producing a maximum of 26.9 ± 1.9% and CT1166 a maximum of 29.2 ± 2.9% both at a 10^{-5} M concentration. When the extent of the bone resorption was assessed by measuring the total surface area of the lacunae it was found that CT1166 and Ro 31-7467 produced a degree of inhibition similar to that for the number of resorption lacunae (Figure 5). This was due to the fact that the mean surface area of the individual resorption lacunae in the inhibitor-treated cultures (CT1166, 547 ± 87 μm²; Ro 31-7467, 602 ± 89 μm²) was similar to the control cultures (573 ± 75 μm²).

To determine whether the inhibitors were exerting a toxic effect on the osteoclast cultures, the cells were inspected both

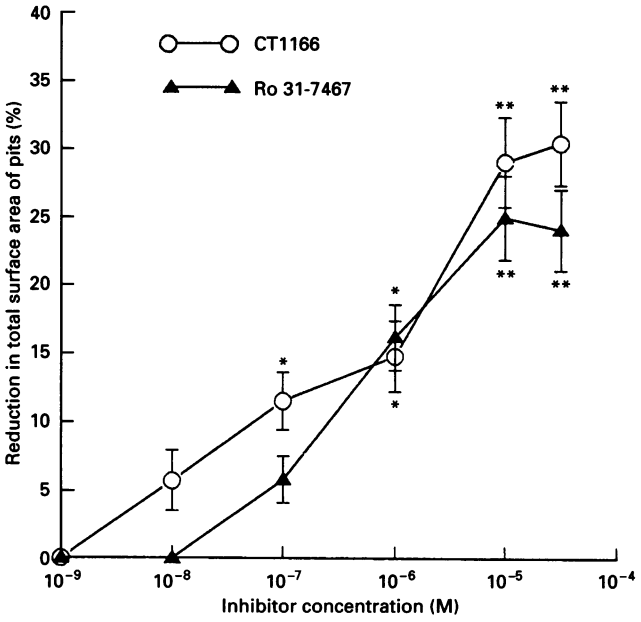

Figure 5 Effects of CT1166 and Ro 31-7467 on IL-1-stimulated rat osteoclast lacunar resorption

The results are expressed as the percentage inhibition of IL-1-stimulated osteoclast resorption lacunae (total surface area) which was arbitrarily set to 100%. Each point is the mean ± S.E.M. of quadruplicate cultures. The number of pits measured in the CT1166-treated cultures was 1234 and 987 in the Ro 31-7467-treated cultures. The inhibitory effects of CT1166 (10^{-7}–5×10^{-5} M) and Ro 31-7467 (10^{-6}–5×10^{-5} M) were statistically significant compared with the control at $P < 0.05$.

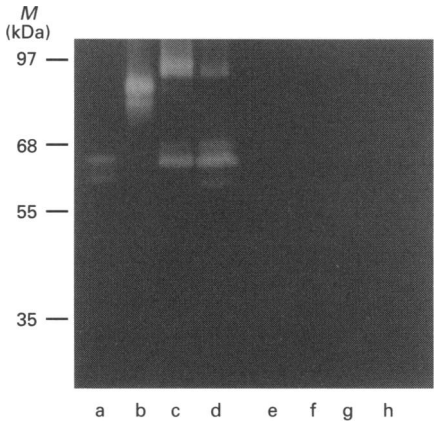

Figure 6 Zymogram analysis of supernatants from mouse calvariae and rat osteoclast cultures

Purified culture supernatants (10 μl) from IL-1-stimulated (10^{-10} M) mouse calvariae and rat osteoclasts were run non-reduced on an 8% polyacrylamide gel with gelatin (0.5 mg/ml) incorporated. Lanes a and e, 10 ng/ml gelatinase A standard; lanes b and f, 10 ng/ml gelatinase B standard; lanes c and g, rat osteoclast; lanes d and h, mouse calvariae. After SDS removal, the gel was cut in half and the two halves incubated at room temperature in: lanes a–d, Tris/HCl buffer (pH 7.9)/30 mM $CaCl_2$; lanes e–h, Ca^{2+}-free Tris/HCl buffer (pH 7.9) + 2 mM 1,10-phenanthroline. The gel was stained and destained as described in the Materials and methods section.

after Neutral Red staining and following fixation and staining for TRAP. In the presence of the MMP inhibitors multinucleated cells took up Neutral Red just as well as controls, suggesting that

