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Human osteoclastomas contain multiple forms of cathepsin B

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During bone resorption, the osteoclast secretes hydrolytic enzymes into the sealing zone which it creates between itself and the bone surface. Since this environment is acidic, proteinases active at low pH must therefore be responsible for degrading the bone matrix, which is largely composed of type I collagen. To investigate these enzymes, we have used human osteoclastomas as the starting material. Sequential chromatography on S-Sepharose, phenyl-Sepharose, heparin-Sepharose and Sephacryl S-200HR resulted in the separation of six cysteine proteinase activities. These proteinases have M_r values ranging from 20000 to 42000. The pH profiles of activity showed optima between 3.5–6.0 for both synthetic substrates and type I collagen. All the proteinases were able to degrade soluble and insoluble type I collagen. The kinetics of hydrolysis using Z-Phe-Arg-NHMec and Bz-Phe-Val-Arg-NHMec as substrates resulted in values within the range expected for cathepsin B. The six activities were all inhibited by the cysteine proteinase inhibitors antipain, chymostatin, leupeptin and E-64. The rate constants of inactivation using Z-Phe-Tyr-(*O-t*-Bu)CHN_2 were also similar to the published rates for cathepsin B. Antibodies to cathepsin B reacted with all activities. These antibodies localised the enzyme activities to the osteoclast within the tumour. Northern blotting using a cDNA probe to cathepsin B revealed three species of mRNA transcripts. These results suggest that multiple forms of cathepsin B-like proteinases are involved in osteoclastic bone resorption.

Introduction

The osteoclast is the effector of bone resorption. Its activity is normally integrated to the requirements of skeletal morphogenesis and remodelling, and to those of calcium homeostasis, but its potential for destruction is demonstrated by the random resorption that occurs in Paget's disease [1]. Osteoclasts destroy bone in metastases and act in collaboration with pannus in the osteoarticular destruction of rheumatoid arthritis [2]. Less conspicuously, but with devastating results, the osteoclast contributes to the development of osteoporosis with levels of osteolysis above those observed in unaffected individuals [3].

Bone resorption is accomplished through the formation by osteoclasts of a circumpherential sealing zone of close adhesion between the osteoclast and bone (clear zone), within which the plasma membrane is thrown into complex folds (ruffled border) [4]. Osteoclasts resorb bone through the formation of an extracellular acidic compartment [5,6]. This acidification results in the solubilisation of the bone mineral. Acid hydrolases are then secreted to degrade the organic matrix, composed predominantly of type I collagen. Inhibitors of cysteine proteinases are capable of inhibiting bone resorption in vitro [7,8]. The cysteine proteinases have been reviewed recently [9,10]. At least three distinct cysteine proteinases capable of degrading collagen have been identified in extracts from bone tissues: cathepsins B, L and a high M_r (70000) cysteine proteinase [8,11].

We have set out to study further the cysteine proteinases responsible for bone resorption. Since the osteoclast is only a minor representative of the cells in bone, osteoclastomas were used as the starting material. These are rare primary tumours of bone which contain large numbers of apparently normal osteoclasts, induced into the tumour by neoplastic osteo-

Abbreviations: –NHMec, 7-(4-methyl)coumarylamide; TLCK, tosyl lysine chloromethyl ketone; TPCK, tosyl phenylalaninine chloromethyl ketone; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; HEDS, 2-hydroxyethyldisulphide; PVDF, poly(vinylidene) difluoride.

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blastic cells [12]. Extracts from these tumours were subjected to conventional liquid chromatography and the cysteine proteinase activities were separated from one another and characterised. The further characterisation of the cysteine proteinases in bone could lead to the development of improved strategies for the detection and inhibition of excessive osteoclastic resorption of bone.

Experimental procedures

Materials

S-Sepharose, phenyl-Sepharose, heparin-Sepharose and Sephacryl S-200HR were obtained from Pharmacia, Milton Keynes, U.K. Aminomethylcoumarin-peptides were obtained from Cambridge BioSciences, Cambridge, UK or Sigma Chemicals, Poole, U.K., and the diazomethanes *Z*-Phe-Tyr-(*O*-*t*-Bu)CHN_2 and *Z*-Tyr-AlaCHN_2 from Dr. Elliot Shaw, Friedrich Miescher-Institut, Basel, Switzerland. Radiochemicals and nitrocellulose membranes were from Amersham U.K. Immobilon-P membranes were purchased from Millipore, Watford, UK. All chemicals were of analytical grade and were obtained from either BDH, Poole, U.K. or Sigma Chemicals. Antibodies to cathepsin B were from Serotec, Oxford, U.K.

Osteoclastomas were collected immediately after surgery and were stored at −70°C.

Determination of cysteine proteinase activity

Enzyme activity was measured fluorimetrically. The mixture containing enzyme, 100 μM *Z*-Phe-Arg-NHMec (or other peptide substrate), 30 mM cysteine and 50 mM sodium citrate (pH 3.6), in a final volume of 0.2 ml, was incubated at 37°C for 1 h. The reaction was terminated by the addition of 0.8 ml of 30 mM sodium acetate/100 mM sodium chloroacetate (pH 4.3). The fluorescence of the aminomethylcoumarin liberated was measured using a Locarte model 8-9 fluorimeter. One unit of enzyme activity corresponds to the hydrolysis of 1 μmol of substrate/min. The effects of inhibitors were determined using this standard assay. Rate constants of inactivation were determined as described by Barrett et al. [13]. The concentrations of *Z*-Phe-Tyr-(*O*-*t*-Bu)CHN_2 ranged from 0.5–6 μM.

