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Original Articles

Expression of the Proteinase Specialized in Bone Resorption, Cathepsin K, in Granulomatous Inflammation

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Abstract

Background: The cysteine proteinase cathepsin K has aroused intense interest as the main effector in the digestion of extracellular matrix during bone resorption by osteoclasts. The enzyme is not a housekeeping lysosomal hydrolase, but is instead expressed with striking specificity in osteoclasts. In this work, we present evidence for the association of cathepsin K with the granulomatous reaction. Granulomas are inflammatory tissue reactions against persistent pathogens or foreign bodies. We came across cathepsin K while working on *Echinococcus granulosus,* a persistent tissue-dwelling, cyst-forming parasite that elicits a granulomatous response.
Materials and Methods: The walls of hydatid cysts from infected cattle were solubilized. Strong proteolytic activity was detected in the extracts. The proteinase responsible was purified by anion exchange and gel filtration. The purified protein was subjected to N-terminal sequencing, and its identity further confirmed by Western blotting, with a cathepsin K-specific antibody. The same antibody was used to localize the proteinase in paraffin-embedded sections of the parasite and the local host response.
Results: A proteinase was purified to near homogeneity from hydatid cyst extracts. The enzyme was unequivocally identified as host cathepsin K. Both the proenzyme and the mature enzyme forms were found. Cathepsin K was then immunolocalized both to the parasite cyst wall and to the epithelioid and giant multinucleated cells of the host granulomatous response.
Conclusions: In the granulomatous response to the hydatid cyst, cathepsin K is expressed by epithelioid and giant multinucleated cells. We propose that, by analogy with bone resorption, cathepsin K is secreted by the host in an attempt to digest the persistent foreign body. Both processes, bone resorption and granulomatous reactions, therefore tackle persistent extracellular material (the bone matrix or the foreign body), and utilize specialized cells of the monocytic lineage (osteoclasts or epithelioid/giant cells) secreting cathepsin K as an effector.

Introduction

The physiological process of bone resorption is carried out by specialized multinucleated cells of monocytic origin called osteoclasts. Osteoclasts bring about both the solubilization of the mineral component of bone and the digestion of its organic matrix. It has become apparent over the last 5 years that the major enzyme responsible for the digestion of this organic matrix is the cysteine proteinase cathepsin K. Part of the evidence for this arises from the distinctive pattern of expression of the enzyme: cathepsin K is present at high levels in osteoclasts and in the closely related chondroclasts, but it is undetectable or expressed at much lower levels in all other tissues and cell types examined from humans, rabbits, and mice (1–6). Within osteoclasts, the enzyme is most abundant next to the surface of the bone being resorbed (5–7). Fur-

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ther, both antisense cathepsin K oligodeoxynucleotide (8) and synthetic inhibitors of the enzyme (9,10) inhibit bone resorption. Most decisively, cathepsin K deficiency in humans causes pycnodysostosis, a skeletal disease characterized by deficient bone resorption (11,12). A similar phenotype arises in cathepsin K-knockout mice (13). Interestingly, the clinical manifestations of deficiency are restricted to bone, in contrast to the accumulation of undigested intracellular material associated with deficiencies in other lysosomal hydrolases.

In this work we present evidence for the association of cathepsin K with a granulomatous reaction. Granulomas are tissue reactions typically elicited by persistent, indigestible pathogens, pathogen products, or inert foreign material. They are characterized by the accumulation of cells of monocytic origin and are thought to be directed both at walling off and at eliminating the persistent foreign body (reviewed in 14–19). The hallmarks of granulomatous reactions are special types of activated macrophages called epithelioid cells and giant multinucleated cells. The functions of these cell types are not well understood. They both have poor or no phagocytic capacity, and are thought, mainly from their morphology, to be highly secretory.

The observations in this work refer to the epithelioid and giant cells surrounding, as a continuous palisading rim, the hydatid cyst of the cestode parasite *Echinococcus granulosus.* This infection of livestock and humans is characterized by the growth of fluid-filled cysts in the parenchyma of various internal organs, mainly the liver and lungs (reviewed in 20). The host tissue reaction to this very persistent pathogen is composed of an inner rim of epithelioid and giant cells, a mononuclear and eosinophil infiltrate, and an outer fibrous layer of fibroblasts and collagen (21–23). Overall, this is a classical T-cell–dependent granuloma that surrounds a larger than usual indigestible body; the cyst can become several tens of centimeters in diameter.

Our observations were carried out on hydatid cysts from bovine lungs. Host-derived cathepsin K was found in the outer structure of the parasite cyst (hydatid cyst wall, HCW), which is closely apposed to the epithelioid and giant cells of the host reaction, and is known to bind certain host proteins in vivo (24). The enzyme was then shown by immunohistochemistry to be expressed by host inflammatory cells.

Materials and Methods

Echinococcus granulosus Material

The hydatid cyst is filled with HCF and bounded by the two-layered parasite-derived HCW. The inner layer of the HCW (germinal layer) is the live parasite tissue. The outer layer (laminated layer) is a tough, elastic, carbohydrate-rich structure that can be up to 2 mm thick. The hydatid material used in this work was from cysts from naturally infected cow's lungs from slaughterhouses in Uruguay. The processing of the hydatid material and the preparation of extracts from the HCW has been described in detail elsewhere (24). In brief, the extraction procedure used consisted of an initial wash with phosphate-buffered saline (PBS), followed by detergent extractions aimed at solubilizing the cellular layer, and then by a pulverization step under liquid nitrogen aimed at disorganizing the resilient laminated layer. A PBS-soluble fraction was obtained (PBS extract). Portions of the insoluble pellet were further extracted with a battery of different solvents, including 2 M NaCl (2 M NaCl extract). A cocktail of protease inhibitors, consisting of Pefabloc SC (4-(2-aminoethyl)-benzenesulfonylfluoride; Pentapharm Ltd., Basel, Switzerland) at 0.5 mM, iodoacetamide at 0.2 mM, EDTA at 5 mM, and pepstatin A (ICN Biomedicals Ltd., Aurora, OH) at 2 μg/ml, was added at all steps during extraction.

