The effects of serine proteinase inhibitors on bone resorption *in vitro*

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Abstract

The aims of this study were to identify the role and sites of action of serine proteinases (SPs) in bone resorption, a process which involves a cascade of events, the central step of which is the removal of bone matrix by osteoclasts (OCs). This resorbing activity, however, is also determined by recruitment of new OCs to future resorption sites and removal of the osteoid layer by osteoblasts (OBs), which enables OCs to gain access to the underlying mineralized bone. The resorption systems we have studied consisted of (i) neonatal calvarial explants, (ii) isolated OCs cultured on ivory slices, (iii) mouse OBs cultured on either radiolabelled type I collagen films or bone-like matrix, (iv) bone marrow cultures to assess OC formation and (v) 17-day-old fetal mouse metatarsal bone rudiments to assess OC migration and fusion.

Two separate SP inhibitors, aprotinin and α_2 -antiplasmin dose-dependently inhibited ^{45}Ca release from neonatal calvarial explants: aprotinin (10^{-6} M) was the most effective SP inhibitor, producing a maximum inhibitory effect of 55.9%.

Neither of the SP inhibitors influenced either OC formation or OC resorptive activity. In contrast, each SP inhibitor dose-dependently inhibited OB-mediated degradation of both type I collagen fibrils and non-mineralized bone matrix. In 17-day-old metatarsal explants aprotinin produced a 55% reduction in the migration of OCs from the periosteum to the mineralized matrix after 3 days in culture but after 6 days in culture aprotinin was without effect on OC migration. Primary mouse osteoblasts expressed mRNA for urokinase type plasminogen activator (uPA), tissue type plasminogen activator (tPA), the type I receptor for uPA, plasminogen activator inhibitor types I and II and the broad spectrum serine proteinase inhibitor, protease nexin I. *In situ* hybridization demonstrated expression of tPA and uPA in osteoclasts disaggregated from 6-day-old mouse long bones. We propose that the regulation of these various enzyme systems within bone tissue determines the sites where bone resorption will be initiated.

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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 subosteoclastic resorption zone (SORZ), a specialized extracellular compartment bounded by the ruffled border of the cell and the mineralized bone matrix (Baron 1989). Osteoclasts acidify the SORZ leading to dissolution of mineral (Blair *et al.* 1989) while the organic matrix is believed to be degraded by lysosomal cysteine proteinases, matrix metalloproteinases (MMPs) and also serine proteinases (Hill *et al.* 1995). Bone resorption is also governed by the recruitment of new osteoclasts from progenitor cells of the mononuclear phagocyte system (Suda *et al.* 1992). The mononuclear precursors are disseminated via the bloodstream and deposited in the

mesenchyme surrounding bone rudiments where they proliferate into preosteoclasts prior to migrating to future resorption sites (Blavier & Delaisse, 1995). Osteoblasts play an accessory role in bone resorption by releasing proteinases that degrade the surface osteoid layer (principally type I collagen), facilitating the access of osteoclasts to mineralized bone (Chambers *et al.* 1985).

The plasminogen activators (PAs), urokinase type PA (uPA) and tissue type PA (tPA), are serine proteinases that catalyse the conversion of the proenzyme plasminogen into a broad spectrum serine protease, plasmin. Plasmin can either directly or indirectly via activation of latent MMPs promote the degradation of all components of the extracellular matrix *in vitro* (Murphy *et al.* 1992, Mignatti & Rifkin 1993). With regard to a possible involvement of serine proteinases in bone turnover, plasminogen has been shown to be present in extracellular matrices

(Knudsen *et al.* 1986). Furthermore, osteoblasts produce PAs in response to agents that promote bone resorption (Fukumoto *et al.* 1992, Allan & Martin 1995) and most recently it has been suggested that serine proteinases are involved in the degradation of non-collagenous proteins of bone (Daci *et al.* 1999). Although these results support the notion that the PA/plasmin system might be involved in bone resorption, results from other studies suggest a limited role (Leloup *et al.* 1994, 1996).

Since plasminogen is the most abundant and best defined substrate for PAs and its activation by PAs results in the generation of plasmin (Vassalli *et al.* 1991), the importance of plasmin activity to the normal sequence of events that leads to bone resorption was investigated in this study. We assessed the contribution of the PA/plasmin system to the different aspects of the bone resorption cascade using selective inhibitors of serine proteinases (SPs) in combination with a variety of *in vitro* models that are specific for the various aspects of the resorption process. Furthermore, an assessment was made of the expression of the SPs and their natural inhibitors in these model systems by RT-PCR and *in situ* hybridization.