In kinetic experiments the concentration of substrate ranged from 20 to 200 μM. The reaction was linear with time and over the range of enzyme concentrations used throughout these experiments. Enzyme activity involving type I collagen as the substrate was determined by measuring the release of ^{3}H-peptides from tritiated rat type I collagen. Type I collagen was tritiated as described by Mookhtiar [14]. The reaction mixture, containing enzyme, collagen (50000 cpm, 10 μg), 30 mM cysteine, 50 mM sodium citrate (pH 3.6), in a final volume of 0.15 ml, was incubated for 3–18 h at 37°C. The reaction was terminated by the addition of 5% BSA (100 μl) and 5% TCA/0.25% tannic acid (250 μl) After standing for 20 min at room temperature followed by centrifugation for 5 min at 12000 rpm, aliquots (350 μl) of the supernatants were measured in a LKB 1211 Rackbeta scintillation counter. Insoluble collagen degradation was determined using the method of Johnson-Wint [15]. Aliquots of tritiated type I collagen (50000 cpm) were plated in 0.5 cm diameter Linbro wells and dried at 37°C. A mixture of enzyme, 40 mM cysteine, 50 mM sodium citrate (pH 3.6) in a volume of 0.15 ml was added to each well. After incubation at 37°C for 1–5 h, radioactivity released from the collagen film was measured by liquid scintillation counting. Undegraded collagen was digested with 50 μg bacterial collagenase overnight, and the radioactivity measured.

Protein determination

Protein concentrations were determined spectrophotometrically at 280 nm or fluorimetrically [16]. The aqueous protein (0.5 ml), mixed with 0.75 ml of 0.1 M sodium borate buffer (pH 9.2) was vortexed with 0.25 ml of fluorescamine (0.2 mg/ml in acetone), and the products measured fluorimetrically. Bovine serum albumin was used as the standard.

Purification of cysteine proteinases from osteoclastomas

All procedures were carried out at 4°C unless stated otherwise. All buffers contained 1 mM HEDS and 0.02% sodium azide. Cysteine proteinase activity was determined using the fluorimetric method with *Z*-Phe-Arg-NHMec as the substrate. Samples were concentrated using an Amicon membrane (PM-10) unless stated otherwise.

Step 1: Homogenisation. Frozen tissue (50 g) was thawed and homogenised in a Waring blender with 8 vol (w/v) of 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.1 mM phenylmethylsulphonyl fluoride. Two volumes of 5% (v/v) Triton X-100 in the same buffer were added to the homogenate, which was then stirred overnight. The homogenate was centrifuged for 1 h at 12000 × *g*, and the supernatant was dialysed exhaustively against 20 mM sodium acetate (pH 5.5), followed by further centrifugation for 1 h at 12000 × *g*.

Step 2: S-Sepharose chromatography. The supernatant from step 1 was applied to a 1.6 × 40 cm S-Sepharose column equilibrated in the same buffer. After thorough washing of the column with 200 ml of the equilibration buffer, the cysteine proteinase activity was eluted with a 300 ml linear gradient of 0.0–0.7 M NaCl. Fractions (5 ml) were collected and assayed for cysteine proteinase activity. Active fractions from each of the three major peaks were pooled, concentrated

and dialysed against 50 mM Tris-HCl (pH 8.0) containing 30% saturated ammonium sulphate.

Step 3: Phenyl-Sepharose chromatography. Each of the three pooled enzyme activities from the previous step was centrifuged at 12000 × g for 30 min and the supernatants applied to a 1 × 20 cm column of phenyl-Sepharose equilibrated in the buffer used for dialysis in step 2. The enzymes were eluted using a 200 ml linear gradient of 30–0% ammonium sulphate in 50 mM Tris-HCl (pH 8.0). Active fractions from each peak were pooled and dialysed against 50 mM Tris-HCl (pH 7.5).

Step 4: Heparin-Sepharose chromatography. Enzyme activities from step 3 were applied to a 1.6 cm × 20 cm column of heparin-Sepharose equilibrated in 50 mM Tris-HCl (pH 7.5), followed by elution using a 300 ml linear gradient of 0.0–1.0 M NaCl in the same buffer.

Step 5: Sephacryl S-200HR chromatography. Each of the enzyme activities was concentrated to 1 ml and applied to a 1.6 × 100 cm column of Sephacryl S-200HR equilibrated in 50 mM Tris-HCl (pH 7.0). Active fractions were pooled and concentrated to volumes of approx. 2 ml.

The purified enzyme activities were stored at 4°C.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed according to the method of Laemmli.

M_r determination by gel filtration

Proteinase activities were chromatographed on a Sephacryl S-200 HR column, as described in purification step 5. The void volume was measured using Dextran Blue and the total volume determined using [^{3}H]leucine. Phosphorylase *b* (97400), bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), trypsin inhibitor (20100) and cytochrome *c* (12400) were used as M_r markers. Elution volumes were determined by measuring the absorbance at 280 nm.

Immunoblotting

Proteins, separated by SDS/polyacrylamide-gel electrophoresis, were transferred to Immobilon PVDF membranes [17] using a Millipore semi-dry blotting apparatus. The membrane was incubated overnight in 3% marvel/PBS/0.2% Tween 20 and then sequentially with sheep anti-cathepsin B (diluted 1:200), biotin-conjugated anti-sheep IgG (1:1000) and avidin-alkaline phosphatase (1:5000). Each incubation was for 2 h and the membrane was washed three times with PBS/0.2% Tween 20 between each change of reagent. The membrane was incubated with 0.1 M ethanolamine-HCl (pH 9.6) for 10 min and then with the substrate solution (100 μg/ml 5-bromo-4-chloro-3-indolyl phosphate, 100 μg/ml nitroblue tetrazolium, 1 mM $MgCl_2$, 0.1 M ethanolamine-HCl pH 9.6). The reaction was terminated by rinsing the membrane with water.