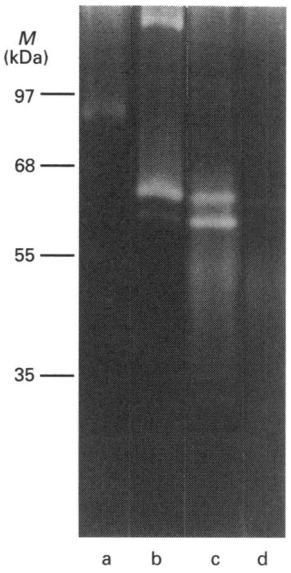

Figure 7 Zymogram analysis of ivory and calvarial bone extracts

Purified and concentrated extracts (10 μl) of ivory and calvarial bone extracts from gelatin–Sepharose chromatography were run non-reduced on an 8% polyacrylamide gel with gelatin (0.5 mg/ml) incorporated. Zones of lysis were revealed as described in the text. Lane a, 10 ng/ml gelatinase B standard; lane b, 10 ng/ml gelatinase A standard; lane c, calvarial extract; lane d, ivory extract. The relative mobilities (as molecular mass) of reduced standard proteins are indicated.

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 motility of the osteoclasts. Furthermore, at the maximum concentration used (10^{-5} M) neither compound had an inhibitory effect on glycolysis, assessed by measuring the amount of lactate produced by the osteoclast cultures (Table 3). Finally, at the highest concentration used neither of the inhibitors altered the number of osteoclasts, as compared with controls. The mean number of TRAP-positive multinucleated cells per 1 cm^2 slice (15 slices per treatment) was 83.2 ± 4.9 for the control, 79.5 ± 3.2 for CT1166 (10^{-5} M) and 87.2 ± 5.1 for Ro 31-7467 (10^{-5} M).

Collagenase and gelatinase activities of mouse calvarial and rat osteoclast culture supernatants

Mouse calvariae and rat osteoclast cultures released 0.28 ± 0.07 and 0.19 ± 0.05 units/ml of 4-aminophenylmercuric acetate (APMA)-activatable (latent) collagenase respectively, into the culture medium. The mouse and rat culture supernatants contained both pro and active forms of gelatinases A and B (Figure 6, lanes c and d). The MMP inhibitor 1,10-phenanthroline (2 mM) abolished zones of lysis on one-half of a zymogram analysis of culture supernatants from mouse calvariae and rat osteoclasts (Figure 6, lanes g and h). This inhibition is further evidence that the enzymes present in the culture supernatants responsible for gelatin degradation are metalloproteinases. Indirect immunofluorescence of IL-1-stimulated mouse osteoclast cultures also demonstrated that these cells synthesize collagenase and gelatinases A/B, the enzymes being localized to the Golgi apparatus and secretory vesicles of the osteoclasts (data not shown).

Collagenase and gelatinase activity of ivory and calvarial bone

Calvarial bone contained 0.154 unit of collagenase activity per 10 mg of tissue, but we detected no collagenase activity in ivory. Similarly calvarial bone contained both gelatinase A and B activity (Figure 7) while ivory contained only a trace of material running as the pro form of gelatinase A.

DISCUSSION

The data presented in this study demonstrate for the first time that potent, selective inhibitors of collagenase and gelatinase prevent (a) bone resorption *in vitro*, as assessed using rodent calvarial explants and isolated osteoclast cultures, and (b) osteoblast-mediated type-I collagenolysis *in vitro*.

The ability of Ro 31-7467 to inhibit calvarial bone resorption and osteoblast-mediated type-I collagenolysis at concentrations selective for the inhibition of collagenase are attributable to the rate-limiting role of this enzyme in type-I collagen degradation (Murphy and Reynolds, 1985), and the fact that only a small proportion of latent collagenase secreted by osteoblasts is activated and therefore available for collagen degradation (Thomson et al., 1987). Based upon the resistance of the native type-I collagen triple helix to degradation by non-specific proteinases and the specificity of collagenase for the substrate, collagenase has been considered to be the principal MMP involved in bone collagen digestion (Delaisse et al., 1985; 1988; Eeckhout et al., 1986; Eeckhout, 1990). However, our data demonstrate that gelatinases A and/or B also participate in calvarial bone resorption and type-I collagen degradation. This view is strengthened by the fact that human osteoblasts produce gelatinase A to a greater extent, both constitutively and in response to osteotropic hormones, than collagenase (Meikle et al., 1992). While collagenase is responsible for the initial cleavage of native type-I collagen, gelatinases A and/or B may play a significant role in the subsequent digestion of the denatured collagen fibrils. This conclusion was confirmed in the present study by the demonstration that a combination of Ro 31-7467 and CT1166 were required to prevent completely calvarial bone resorption during the 96 h culture period.