Materials and Methods

Materials

Cell culture reagents and the SP inhibitors, aprotinin and α_2 -antiplasmin, were purchased from Sigma Chemical Co. (Poole, Dorset, UK). $^{45}\text{CaCl}_2$, ^{14}C and ^3H -amino acid mixture were purchased from Amersham International (Aylesbury, Bucks, UK). Enzymes and reagents for RT-PCR were purchased as a kit from Applied Biosystems (Foster City, CA, USA). The MMP inhibitor, CT1399, and the cysteine proteinase inhibitor, Ep453, were gifts from Dr A Docherty (Celltech, UK) and Dr M Murata (Research Centre, Taisho Pharmaceuticals, Japan) respectively.

Methods

Preparation of acid-treated serum To destroy serum inhibitors of neutral proteinases, aliquots (20 ml) of heat-inactivated calf serum (GlobePharm, Esher, Surrey, UK) 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. This serum was used in all the assays.

Neonatal calvarial assay Bone resorption was assessed by analysing $^{45}\text{CaCl}_2$ release from prelabelled cultured neonatal mouse calvarial bones (Reynolds & Dingle 1970). Briefly, 1-day-old mice were injected s.c. with 0.1 megabecquerels $^{45}\text{CaCl}_2$. Six days later calvarial bones were excised and cultured in pairs in CMRL 1066 medium (2 ml) containing 5% acid-treated fetal calf serum (FCS)

for up to 4 days with media changes every two days. 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 $^{45}\text{Ca}^{2+}$ release due to passive exchange of isotope with cold Ca^{2+} in the culture medium, two bones from each litter were devitalized by three cycles of freeze-thawing. The percentage release from the devitalized bone was subtracted from each living bone to give the amount of cell-mediated resorption. The bones were stimulated with either parathyroid hormone (PTH) (10^{-8} M) or 1,25-dihydroxyvitamin D_3 ($1,25\text{-(OH)}_2\text{D}_3$) (10^{-8} M).

Isolated osteoclast assay The osteoclast bone resorption assay is based on the ability of isolated osteoclasts to resorb devitalized cortical bone, dentine, or ivory slices *in vitro* (Boyde *et al.* 1984). Ivory slices (200 μm) were cut with a low speed water-cooled diamond saw (Isomet, Buehler, Coventry, UK) from a 1-cm² rod. Ivory slices were chosen because they are free of vascular systems and pre-existing resorbing surfaces; osteoclasts can resorb ivory. Osteoclasts were prepared from femurs of 2- to 3-day-old mice. Osteoclasts were allowed to settle for 20 min at 37 °C. The substrate was then washed free of nonadherent cells, and the slices were incubated for 24 h in a humidified atmosphere of 5% CO_2 -95% air at 37 °C in 500 μl α -MEM supplemented with acid-treated 5% FCS, 2.0 g/l NaHCO_3 , 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Due to the variability in the number of osteoclasts isolated from each mouse, a single experiment consisted of 6 ivory slices bearing the cells from one mouse, with 3 slices for each control and test variable. Each experimental variable was repeated 4 times, and the results were expressed as the treated-to-control ratio.

At the completion of the culture period, the cells were removed from the ivory slices. The method used for the precise quantitation of the resorptive capacity involved estimating the surface area of the resorption lacunae by image analysis (TC Image, Foster Finlay Associates, UK).

Murine bone marrow cultures A mouse bone marrow culture system was used to assess osteoclast differentiation (Takahashi *et al.* 1988). In brief, tibiae were removed from 5-week-old mice and marrow cells were plated in 24-well dishes and incubated in the presence of $1,25\text{-(OH)}_2\text{D}_3$. The latter agent has been shown to stimulate the formation of tartrate-resistant acid phosphatase (TRAP)-positive (TRAP+ve) osteoclast-like multinucleate cells (MNCs). After 8 days, the cultures were stained for TRAP and the cells counted.

Metatarsal long bone assay The three middle metatarsals of each hindlimb of 17-day-old mouse embryos (day of vaginal plug discovery equals day 0 of gestation) were

dissected as a triad (Blavier & Delaisse 1995). One triad of each pair was cultured as a control, the other as a test. Each triad was cultured in 1 ml CMRL 1066 medium supplemented with glutamine (200 mg/l), 10% acid-treated FCS with 1,25-(OH)₂D₃ (10⁻⁸ M). The cultures were run for 3 and 6 days and were then fixed in 4% paraformaldehyde overnight at 4 °C. Specimens were then washed in PBS, decalcified in 5% EDTA overnight, dehydrated through a graded series of ethanol and embedded in paraffin. Sections of 5 µm were cut and transferred to sialinized glass slides (Sigma). Sections were stained for TRAP and counterstained with haematoxylin.