Immunofluorescence techniques

Immunofluorescence staining of frozen sections of osteoclastomas with antibodies to cathepsin B was carried out as described previously [18].

Northern blotting

Total RNA was isolated from osteoclastomas by the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi [19]. Samples, 20 μg, were electrophoresed through 1% agarose gels containing formaldehyde [20]. Gels were rinsed with water and vacuum blotted onto nitrocellulose membranes in 10 × SSC (SSC = 0.3 M NaCl, 0.03 M sodium citrate pH 7.0). After blotting, the membranes were heated to 80°C for 2 h in a vacuum oven. The blots were then wetted in 6 × SSC and prehybridised for 4 h in 5 × SSC, 50 mM sodium phosphate (pH 6.5), 3 mg/ml Ficoll, 3 mg/ml polyvinylpyrolidone, 3 mg/ml bovine serum albumin and 250 μg/ ml calf thymus DNA at 68°C. cDNA probes labelled to a specific activity of $1 \cdot 10^9$ dpm/μg using the method of Feinberg and Vogelstein [21] were added to the prehybridised blots and incubated overnight at 68°C ($1 \cdot 10^5$ dpm per ml of solution). Blots were washed in 2 × SSC/0.1% SDS for 10 min at room temperature followed by incubation at 55°C in 1 × SSC/0.1% SDS for 2 h. After washing, the membranes were sandwiched between Saran wrap and subjected to autoradiography. cDNA probes for rat cathepsin B [22], and human cathepsins L [23] and H [24] were used.

Results

Separation of proteinase activities

The cysteine proteinase activity of human osteoclastomas was separated into three fractions by chromatography on S-Sepharose (Fig. 1). The second and third activities to be eluted were each separated into two activities by phenyl-Sepharose chromatography (Fig. 2). The five activities were further purified by chromatography on heparin-Sepharose, where the first peak from the S-Sepharose column separated into two activities (Fig. 3). Each activity chromatographed as a single peak on Sephacryl S-200 HR. The designation of these activities (enzymes 1–6) is explained in the figure legends. A schematic diagram showing the origins of the enzymes is given in Fig. 4. One or two osteoclastomas were used in each purification. The six activities were observed in eight out of ten preparations. The final specific activities of the enzyme were comparable in each preparation.

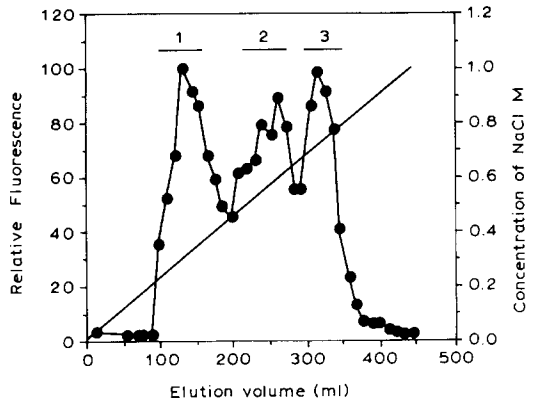

Fig. 1. Chromatography of an osteoclastoma extract on S-Sepharose. Activities were measured using Z-Phe-Arg-NHMec as substrate. Fractions indicated by bars were pooled. Peaks 1, 2 and 3 eventually gave rise to enzymes 1 and 2, 3 and 4, and 5 and 6, respectively.

The specific activities of the purified enzymes ranged from 25.5 to 160 nmol/min per mg. The purification summaries are shown in Table I. The value for the enzyme activity of the crude extract is probably low due to the presence of endogenous inhibitors of cysteine proteinases. As the enzymes cannot be assayed separately in the homogenate, the 'recovery' and 'purification' of each activity cannot be calculated. Gel filtration on Sephacryl S-200 HR indicated that the molecular weights of the proteinases ranged from 20000–42000 (Table II).

SDS-polyacrylamide gel electrophoresis demonstrated that the enzymes were not homogeneous with respect to protein content. However, the osteoclastoma cysteine proteinases are probably enzymatically homo-

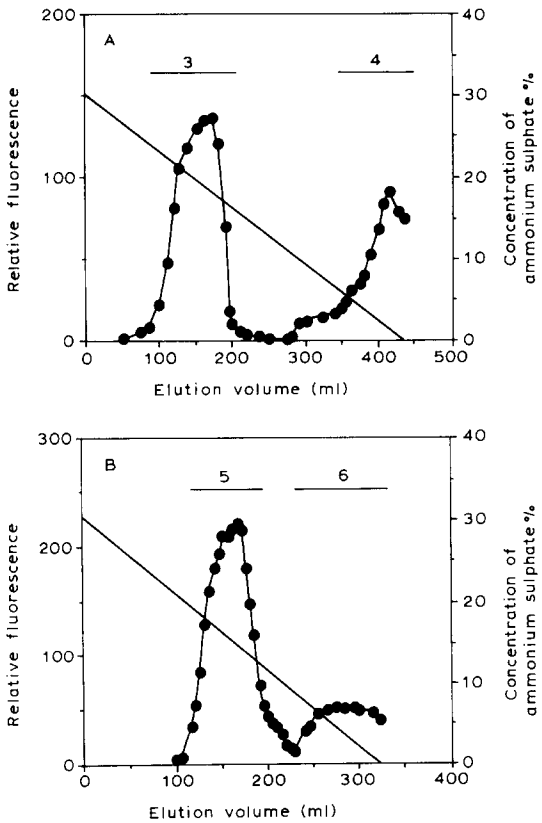

Fig. 2. Chromatography of S-Sepharose fractions on phenyl-Sepharose. (A) Peak 2 and (B) Peak 3 after S-Sepharose chromatography are shown. Peak 1 (not shown) chromatographed as a single peak on phenyl-Sepharose. Activities were measured using Z-Phe-Arg-NHMec as substrate. Fractions indicated by bars were pooled. The two peaks of activity eluting in (A) are designated enzymes 3 and 4, and the two peaks of activity eluting in (B) are designated enzymes 5 and 6.