Our data have also shown that MMPs participate in collagen degradation within the sub-osteoclastic resorption zone, in agreement with morphological data (Everts et al., 1992). Because the present investigation has shown that ivory contains no detectable collagenase or gelatinase activity, we conclude that the osteoclast is a source of MMPs. This view is supported by our demonstration that rodent osteoclasts synthesize collagenase and gelatinases A/B. Since the MMP inhibitors were only effective in preventing stimulated rather than unstimulated osteoclast cultures, it is likely that the cells only produce the MMPs under certain states of activation. This may be similar to the situation in mononuclear phagocytes (MNPs), cells that are ontogenetically related to osteoclasts (Chambers, 1989). It has been shown that MNPs secrete greater quantities and a wider spectrum of MMPs in response to cytokine stimulation and according to their degree of cellular differentiation (Cury et al., 1988; Shapiro et al., 1990; Welgus et al., 1990). We also know from our work with osteoblasts (Meikle et al., 1992) that apart from gelatinase-A, TIMP-1 and TIMP-2, which are constitutively synthesized, that MMP synthesis is very low unless the cells are stimulated.

Since we have shown that gelatinase A and B, in addition to collagenase, are present in bone, in contrast to ivory, it is conceivable that we have underestimated the contribution of the MMPs to the sub-osteoclastic degradation of bone collagen. This will only be resolved once it is possible to quantify accurately the extent of osteoclast lacunar resorption in living bone to a similar degree to that in a homogenous substrate such as ivory.

Both compounds only affected isolated osteoclast resorptive activity at concentrations in which they act as general inhibitors of MMPs. This could be due to limited access of the compounds to the sub-osteoclastic resorption zone, or to unfavourable MMP:inhibitor ratios within the zone itself. A more likely explanation is that lysosomal CPs play a more significant role at this stage of the resorptive cascade (Delaisse et al., 1987; Everts et al., 1988, 1992; Rifkin et al., 1991). Using selective inactivators of CPs we have recently identified cathepsins B, L and/or S as the lysosomal CPs that participate in bone resorption in calvarial explants and osteoclast cultures (Hill et al., 1994). While the current MMP inhibitors were found to be as effective as CP inactivators in calvarial explant cultures, the CP inactivators produced a more complete inhibition of lacunar resorption in the isolated osteoclast assay (Hill et al., 1994). Although this would indicate that CPs play a more prominent role than MMPs in osteoclastic bone resorption, we propose that both classes of enzyme act in concert, resulting in a more efficient degradation of both collagen and non-collagenous matrix components within the sub-osteoclastic resorption zone. Co-operation between the enzymes may also occur at the level of zymogen activation: in the presence of cathepsin B, stromelysin, which has been detected in osteoclasts (Case et al., 1989), can activate procollagenase (Murphy et al., 1992c). There is also evidence to suggest that the collagenolytic activities of the MMPs and CPs are enhanced by high concentrations of Ca^{2+} generated within the bone-resorbing compartment during mineral dissolution (Etherington and Birkedal-Hansen, 1987; Eeckhout, 1990). Stromelysin retains activity at the acidic pH values encountered within the resorption lacunae (Galloway et al., 1983) but there is a significant loss in the activity of collagenase (Vaes, 1972) and gelatinase (Seltzer et al., 1981) below pH 6. Therefore, one might envisage that the latter two enzymes do not participate in the sub-osteoclastic degradation of collagen. However, the pH within the resorption zone will depend upon the efficiency of osteoclast acid production and the buffering potential of the bone salts released during mineral dissolution. The differences in pH optima of the lysosomal CPs (acidic) and MMPs (neutral) may extend the pH range over which osteoclast bone resorption can occur. Furthermore, these variations in pH may result in spatial and temporal differences in the actions of the various enzymes within this region. The lysosomal CPs may be secreted concurrently with release of H^+ ions, while the MMPs may be active at a later stage in the resorption process once the acid has been neutralized by the released bone salts.

In conclusion, our findings suggest that not only collagenase but also gelatinases A and/or B play an important role in bone resorption. Furthermore, our data indicate that osteoclast-derived MMPs participate in the sub-osteoclastic degradation of bone collagen, in addition to their role in osteoblast-mediated osteoid degradation. It should be noted that the results obtained in this study using the MMP inhibitors rely on the extrapolation of the IC_{50} values for human enzymes to those of rodents. Finally, since collagenase and gelatinases A and B seem to play an important role in bone remodelling and an imbalance of either of these MMPs over their natural inhibitor, TIMP, could be responsible for the increased bone loss in pathological conditions.