Histomorphometry The number of TRAP-positive cells and their nuclei were determined in 10 evenly spaced longitudinal sections per long bone rudiment. According to their location they were scored as (1) lying in the developing marrow cavity, that is the area of resorbing calcified cartilage surrounded by the thin bone collar or (2) in the periosteum-perichondrium, that is the soft tissue around the bone rudiment. The few cells lying within the (thin) bone collar were equally divided over the two compartments.

Preparation of osteoblasts from neonatal mouse calvariae Calvarial osteoblasts were prepared and characterized as previously described (Heath *et al.* 1984).

Preparation of collagen films

Radiolabelled collagen films were prepared as described previously (Gavrilovic *et al.* 1985).

Culture of osteoblasts on collagen films Osteoblasts (1 × 10⁵/well) were settled onto collagen films in 1 ml DMEM plus 10% acid-treated 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% acid-treated serum as described above. Then 1,25-(OH)₂D₃ (10⁻⁸ M) (added in 5 µl ethanol) alone or 1,25-(OH)₂D₃ plus either aprotinin or CT1399, or ethanol alone was added to the wells and the cultures were maintained at 37 °C for 48 h. The basal release of ¹⁴C by unstimulated osteoblasts was subtracted from the 1,25-(OH)₂D₃-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, 1200 g) to remove any collagen fibrils, and radioactivity released during collagen degradation was quantified by liquid scintillation counting. 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.

Formation of ³H-labelled extracellular bone matrix The murine calvaria-derived cell line, MC3T3-E1, is a

well characterized osteoblast culture system providing a suitable model of osteogenesis analogous to *in vivo* bone formation (Quarles *et al.* 1992). In the present study, an extracellular bone-like matrix was produced as described by Roday *et al.* (1997). MC3T3-E1 cells were plated at a density of 1 × 10⁴ cells/well on collagen-coated 24-well plates (Becton Dickinson, Bedford, MA, USA) and cultured in MEM supplemented with 10% FBS, 50 µg/ml ascorbic acid. After 4–5 days, when the cultures had reached confluence and the formation of an extracellular matrix had started, fresh medium was added containing 1 µCi/ml ³H-amino acid mixture (Amersham International) to create a non-mineralized radiolabelled extracellular bone matrix. After 14 days, cells were lysed with 0.5 ml/well Triton X-100 (0.5% v/v in PBS). The cytoskeleton was removed by 25 mM NH₄OH treatment and 1 mM phenylmethylsulphonyl fluoride (0.5 ml/well) treatment was used to block proteinase activity. Matrices were washed with H₂O and 75% (v/v) ethanol to remove unincorporated ³H-radiolabelled amino acids, dried and stored at -20 °C.

Bone matrix degradation assay To assay for matrix degradation, osteoblasts were plated onto the matrices as for the collagen film assays. After 72-h incubation, the media were removed and the extent of degraded ³H-radiolabelled matrix released into the medium was determined by liquid scintillation counting. The remaining matrix was degraded with 0.25% (w/v) trypsin, 0.1% (w/v) collagenase in PBS for 1 h at 37 °C, and the amount of radioactivity in the matrix similarly assessed. Matrix degradation was expressed as ³H released in the medium as a percentage of the total amount of ³H released by the cells during the 72-h culture period, plus that solubilized from the remaining matrix by trypsin/collagenase treatment.

Reverse transcriptase-polymerase chain reaction (RT-PCR) RNA was extracted using an RNA isolation kit (Stratagene, Amsterdam, The Netherlands). RT-PCR reactions were performed using the GeneAmp rTth Reverse Transcriptase RNA PCR Kit (Applied Biosystems, St. Louis, MO, USA) and a Stratagene Robocycler Gradient 96 PCR machine (Stratagene). Synthetic oligonucleotide sequences specific for tPA, uPA, uPA receptor (uPAR), plasminogen activator inhibitor types I and II (PAI-1 and PAI-2) and protease nexin 1 (PN-1) were synthesized by Life Technologies Ltd (Paisley, Strathclyde, UK) using previously published sequence data (Yang *et al.* 1997) and are shown in Table 1.

Band size was checked against molecular weight standards (X174 HaeIII digest) and sequenced to confirm authenticity.

Preparation of uPA and tPA riboprobes The expected DNA fragment for tPA was excised and purified from a TAE gel using a QIAquick Gel Extraction Kit (Qiagen).