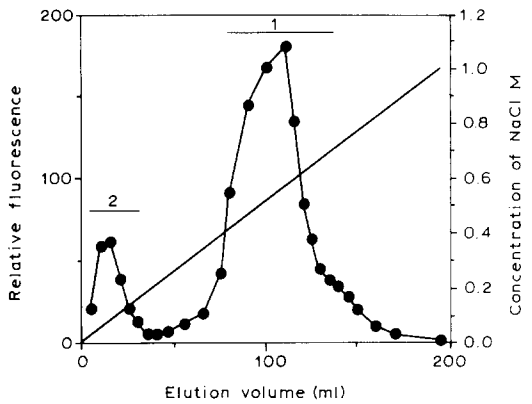

Fig. 3. Chromatography of S-Sepharose peak 1 on heparin-Sepharose. The first peak of activity eluting from the S-Sepharose column was further purified on phenyl-Sepharose and then chromatographed on heparin-Sepharose. Activities were measured using Z-Phe-Arg-NHMec as substrates. Fractions indicated by bars were pooled. The first peak to elute from the column is designated enzyme 2, whereas the later, larger peak is designated enzyme 1. Enzymes 3–6, obtained after phenyl-Sepharose chromatography eluted as single peaks on heparin-Sepharose (not shown).

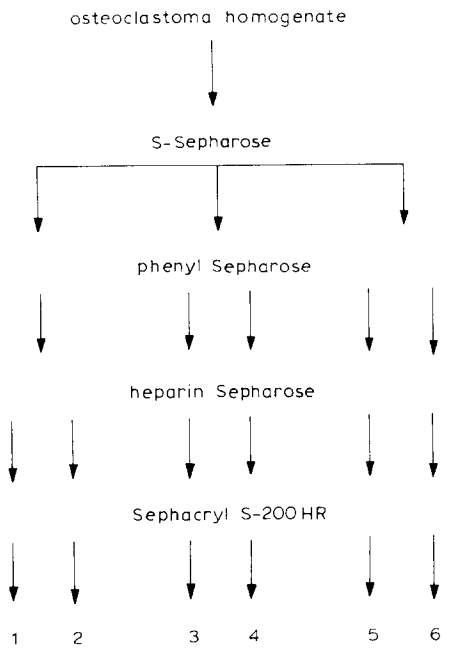

Fig. 4. A schematic representation of the purification procedures used to separate osteoclastoma cysteine proteinase activities.

TABLE I

Summary of the purification of osteoclastoma cysteine proteinases

Activity was measured using *Z*-Phe-Arg-NHMec as substrate.

Enzyme	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)
Crude extract *	11876	207	0.017
1	2.3	182	80
2	0.8	21	25.5
3	2.2	118	54.6
4	0.9	149	160
5	0.8	77	97
6	0.8	53	65

* The value of the enzyme activity of the crude extract is probably low due to the presence of endogenous inhibitors of cysteine proteinases.

TABLE II

Determination of the molecular weights of osteoclastoma cysteine proteinases by gel filtration on Sephacryl S-200 HR

Enzyme	M_r
1	27000
2	20000
3	25000
4	33000
5	42000
6	20000

geneous as attempts at further fractionation, using ion-exchange chromatography and chromatofocusing, were unsuccessful (data not shown).

The initial homogenisation and overnight incubation were carried out at pH 8 because at pH 5 there was considerable loss of activity, probably due to autolysis. Although cathepsin B, and to a lesser extent cathepsin L, have been reported to be inactivated when incubated at pH 8 and 37°C [10], the osteoclastoma cathepsin activity was relatively stable at pH 8 and 4°C. Homogenisation of an osteoclastoma at pH 5 followed immediately by chromatography on S-Sepharose resulted in an identical activity profile to that observed when the homogenisation was carried out at pH 8.