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REFERENCES

Achord, D. T., Brot, F. E., Bell, C. E. and Sly, W. S. (1978) Cell **15**, 269–278

Barhan, D. and Trinder, P. (1972) Analyst **97**, 142–144

Baron, R. (1989) Anat. Rec. **224**, 317–324

Blair, H. C., Kahn, A. J., Crouch, E. C., Jeffrey, J. J. and Teitelbaum, S. L. (1986) J. Cell Biol. **102**, 1164–1172

Blair, H. C., Teitelbaum, S. L., Ghiselli, R. and Gluck, S. (1989) Science **245**, 855–857

Boyde, A., Ali, N. N. and Jones, S. J. (1984) Br. Dent. J. **156**, 216–220

Case, J. P., Sano, H., Lafyatis, R., Remmers, E. F., Kumkumian, G. K. and Wilder, R. L. (1989) J. Clin. Invest. **84**, 1731–1740

Chambers, T. J. (1989) in Bone and Mineral Research: The Origin of the Osteoclast (Peck, W. A., ed.), pp. 1–85, Elsevier Science Publishers BV, Amsterdam

Chambers, T. J., Thomson, B. M. and Fuller, K. (1984) J. Cell Sci. **70**, 61–71

Chambers, T. J., McSheehy, P. M. J., Thomson, B. M. and Fuller, K. (1985) Endocrinology **116**, 234–239

Cury, J. D., Campbell, E. J., Lazarus, C. J., Albin, R. J. and Welgus, H. G. (1988) J. Immunol. **141**, 4306–4312

Delaisse, J.-M., Eeckhout, Y., Sear, C., Galloway, A., McCullagh, K. and Vaes, G. (1985) Biochem. Biophys. Res. Commun. **133**, 483–490

Delaisse, J. M., Boyde, A., Maconnachie, E., Ali, N. N., Sear, C. H. J., Eeckhout, Y., Vaes, G. and Jones, S. J. (1987) Bone **8**, 305–313

Delaisse, J.-M., Eeckout, Y. and Vaes, G. (1988) Endocrinology **123**, 264–276

Delaisse, J.-M., Ledent, P. and Vaes, G. (1991) Biochem. J. **279**, 167–174

Docherty, A. J. P., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J. R., Murphy, G. and Reynolds, J. J. (1985) Nature (London) **318**, 66–69

Eeckhout, Y. (1990) Biochem. J. **272**, 529–532

Eeckhout, Y., Delaisse, J.-M. and Vaes, G. (1986) Biochem. J. **239**, 793–796

Etherington, D. J. and Birkedal-Hansen, H. (1987) Collagen Rel. Res. **7**, 185–199

Everts, V., Beertsen, W. and Schroder, R. (1988) Calcif. Tissue Int. **43**, 172–178

Everts, V., Delaisse, J.-M., Korper, W., Niehof, A., Vaes, G. and Beertsen, W. (1992) J. Cell. Physiol. **150**, 221–231

Galloway, W. A., Murphy, G., Sandy, J. D., Gavrilovic, J., Cawston, T. E. and Reynolds, J. J. (1983) Biochem. J. **209**, 741–752

Gavrilovic, J., Reynolds, J. J. and Murphy, G. (1985) Cell Biol. Int. Rep. **9**, 1097–1107

Heath, J. K., Atkinson, S. J., Meikle, M. C. and Reynolds, J. J. (1984) Biochim. Biophys. Acta **802**, 151–154

Hembry, R. M., Murphy, G. and Reynolds, J. J. (1985) J. Cell Sci. **73**, 105–119

Hembry, R. M., Murphy, G., Cawston, T. E., Dingle, J. T. and Reynolds, J. J. (1986) J. Cell Sci. **81**, 105–123

Heussen, C. and Dowdle, E. B. (1980) Anal. Biochem. **102**, 196–202

Hill, P. A., Reynolds, J. J. and Meikle, M. C. (1993) Biochim. Biophys. Acta **1177**, 71–74

Hill, P. A., Buttle, D. J., Jones, S. J., Boyde, A., Murata, M., Reynolds, J. J. and Meikle, M. C. (1994) J. Cell. Biochem. **56**, 118–130

Johnson, W. H., Roberts, N. A. and Borkakoti, N. (1987) J. Enzyme Inhibition **2**, 1–22