Table 1 Oligonucleotide RT-PCR primer sequences for mouse PAs

	Primer sequence	Expected product length (bp)
Gene		
tPA	F5'-GACGATACTTATGACAACGAC-3' R5'-TATTAACAGATGCTGTGAGG-3'	255
uPA	F5'-CGAATACTACAGGAAGAC-3' R5'-GACATTTTCAGGTTCTTTGG-3'	219
uPAR1	F5'-ATTGCTCTCTGCTCCTGAC-3' R5'-GAGACCCAACCTTATTCACCTG-3'	331
PAI-1	F5'-ATCCTGCCTAAGTTCTCTCTG-3' R5'-ATTGTCTCTGTCGGGTTGTG-3'	290
PAI-2	F5'-CAAAGCTGAACATTGGATAC-3' R5'-ACCACAACATCATCTTCATC-3'	205
PN-1	F5'-GCGATATAATGTAACGGAG-3' R5'-CAAAAATTGATGGACTCAGAG-3'	225
GAPDH	F5'-CCACGAGAAATATGACAAC-3' R5'-GATGCAGGGATGATGTTTC-3'	222

F, forward; R, reverse.

The fresh PCR product was ligated to pGEM-T vector (Promega) using T4 DNA ligase (Promega). The probe for uPA was a generous gift from Dr J D Vassalli (University of Geneva) and consisted of a 660 base pair fragment cloned into the PstI/HindIII site of plasmid pSP64. The plasmids were transformed to competent JM109 *E. coli* plasmid DNA extracted using a method based on Qiagen Maxiprep DNA purification (Qiagen). Plasmids were linearized with BamHI for synthesis of digoxigenin-11-UTP riboprobes. Plasmids were linearized with NcoI and SpeI for synthesis of digoxigenin-11-UTP-labelled riboprobes. The probe for MMP-9 was a generous gift from Dr K Tryggvason (Karolinska Institute, Sweden).

Statistical analysis Data are expressed as means $\pm$ S.E.M. Differences between control and treatment groups were determined by the Mann-Whitney U test. Significant differences from control are represented by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

Calvarial bone resorption

To determine the role of SPs in bone resorption, a neonatal calvarial assay was used. This assay simultaneously screens for activities influencing various aspects of the bone resorption process including osteoclast fusion and migration to resorption sites, osteoclast activity and osteoblast-mediated degradation of type I collagen. When the calvarial explants were cultured in normal FCS, the SP inhibitors did not prevent bone resorption (data not shown). However, when the calvarial explants were cultured in acid-treated FCS which is devoid of inhibitors

Figure 1 Effects of SP inhibitors on the PTH-stimulated release of $^{45}\text{Ca}^{2+}$ from calvarial bones after a 48-h incubation period. The results are expressed as percentage inhibition of PTH-stimulated ^{45}Ca release which was arbitrarily set at 100%. Each value is the mean $\pm$ S.E.M. of 5 pairs of bones. The inhibitory effects of aprotinin (10^{-8} – 10^{-5} M) and α_2 -antiplasmin (10^{-8} – 10^{-5} M) were statistically significant (* $P < 0.05$, ** $P < 0.01$). The percentage release of ^{45}Ca from PTH-stimulated bones was 25.6 ± 4.3 for aprotinin and 23.6 ± 3.6 for α_2 -antiplasmin. Both inhibitors dose-dependently inhibited release of ^{45}Ca .

of neutral proteinase activity each SP inhibitor dose-dependently inhibited ^{45}Ca release, in the range 10^{-10} to 10^{-5} M (Fig. 1). During the 4 days of culture, aprotinin and α_2 -antiplasmin produced a maximal inhibition of approximately 50% of ^{45}Ca release at a concentration of 10^{-5} M. To determine whether the inhibitory effect was reversible, a recovery experiment was carried out. Calvarial bones were treated with PTH (10^{-9} M) and either aprotinin (10^{-5} M) or α_2 -antiplasmin (10^{-5} M) for the first 48 h and then cultured in the presence of PTH for 48–96 h. The inhibitory effects of the compounds seen during the initial culture period were subsequently lost (Table 2). PTH-stimulated release of ^{45}Ca returned to levels observed without addition of inhibitors during the 0–48 h culture period.

Osteoclast pit formation on ivory slices

Figure 2 shows that aprotinin did not prevent osteoclast resorptive activity on ivory slices, producing only a 2% inhibition in pit number and a 5% inhibition in surface area resorbed. In contrast, a CP inhibitor, Ep453 (10^{-5} M), produced significant inhibition of osteoclast activity of approximately 80% for both number of pits and the surface area resorbed. Similarly, the MMP inhibitor,

Table 2 Recovery from the inhibitory effects of aprotinin and α_2 -antiplasmin on the PTH-stimulated release of ^{45}Ca from mouse calvarial bones in culture. Values are means $\pm$ S.E.M. for five calvarial bones prelabelled with 0.37 Mbq ^{45}Ca

Treatment [†]		Cell-mediated ^{45}Ca release (%)	
0–48 h	49–96 h	0–48 h	49–96 h
PTH	PTH	17.2 $\pm$ 2.1	13.3 $\pm$ 2.6
PTH + aprotinin	PTH	9.1 $\pm$ 1.5*	15.2 $\pm$ 2.1
PTH + α_2 -antiplasmin	PTH	8.3 $\pm$ 2.3*	12.6 $\pm$ 1.6

[†]PTH, aprotinin and α_2 -antiplasmin were added at final concentrations of 10^{-9} M, 10^{-5} M, and 10^{-5} M respectively. *Significantly different from PTH alone at $P < 0.05$.