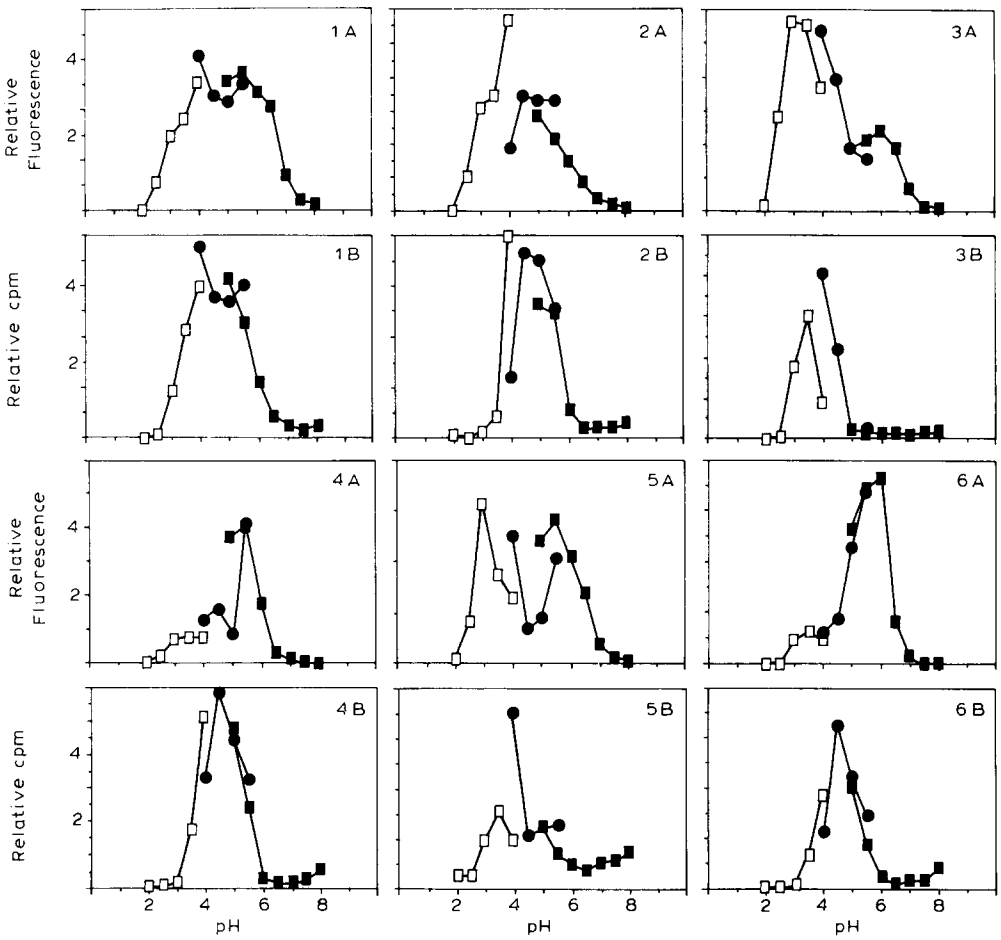

Fig. 5. Determination of the pH-profiles of activity of osteoclastoma cysteine proteinases. The pH-profiles of activity of enzymes 1–6 were determined using either (A) *Z*-Phe-Arg-NHMec or (B) [^{3}H]collagen as substrates. Buffers used were 100 mM sodium citrate (□), 100 mM sodium acetate (●) or 100 mM sodium phosphate (■).

TABLE III

Relative action of osteoclastoma cysteine proteinases on synthetic substrates

Enzyme assays were performed as described in the text. Substrate concentrations were 100 μM. Activities are expressed as % relative hydrolysis/mol of enzyme.

Substrate	Relative hydrolysis %					
enzyme:	1	2	3	4	5	6
Z-Phe-Arg-NHMec	30	4	100	19	26	13
Z-Arg-Arg-NHMec	2	0	0	2	0	0
Bz-Phe-Val-Arg-NHMec	19	4	6	1	0	0

TABLE V

Kinetics of hydrolysis of synthetic substrates by osteoclastoma cysteine proteinases

See text for details of assay procedures. Values for cathepsins B, L and S are taken from reference [27].

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
(a) Hydrolysis of Z-Phe-Arg-NHMec			
1	15	0.10	150
2	4	0.67	6
3	156	0.27	578
4	40	0.02	2000
5	35	0.27	130
6	20	0.02	1000
Cathepsin B	364	0.223	1632
Cathepsin L	17.6	0.0028	6286
Cathepsin S	4.3	0.0147	320
(b) Hydrolysis of Bz-Phe-Val-Arg-NHMec			
1	79	0.21	376
2	21	0.20	105
3	28	0.10	280
Cathepsin B	17.5	0.029	603
Cathepsin L	1.1	0.0018	611
Cathepsin S	13	0.0081	1605

TABLE VI

Effects of inhibitors on the hydrolysis of Z-Phe-Arg-NHMec by osteoclastoma cysteine proteinases

Inhibitor	(μM)	% Inhibition					
	enzyme:	1	2	3	4	5	6
Antipain	(1)	59	51	16	18	16	3
	(10)	78	92	61	21	62	ND [a]
Chymostatin	(1)	20	36	23	0	6	0
	(10)	72	89	76	2	80	10
Leupeptin	(1)	76	92	75	10	82	17
E-64	(0.1)	57	92	15	70	22	54
Cystatin	(0.1)	70	98	98	47	98	90
TLCK	(1)	24	37	2	0	2	0
	(10)	62	81	30	0	40	0
TPCK	(10)	5	13	3	31	8	3
	(100)	16	21	13	24	25	30
Z-Phe-Tyr-(O-t-Bu)CHN_2	(1)	36	17	42	87	48	86
	(10)	83	79	97	97	99	100
Z-Tyr-Ala-CHN_2	(1)	76	29	82	47	81	49
	(10)	98	83	98	91	99	84

[a] ND, not determined.

Substrate specificity

The pH profiles of activity showed optima ranging from 3.5 to 6.0 using the synthetic substrate Z-Phe-Arg-NHMec and type I collagen, although the two optima for each enzyme were not always the same (Fig. 5). The relative hydrolysis of synthetic substrates is shown in Table III. The preferred substrate for all enzymes was Z-Phe-Arg-NHMec, although some were more active than others. The rates of hydrolysis of type I collagen in both soluble and insoluble forms at 25°C and 37°C are shown in Table IV. The concentrations of active enzymes were calculated by active site titration using Z-Tyr-Ala-CHN_2 [13]. The active concentrations were 1.45, 6.20, 0.62, 2.00, 0.52 and 1.50 μM for activities 1–6 respectively.

The kinetics of hydrolysis of Z-Phe-Arg-NHMec and Bz-Phe-Val-Arg-NHMec, together with published data for cathepsins B, L and S, are given in Table V.

TABLE IV

Relative action of osteoclastoma cysteine proteinases on type I collagen

The degradation of type I collagen was assayed as described in the text.