Johnson-Wint, B. (1980) Anal. Biochem. **104**, 175–181

Knight, C. G., Willenbrock, F. and Murphy, G. (1992) FEBS Lett. **296**, 263–266

Laemmli, U. K. and Favre, M. (1973) J. Mol. Biol. **80**, 575–599

Lerner, U. H. (1987) J. Bone Min. Res. **2**, 375–383

Ljunggren, O., Ransjo, M. and Lerner, U. H. (1991) J. Bone Min. Res. **6**, 543–550

Lorenzo, J. A., Pilbeam, C. C., Kalinowski, J. F. and Hibbs, M. S. (1992) Matrix **12**, 282–290

Meikle, M. C., Bord, S., Hembry, R. M., Compston, J., Croucher, P. I. and Reynolds, J. J. (1992) J. Cell Sci. **103**, 1093–1099

Moore, W. M., Spilburg, C. A., Hirsch, S. K., Evans, C. L., Wester, W. N. and Martin, R. A. (1986) Biochemistry **25**, 5189–5195

Morrison, J. P. and Walsh, C. T. (1988) Adv. Enzymol. Rel. Areas Mol. Biol. **61**, 201–301

Murphy, G. and Reynolds, J. J. (1985) BioEssays **2**, 55–60

Murphy, G. and Reynolds, J. J. (1993) in Connective Tissue and its Heritable Disorders: Extracellular Matrix Degradation (Royce, P. M. and Steinmann, B., eds.), pp. 287–316, Wiley-Liss, Inc., New York

Murphy, G., McAlpine, C. G., Poll, C. T. and Reynolds, J. J. (1985) Biochim. Biophys. Acta **831**, 49–58

Murphy, G., Hembry, R. M., McGarrity, A. M. and Reynolds, J. J. (1989) J. Cell Sci. **92**, 487–495

Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D. and Docherty, A. J. P. (1992a) Biochem. J. **283**, 637–641

Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. I., O'Connell, J. P. and Docherty, A. J. P. (1992b) J. Biol. Chem. **267**, 9612–9618

Murphy, G., Ward, R., Gavrilovic, J. and Atkinson, S. (1992c) Matrix **1** (Suppl.), 224–230

O'Connell, J. P., Willenbrock, F., Docherty, A. J. P., Eaton, D. and Murphy, G. (1994) J. Biol. Chem. **269**, 14967–14973

Otsuka, K., Sodek, J. and Limeback, H. (1984) Eur. J. Biochem. **145**, 123–129

Reynolds, J. J. and Dingle, J. T. (1970) Calc. Tissue Res. **4**, 339–349

Rifas, L., Halstead, L. R., Peck, W. A., Avioli, L. V. and Welgus, H. G. (1989) J. Clin. Invest. **84**, 686–694

Rifkin, B. R., Vernillo, A. T., Kleckner, A. P., Auszmann, J. M., Rosenberg, L. R. and Zimmerman, M. (1991) Biochem. Biophys. Res. Commun. **179**, 63–69

Sakamoto, S. and Sakamoto, M. (1984) Biomed. Res. **5**, 39–46

Sakamoto, S. and Sakamoto, M. (1986) in Bone and Mineral Research: Bone Collagenase, Osteoblasts and Cell-Mediated Bone Resorption (Peck, W. A., ed.), vol. 4, pp. 49–102, Elsevier Science Publishers B. V., Amsterdam

Sellers, A. and Reynolds, J. J. (1977) Biochem. J. **167**, 353–360

Sellers, A., Reynolds, J. J. and Meikle, M. C. (1978) Biochem. J. **171**, 493–496

Seltzer, J. L., Adams, S. A., Grant, G. A. and Eisen, A. Z. (1981) J. Biol. Chem. **256**, 4662–4668

Shapiro, S. D., Campbell, E. J., Kobayashi, D. K. and Welgus, H. G. (1990) J. Clin. Invest. **86**, 1204–1210

Stack, M. S. and Gray, R. D. (1989) J. Biol. Chem. **264**, 4277–4281

Stetler-Stevenson, W. G., Brown, P. D., Onisto, M., Levy, A. T. and Liotta, L. A. (1990) J. Biol. Chem. **265**, 13933–13938

Thomson, B. M., Atkinson, S. J., Reynolds, J. J. and Meikle, M. C. (1987) Biochem. Biophys. Res. Commun. **148**, 596–602

Vaes, G. (1972) Biochem. J. **126**, 275–289

Welgus, H. G., Campbell, E. J., Cury, J. D., Eisen, A. Z., Senior, R. M., Wilhelm, S. M. and Goldberg, G. I. (1990) J. Clin. Invest. **86**, 1496–1502

Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A. and Goldberg, G. I. (1989) J. Biol. Chem. **264**, 17213–17221

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