Figure 2 Effects of proteinase inhibitors on the number and the total surface area of mouse osteoclast lacunae. Each value is the percentage inhibition in interleukin (IL)-1 α -stimulated osteoclast lacunar resorption arbitrarily set at 100%. The values represent the means $\pm$ S.E.M. from five individual experiments representing 15 slices for each variable. The number and surface area of pits on the Ep453- and CT1399-treated slices were significantly different from control (** $P < 0.01$). The number of lacunae in the control cultures was 1021 (Ep453), 1293 (CT1399) and 879 (aprotinin).

CT1399, produced a significant decrease in osteoclast activity of approximately 45% for pit number and surface area resorbed.

Osteoclast formation in vitro

Murine bone marrow cells stimulated with 1,25-(OH) $_2$ D $_3$ were used to assess the effects of the SPs on osteoclast formation. Maximal numbers of osteoclasts, identified as TRAP+ve MNCs were formed by 1,25-(OH) $_2$ D $_3$ at a concentration of 10^{-8} M. The addition of either aprotinin

Table 3 The effects of SP inhibitors on TRAP+ve MNC formation. Results are expressed as means $\pm$ S.E.M. of 6 wells from 2 experiments

Treatment	Number of TRAP-positive MNCs per well
1,25-(OH) $_2$ D $_3$	65 $\pm$ 12
1,25-(OH) $_2$ D $_3$ + CT1399	69 $\pm$ 8
1,25-(OH) $_2$ D $_3$ + aprotinin	73 $\pm$ 13
1,25-(OH) $_2$ D $_3$ + α_2 -antiplasmin	62 $\pm$ 10

Mouse bone marrow cells were cultured as described in Materials and Methods in the presence of 1,25-(OH) $_2$ D $_3$ (10^{-8} M). Aprotinin or α_2 -antiplasmin were added at a concentration of 10^{-5} M at the beginning of the culture. After 8 days the number of TRAP+ve MNCs were counted.

(10^{-5} M), or α_2 -antiplasmin to the cultures did not effect the number of TRAP+ve MNCs formed (Table 3).

Osteoclast migration and fusion

The effects of the proteinase inhibitors on the number of TRAP+ve cells in both the periosteum and mineralized matrix were determined at the beginning of the experiment and after 3 and 6 days of culture (Fig. 3). As the TRAP cells were often multinucleate, especially in the excavating marrow cavity, both TRAP cell profiles and nuclei were counted. The SP inhibitor, aprotinin, produced a significant reduction of approximately 55% in the invasion of the mineralized matrix by TRAP+ve cells after 3 days with a concomitant accumulation of TRAP+ve cells in the periosteum, thus showing that the SP inhibitors do not stop the formation of new TRAP+ve cells. A similar situation was found for the number of nuclei per TRAP+ve cell. However, after 6 days culture with aprotinin the numbers of TRAP+ve cells and nuclei per TRAP+ve cell were similar to the control cultures (Fig. 3). In contrast, the MMP inhibitor, CT1399, completely prevented the invasion of the mineralized matrix by TRAP+ve cells after 3 days, with a concomitant increase in the number of TRAP+ve cells in the periosteum. After 6 days, CT1399 still produced a significant reduction in the migration of TRAP+ve cells although its effects were incomplete (Fig. 3). None of the inhibitors appear to affect the intrinsic ability of cells to fuse.

Degradation of bone-like matrix

To determine whether the PA system is involved in the degradation of non-mineralized bone matrix, primary osteoblasts were cultured on ^3H -labelled bone-like matrix produced by MC3T3-E1 cells. Mouse osteoblasts cultured for 72 h in the absence of serum produced neither basal nor 1,25-(OH) $_2$ D $_3$ -stimulated matrix breakdown, but the stimulated osteoblasts produced a threefold increase in