Substrate	mg collagen degraded/mmol enzyme per min					
enzyme:	1	2	3	4	5	6
Type I collagen (37°C)						
soluble	383	123	71	111	12	93
insoluble	601	164	904	228	128	0
Type I collagen (25°C)						
soluble	61	18	45	53	12	41
insoluble	57	6	35	0	12	0

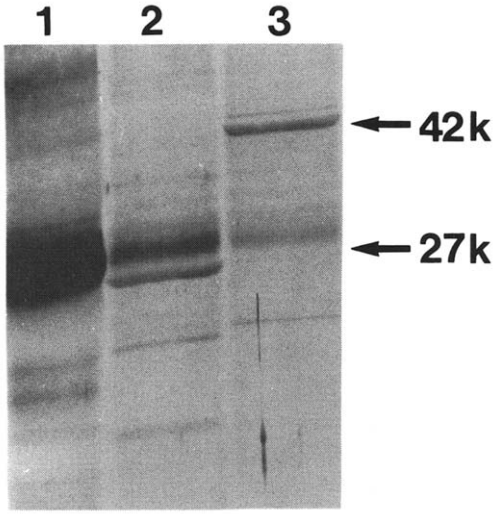

Fig. 6. Western blot of partially purified osteoclastoma cysteine proteinases using an antibody to cathepsin B. The three peaks of activity eluting from the S-Sepharose column were subjected to SDS/polyacrylamide-gel electrophoresis and transferred to a PVDF-membrane. The membrane was incubated with an antibody to cathepsin B and positive bands identified using biotin-avidin techniques (see text).

Values were calculated using Lineweaver-Burke plots. However, such comparisons should be treated with caution because, although assays were carried out at the pH optima, each enzyme has a different pH optimum.

TABLE VII

Rate constants of inactivation of osteoclastoma cysteine proteinases by Z-Phe-Tyr-(O-t-Bu)CHN$_2$

See text for details. Values for cathepsins B and L are taken from reference [32].

Enzyme	Rate of inactivation ($M^{-1} min^{-1}$)
1	$0.8 \cdot 10^{-3}$
2	$10.6 \cdot 10^{-3}$
3	$7.8 \cdot 10^{-3}$
4	$0.4 \cdot 10^{-3}$
5	$15.0 \cdot 10^{-3}$
6	$31.8 \cdot 10^{-3}$
Cathepsin B	$0.5 \cdot 10^{-3}$
Cathepsin L	$1.0 \cdot 10^{-7}$

Inhibition studies

The effects of inhibitors of cysteine proteinases antipain, chymostatin, the diazomethanes, *Z*-Phe-Tyr-(*O*-*t*-Bu)CHN$_2$ and *Z*-Tyr-Ala-CHN$_2$, leupeptin, TLCK, TPCK, cystatin and E-64 are reported in Table VI.

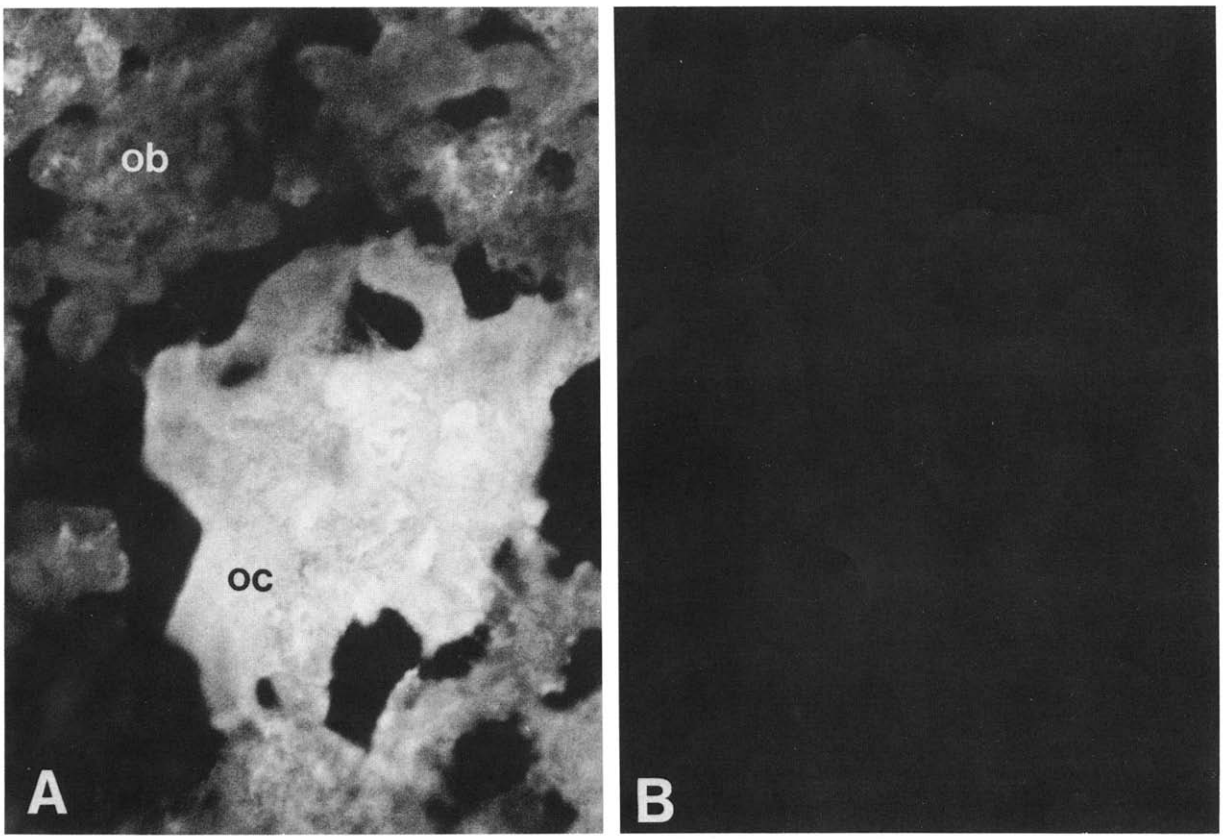

Fig. 7. Immunofluorescence localisation of cathepsin B in osteoclastomas. Sections of osteoclastomas were incubated with either (A) a sheep antibody to cathepsin B or (B) nonimmune sheep serum. Osteoclasts (oc) stain strongly with the antibody whereas the surrounding osteoblastic cells (ob) are only weakly stained. Control sections were devoid of staining. Magnification ×500.