Figure 3 Effects of proteinase inhibitors on the migration of TRAP+ve cells to the calcified cartilage in metatarsal explants. Metatarsals were obtained from 3 litters of 17-day-old fetal mice. The metatarsal triads of the left limb were cultured in control conditions with 1,25-(OH) $_2$ D $_3$ and those of the corresponding right limbs were cultured in the presence of 1,25-(OH) $_2$ D $_3$ and either CT1399 (10^{-6} M) or aprotinin (10^{-6} M) for the indicated times. The number of TRAP+ve cells and their nuclei localized inside and outside the calcified cartilage were counted. Counts inside the calcified cartilage are shown to the right of the '0' axis and those within the periosteum are shown to the left. Thus each bar (left and right) expresses the total numbers in one metatarsal. Counts at day 0, 3 and 6 are the means $\pm$ S.E.M. of, respectively, 18, 15, and 21 metatarsals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with controls.

matrix degradation when plasminogen was added to the cultures (Fig. 4). Plasminogen-dependent matrix breakdown was significantly inhibited by the SP inhibitors, aprotinin (10^{-5} M) and α_2 -antiplasmin (10^{-5} M). Furthermore, plasminogen-dependent breakdown was inhibited by the MMP inhibitor, CT1399 (10^{-6} M).

Degradation of type I collagen films

To confirm a role for the PA system in the degradation of type I collagen, primary osteoblasts were cultured on 14 C-labelled type I collagen films for 48 h. Aprotinin dose-dependently inhibited 1,25-(OH) $_2$ D $_3$ -stimulated collagen breakdown by mouse osteoblasts (Fig. 5). CT1399 produced a complete inhibition whilst the SP inhibitors only produced about a 55% reduction in type I collagenolysis.

Figure 4 Effects of proteinase inhibitors on the degradation of non-mineralized bone matrix. Primary mouse osteoblasts were cultured for 72 h on 3 H-labelled extracellular matrices and stimulated with 1,25-(OH) $_2$ D $_3$ (10^{-8} M) in the presence of 5% serum, in serum-free medium or in serum-free medium supplemented with 5 μ g/ml plasminogen. Aprotinin (10^{-5} M), α_2 -antiplasmin (10^{-5} M) or CT1399 (10^{-6} M) were added to cultures containing 5 μ g/ml plasminogen. The results are expressed as percentage degradation of 3 H-labelled bone matrix. Each bar represents the mean $\pm$ S.E.M. of 6 wells. Aprotinin, α_2 -antiplasmin and CT1399 inhibited degradation of 3 H-labelled bone matrix. ** $P < 0.01$ compared with the control.

Expression of PAs and their inhibitors in osteoblasts

RT-PCR analysis was used to establish expression of mRNA for the plasminogen activators and their inhibitors in unstimulated primary mouse osteoblasts using specific oligonucleotide primers. As shown in Fig. 6, RT-PCR analysis showed that osteoblasts express mRNA transcripts for tPA, uPA and the type-I receptor for uPA (uPAR-I), PAI-1, PAI-2 and the broad spectrum inhibitor, PN-1. The authenticity of the sequences was verified by automated sequencing. The primers for PAI-2 generated PCR fragments between 194 and 420 bp. The band at 194 was verified as PAI-2.

Expression of PAs in osteoclasts

Expression of mRNA for tPA and uPA was investigated by *in situ* hybridization studies on osteoclasts isolated from the long bones of 6-day-old mice. Figure 7A shows a typical multinucleate showing expression of tPA mRNA. For comparison, the sense probe for tPA was used as a negative control and showed negligible hybridization to

Figure 5 Effects of proteinase inhibitors on the degradation of ^{14}C -labelled type I collagen films by mouse osteoblasts. Primary mouse osteoblasts were stimulated by $1,25\text{-(OH)}_2\text{D}_3$ (10^{-8} M) after 48 h. The results are expressed as percentage inhibition of $1,25\text{-(OH)}_2\text{D}_3$ -stimulated ^{14}C release, which was arbitrarily set to 100%. Each point is the mean $\pm$ S.E.M. of six wells. The inhibitory effects of CT1399 (10^{-8} – 10^{-5} M) and aprotinin (10^{-8} – 10^{-5} M) were statistically significant, $**P < 0.01$ compared with control. The percentage release of isotope by $1,25\text{-(OH)}_2\text{D}_3$ -stimulated mouse osteoblasts was 64.7 ± 5.9 which was obtained after subtracting the unstimulated release of isotope (23.7 ± 3.7).

tPA mRNA (Fig. 7B). Osteoclasts cultured on type I collagen also exhibited expression of uPA as shown in Fig. 7C. Osteoclasts hybridized with the sense probe as a comparison exhibited only a weak signal compared with the antisense probe (Fig. 7D). Since MMP-9 is expressed at high levels in osteoclasts this was used as an additional positive control. As shown in Fig. 7E, osteoclasts exhibited

a strong signal for the MMP-9 antisense riboprobe. The sense probe did not hybridize with MMP-9 mRNA (Fig. 7F).

Discussion

The data presented demonstrate that the PA/plasmin system plays a significant role in the bone resorption cascade. Using selective proteinase inhibitors we have shown that the PA/plasmin system is involved in both the migration of osteoclasts to future resorption sites and in osteoblast-mediated degradation of type I collagen. The PA/plasmin system, however, is not involved in either osteoclast formation or osteoclast resorptive activity. Furthermore, we have also demonstrated the expression of various components of the PA/plasmin system in primary mouse osteoblasts and osteoclasts.

Aprotinin is a small extremely stable peptide that reacts rapidly with plasmin to form high-affinity complexes (with a K_d of 10^{-9} – 10^{-10} M). In contrast to α_2 -antiplasmin and α_1 -antitrypsin, the main inhibitor present in plasma, aprotinin also inhibits plasmin when it is bound to the plasminogen/plasmin surface receptors (Stephens *et al.* 1989, Bizik *et al.* 1990) found on many cell types (Mignatti *et al.* 1986). Our demonstration that two separate inhibitors of the PA/plasmin system partially inhibited bone resorption in neonatal calvarial explants that were cultured in serum devoid of neutral proteinase inhibitor activity is similar to the findings of Leloup *et al.* (1994) who found that aprotinin and α_2 -antiplasmin only inhibited bone resorption in fetal bone explants cultured under similar serum conditions. This suggests that proteinase inhibitors present in serum neutralize SP activity produced by osteoclasts and osteoblasts. Neonatal calvarial bone resorption reflects mainly post-mitotic events, namely the fusion of preosteoclasts, the migration of osteoclast to resorption sites, the activation of fully developed mature osteoclasts and osteoblast degradation of collagen fibrils whilst it is virtually independent of proliferation of

Figure 6 RT-PCR of PAs and their inhibitors in osteoblasts. Primary mouse osteoblasts were cultured as described in Materials and Methods in 75-cm^2 tissue culture flasks in the presence of 10^{-8} M PTH. Total RNA was isolated and RT-PCR performed with primers specific for: tPA, uPA, uPAR-1, PAI-1, PAI-2 and PN-1. The housekeeping gene, GAPDH, was used as a positive control. PCR was performed for 35 cycles in a $100\text{ }\mu\text{l}$ reaction and $10\text{ }\mu\text{l}$ were analysed by gel electrophoresis on a 2% agarose gel. A, DNA size marker.

Figure 7 *In situ* hybridization of tPA and uPA in isolated osteoclasts. Osteoclasts were prepared as described in Materials and Methods and coated on rat tail type I collagen-coated coverslips. Coverslips were hybridized with (A) tPA antisense riboprobe, (B) tPA sense riboprobe, (C) uPA antisense riboprobe, (D) uPA sense riboprobe, (E) osteoclasts hybridized with MMP-9 antisense and (F) MMP-9 sense riboprobe. Bar=50 μ m. Osteoclasts exhibited expression of uPA and tPA when hybridized with the respective antisense riboprobes and were positive for MMP-9 mRNA expression.

osteoclast progenitors. This probably explains why the SP inhibitors, aprotinin and α_2 -antiplasmin, only produced a partial inhibition in calvarial bone resorption, since SP activity would appear to be limited to the osteoblast-mediated degradation of the osteoid layer in this culture system and osteoclast migration.

It is widely believed that prior to osteoclastic resorption, the bone surface is freed of a thin investing layer of non-mineralized collagen fibrils. Although osteoblast-derived MMPs, in particular collagenase, have been implicated in this process, it has been suggested that the PA/plasmin system may also be involved in this process

(Thomson *et al.* 1989). Our findings that osteoblast-mediated degradation of both type I collagen and bone-like matrix is dependent on the presence of plasminogen and that the SP inhibitor, aprotinin, partially prevented osteoblast-mediated degradation of these substrates supports the concept that the PA/plasmin system is involved in this aspect of the bone resorption process. Consistent with a role for the PA/plasmin system in osteoid degradation it has been shown that degradation of nonmineralized matrix by cocultures of osteoblasts and osteoclasts is decreased by combined inactivation of uPA and tPA genes (Daci *et al.* 1999). Furthermore, our demonstration that osteoblasts express tPA and uPA is in accordance with previous studies that have shown that a variety of bone resorbing agents upregulate PA activity in osteoblasts (Hamilton *et al.* 1984, 1985, Allan & Martin 1995). Although we have not demonstrated the precise mechanism of action of the PA/plasmin system in collagen degradation it is known that plasmin will activate MMPs, particularly prostromelysin-1 and -2. In concert, plasmin and stromelysins can activate other osteoblast-derived MMPs (Meikle *et al.* 1992), in particular collagenase and gelatinase B (Murphy & Knauper 1997), which may be directly responsible for the type I collagen degradation (Hill *et al.* 1995). RT-PCR analysis also revealed expression of the serine proteinase inhibitors, PAI-1 and PN-1, in primary mouse osteoblasts. Previous studies have demonstrated that PAI-1 mRNA is decreased in rat osteoblasts by PTH and increased by treatment with transforming growth factor- β (TGF- β) (Allan *et al.* 1991, Fukumoto *et al.* 1992) thus highlighting the importance of PAI-1 in regulating the PA/plasmin system in rat osteoblasts. Allan and Martin (1995) demonstrated that expression of PAI-2 and PN-1 in rat calvarial osteoblasts was not modulated by prostaglandin E₂ whereas PAI-1 was modulated in a biphasic manner.