Enzymes were assayed using the substrate *Z*-Phe-Arg-NHMec in the presence of the inhibitors. The rate constants of inactivation using *Z*-Phe-Tyr-(*O*-*t*-Bu) CHN_2 were determined for each of the six enzymes and compared to published values for cathepsins B and L (Table VII).

Immunoblotting experiments

Proteins present in the three peaks of activity obtained by chromatography on S-Sepharose were separated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was probed with an antibody to cathepsin B (Fig. 6). Fraction 1, containing enzymes 1 and 2, showed a broad band with a M_r of around 27000. Fraction 2, containing enzymes 3 and 4, gave two positive bands with M_r values of 25000 and 27000, and a faint band at about 34000. Fraction 3, containing enzymes 5 and 6, showed two bands with M_rs of 27000 and 42000. Controls, in which the PVDF-membrane was incubated with non-immune sheep serum, were devoid of staining (not shown).

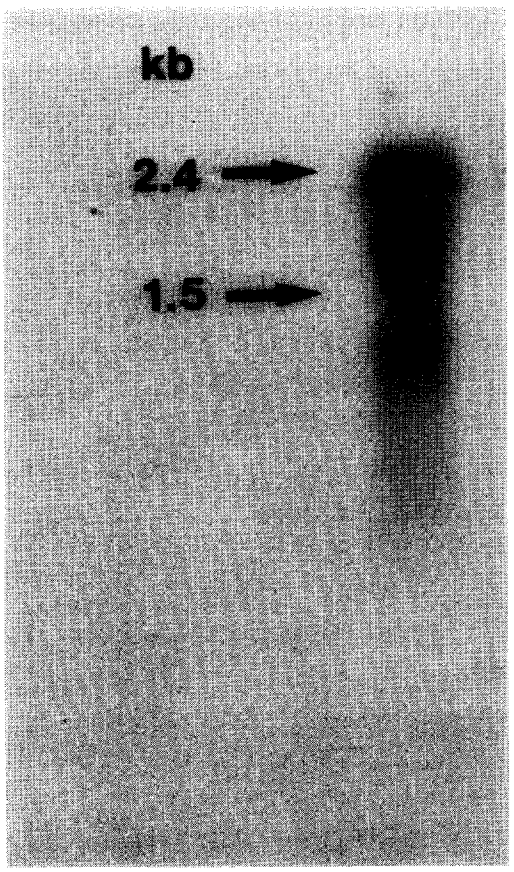

Fig. 8. Determination of the size of cathepsin B mRNA in osteoclastomas. Northern blot of 20 μg of total RNA isolated from osteoclastomas hybridized with a cathepsin B cDNA. Bands were visualised by autoradiography.

Immunofluorescence staining

A section of osteoclastoma stained with an antibody to cathepsin B is shown in Fig. 7. The large, multinucleate osteoclasts stained strongly with the antibody, whereas the smaller, neoplastic osteoblastic cells were only weakly stained.

Northern blotting

Northern blots of osteoclastoma RNA with a cDNA probe to cathepsin B revealed three bands after autoradiography (Fig. 8). The approximate sizes of the cathepsin B-specific transcripts were 2.4, 1.9 and 1.2 kb. No bands were visible when cDNA probes for cathepsins L and H were used (not shown).

Discussion

Six thiol-dependent proteinase activities active at acid pH have been purified from osteoclastoma tissue. These enzymes are typical of cathepsins which are small proteinases, 20–35 kDa in size [9,10]. Since cathepsins B and L are the most abundant and most highly studied cathepsins in tissues, efforts were concentrated on comparing the six osteoclastoma activities with the reported properties of these cathepsins. The published data on cathepsins H, S and N were also compared using kinetic studies, reaction with antibodies and Northern blotting. The preferred synthetic substrate was *Z*-Phe-Arg-NHMec for all enzymes. Both cathepsins B and L are active on this substrate. However, L is more active mol per mol of enzyme than cathepsin B [25]. When comparing relative rates of hydrolysis for *Z*-Phe-Arg-NHMec enzyme 3 was the most active.

Enzymes 1–4 also hydrolysed Bz-Phe-Val-Arg-NHMec, a substrate which is hydrolysed at a greater rate by cathepsin B than cathepsin L on a molar basis [13]. However, cathepsin B should be equally active on this substrate, as it is on *Z*-Phe-Arg-NHMec, whereas cathepsin L only hydrolyses it at approximately 5% the rate at which it hydrolyses *Z*-Phe-Arg-NHMec [25]. Cathepsin N is more active on Bz-Phe-Val-Arg-NHMec than it is on *Z*-Phe-Arg-NHMec [25]. Taking this into account, enzymes 1 and 2 are more B-like in their behaviour and enzymes 3 and 4 are more L-like. However, when incubated with *Z*-Arg-Arg-NHMec, a substrate hydrolysed approx. 10-times more rapidly by B than L, only enzymes 1 and 4 showed some hydrolysis. None of the enzymes hydrolysed Arg-NHMec or Bz-Arg-NHMec, which are substrates for cathepsin H [26]. Cathepsin N, purified from rabbit spleen, has a molecular weight of 34000 and relatively low activity against *Z*-Arg-Arg-NHMec [25] but kinetic details are not yet available.