Our finding that aprotinin prevented the migration of osteoclasts from the periosteum to the mineralized matrix in 17-day-old fetal metatarsals supports the findings of Hoekman *et al.* (1992), who demonstrated that tPA stimulated osteoclastic resorption in these explants, an event that is indicative of osteoclast migration to the calcified matrix. Leloup *et al.* (1994) also demonstrated that uPA is the main PA present in extracts of cultured fetal mouse metatarsals. Although Leloup *et al.* (1994) reported that inhibitors of plasmin did not influence metatarsal bone resorption, they found that when the explants were cultured in serum depleted of plasmin inhibitors there was enhanced bone resorption suggesting that the PA/plasmin system is involved. Furthermore, LeLoup *et al.* (1996) subsequently demonstrated that in mice with an inactivated uPA gene, bone resorption was reduced at the commencement of culture in the metatarsal explants whereas inactivation of the tPA gene had no effect on bone resorption in fetal metatarsal explants.

The inhibitory activity of aprotinin against the invasion of preosteoclasts and the degradation of type I collagen by osteoblasts is comparable with the inhibitory activity of aprotinin in several other culture models in which it contributed to establish the role of the PA/plasmin system in either the invasion or the degradation of extracellular matrices (Mignatti *et al.* 1986, Cajot *et al.* 1989, Quax *et al.* 1991).

The expression of tPA and uPA has been identified in osteoclasts both at the message level by RT-PCR (Yang *et al.* 1997), and at the protein level by immunocytochemistry (Grills *et al.* 1990). However, our demonstration that aprotinin had no inhibitory effect on osteoclast lacunar resorptive activity suggests that, at least under the *in vitro* conditions used in this study, the PA/plasmin system is not directly involved in this aspect of the resorption cascade. These findings are consistent with the results reported by Daci *et al.* (1999) in which osteoclasts derived from mice with a combined inactivation of both uPA and tPA were still able to resorb dentine. Nonetheless, there are several potential roles for the osteoclast-derived PAs in bone. These include the activation of latent proteases (Kwaan 1992), activation of latent growth factors (Martin *et al.* 1993, Lalou *et al.* 1994), and a nonproteolytic role as a mitogenic agent (Kirchheimer *et al.* 1987, Rabbani *et al.* 1990). The activation of various proenzymes by the PA system in osteoclasts, including enzymes that degrade extracellular matrix proteins such as prostromelysin and procollagenase, could aid in the resorption of bone (Delaisse & Vaes 1992). Thus, since there are various proteolytic enzymes that appear to have overlapping functions in the activation of proenzymes or paracrine factors involved in bone resorption, the PA system may be one of several redundant mechanisms involved in this process. Another function of the PA system in osteoclasts may be to activate paracrine factors involved in the regulation of bone remodelling (Martin *et al.* 1993). Plasmin has been shown to dissociate insulin-like growth factor-I from its binding protein in human osteosarcoma cells and to activate the interleukin-1 β precursor and latent TGF- β (Kwaan 1992, Martin *et al.* 1993). In this study, the SP inhibitors had no effect on the formation of TRAP+ve MNCs. This is supported by Daci *et al.* (1999) who reported that cocultures of primary osteoblasts and bone marrow cells derived from mice with a combined inactivation of uPA and tPA genes were both capable of forming TRAP+ve MNCs.

In summary, we demonstrate that serine proteinases are involved in osteoblast-mediated degradation of type I collagen and osteoclast migration to future resorption sites. Osteoclasts and osteoblasts express both tPA and uPA mRNA. However, serine proteinases do not appear to play a role in osteoclast formation or osteoclast resorptive activity. It would seem that these serine proteinases may play an important role in the invasive and migratory

activities of osteoclasts and in facilitating osteoclast access to mineralized bone matrix.

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