Studies of the kinetics of hydrolysis of *Z*-Phe-Arg-NHMec gave results more like cathepsin B than L.

Several of the enzymes gave values in the range expected for cathepsin S [27]. However, when the substrate Bz-Phe-Val-Arg-NHMec was investigated none of the enzymes gave values similar to cathepsin S: all were in the range expected for B and L.

Most of the enzymes were capable of degrading both soluble and insoluble type I collagen. The rates of degradation of insoluble collagen at 25°C were less than 10% of the rates at 37°C. It has been reported that at 25°C cysteine proteinases preferentially degrade the telopeptide extensions [28]. The maximum rate of collagen degradation by osteoclastoma cysteine proteinases was approximately a thousand times lower than the rate reported for rat liver cathepsin L [28]. The collagen used in these experiments was completely resistant to trypsin digestion at 25°C indicating that it was largely in the native state.

All the enzymes were inhibited by the aldehydes antipain, chymostatin and leupeptin, which inhibit serine as well as cysteine proteinases [9]. The aldehydes are powerful inhibitors of cathepsins B and L but less effective against H. The six enzymes were all inhibited by 60–90% at concentrations of 10 μM antipain or chymostatin, except for enzymes 4 and 6, which were only inhibited between 2–21%. All were inhibited by the peptidyl chloromethanes Tos-Phe-CH_2Cl and Tos-Lys-CH_2Cl, which are also inhibitors of serine and cysteine proteinases [29]. E-64 at 10 μM has been reported to rapidly inactivate cathepsins B, H and L [13]. Since all enzymes were totally inhibited at this concentration, the effect of E-64 at 0.1 μM was investigated. Enzymes 1, 2, 4 and 6 were inhibited between 54–92% whereas enzymes 3 and 5 were only inhibited by about 20%.

Peptidyl diazomethanes are specific inhibitors of cysteine proteinases [29–32] and are selective against individual cysteine proteinases [33]. The relative rate constants of inactivation using *Z*-Phe-Tyr-(*O*-*t*-Bu)CHN_2 were of the order of 10^3 M^{-1} min^{-1}, which was similar to the expected value for cathepsin B. Thus, most of the kinetic data derived from inhibitor studies suggests that the osteoclastoma cysteine proteinases represent multiple forms of cathepsin B. This conclusion is also supported by the low activity of these enzymes on native collagen and the kinetic studies with synthetic substrates. However, unlike most previously characterised cathepsin Bs, the osteoclastoma enzymes had little activity on *Z*-Arg-Arg-NHMec and four of the enzymes had little activity on Bz-Phe-Val-Arg-NHMec.

The similarity of the osteoclastoma cysteine proteinases to cathepsin B-like enzymes was further indicated by Western blotting. Analysis of the three major peaks of activity separated by chromatography on S-Sepharose indicated that the molecular weights of the immunoreactive cathepsin B-like enzymes were generally in agreement with those obtained by gel filtration experiments. Immunoreactive bands corresponding to enzymes 2 and 6 (both M_r 20000) were not observed. However, these two enzymes are present in low amounts and might possibly be degradation products of enzymes 1 and 5. Multiple forms of cathepsin B have been found in a variety of normal tissues and, more especially, tumours. For example, in human kidney there are two forms of cathepsin B with M_r of 32000 and 24000 [34]; two isoforms with M_r of 30000 were identified in bovine liver [35] and two isoforms with M_r of 28000 and 20000 in porcine spleen [36]. Higher M_r variants are commonly observed in tumours and are secreted by normal and tumour-derived cell lines. For example, a rat insulinoma contained two forms of cathepsin B with M_r of 39000 and 31500 [37] and a variety of cell lines established from colorectal tumours secreted a form of cathepsin B with an M_r of 38000 [38]. Normal fibroblast cell lines and cultured macrophages secrete forms of cathepsin B with M_r of 46000 and 39000, respectively [39,40]. In vitro experiments have indicated that cathepsin B is synthesised as a precursor with an M_r of 39000–46000 (procathepsin B) which undergoes progressive proteolytic cleavage giving rise to the mature form of the enzyme which has an M_r of 29000 [41]. In some tissues there is further cleavage of a single polypeptide bond to give a disulphide bonded two subunit protein with M_r of 24000 and 5000 [42]. The proteolytic processing to the mature form may occur within the lysosomes [43] which would explain why cells that secrete a relatively high proportion of their cathepsin B (e.g., cells in culture and tumour cells) contain more higher M_r forms of the enzyme than normal tissues.

The complexity of cathepsin B synthesis in osteoclastomas was further illustrated by the demonstration of three specific mRNA transcripts. Most normal tissues contain a single major transcript of 2.3 kb [44]. However, a variety of murine tumours contain multiple mRNA transcripts for cathepsin B [45,46].

It is difficult to rule out the possibility that the complexity of cathepsin B species results from the variety of cell types found in osteoclastomas. However, immunolocalisation experiments demonstrated that most of the cathepsin B was present in osteoclasts rather than the neoplastic osteoblasts. It is, perhaps, surprising that the major cysteine proteinases in osteoclasts resemble cathepsin B rather than cathepsin L. Cathepsin L has been reported to be more active on both native and denatured collagen than cathepsin B. However, other considerations may be important, such as stability of the enzyme in the acidic pH of the subosteoclastic compartment and the requirement for the degradation of non-collagenous bone proteins.

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