Proteolytic Activity Assays

These used as a substrate ^{125}I-labeled amidated bovine complement C3 (^{125}I-C3(NH$_3$)), prepared as described previously (24). The discovery of cathepsin K in this context was a by-product of a study of parasite interactions with host complement. The initial fortuitous detection of the activity, as well as the functional assays during the purification of the proteinase, were carried out under conditions in which the proteolytic event took place after addition of the SDS-PAGE sample buffer. Briefly, samples were incubated at 37°C with ^{125}I-C3(NH$_3$) (around 20000 cpm/tube) in 10 mM of sodium phosphate, 0.5 mM of EDTA, at a pH of 7.0, containing 0.01% w/v Triton X-100 (as a means of minimizing adsorption of the labeled material to the tube walls) and 20 μg/ml of soybean trypsin inhibitor (type I-S, Sigma Chemical Co., Poole, Dorset, U.K.). An equal volume of SDS-PAGE sample buffer (0.2 M of

Tris-HCl, 8 M of urea, 2% w/v SDS, and 20 mM of dithio-treitol, at a pH of 8.0) was added and mixtures further incubated for 20 min at 37°C. Samples were run on 7.5% w/v acrylamide SDS-PAGE gels, then gels were dried and subjected to autoradiography using x-ray films and intensifying screens.

Purification of Proteinase Activity

Forty milliliters of the HCW PBS extract, corresponding to 3.8 mg of total protein, were loaded on a 1-ml resource Q FPLC column (Pharmacia, Uppsala, Sweden), in 20 mM of Tris-HCl, 0.5 mM of EDTA, 0.1% w/v poly (oxyethylene) 10-tridecyl ether (Emulphogene, Sigma), at a pH of 8.0, and eluted with a 30-ml linear NaCl gradient (0–500 mM). The active fractions were refractionated on a Superose 12 HR 10/30 FPLC column (Pharmacia) in PBS and 5 mM of EDTA.

Anti-cathepsin K Antibodies

Purified recombinant human procathepsin K (amino acids 13–329, produced in *E. coli*) and two rabbit antisera against human cathepsin K (one against the recombinant proenzyme, and the other one against a KLH-coupled peptide corresponding to the last 18 amino acid residues of the enzyme) were a kind gift of Dr. A. Punturieri and Dr. S. J. Weiss (Division of Hematology and Oncology, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI). The antisera do not crossreact with the related human cathepsins S, L, or B.

Western Blots

Samples were run in SDS-PAGE (10% w/v acrylamide; reducing conditions), transferred to nitrocellulose membranes (Hybond C, Amersham, Bucks, U.K.), and blocked overnight at 4°C in PBS, 0.1% w/v Tween 20 and 5 mM of EDTA. Free sulfhydryl groups in the electrotransferred proteins were blocked by treatment of the membranes with 2 mM of iodoacetamide in 20 mM of Tris-HCl, at a pH of 8.65. Blots were then probed with the antisera or normal rabbit serum diluted 1/1000 in PBS (Oxoid, Unipath Ltd., Basingstoke, Hants., U.K.) containing 0.05% w/v Tween 20 and 5 mM of EDTA, for 2 hr at room temperature. After washing (in PBS, 0.05% w/v Tween 20 and 5 mM of EDTA), blots were incubated for 90 min at room temperature with alkaline phosphatase-conjugated affinity isolated goat immunoglobulin G (IgG) against rabbit IgG (Sigma, A3812) diluted 1/10000 in the same buffer. Development used nitro blue tetrazolium/5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP; Sigma) substrate.

Amino Acid Sequencing

Samples were run on SDS-PAGE (12.5% w/v acrylamide) and then electroblotted to ProBlott membrane (Perkin-Elmer, Applied Biosystems Division, Warrington, Ches., U.K.). This was stained with Coomassie Brilliant Blue. The bands of interest were excised and sequenced using an Applied Biosystems 494A "Procise" sequencer.

Immunohistochemistry

The host tissue adjacent to hydatid cysts, with the corresponding area of HCW attached, was removed from the infected bovine lungs, fixed in formal saline, and embedded in paraffin using standard procedures. Microtome slices (0.5 mm thick) were dewaxed and, except when indicated, subjected to an antigen retrieval procedure consisting of boiling for 15 min in 10 mM of citrate buffer at a pH of 6. Slides were treated with 3% w/v H_2O_2 for 5 min, and after blocking with 20% w/v normal pig serum in Tris-buffered saline (TBS) for 1 hr, incubated overnight at 4°C with the rabbit antisera against human cathepsin K or normal rabbit serum, diluted 1/200 or 1/300 in TBS containing 1% w/v bovine serum albumin. Development was carried out using biotinylated pig IgG against rabbit IgG (E0431; Dakopatts, Glostrup, Denmark) at 1/400 dilution in TBS for 40 min, then avidin-biotin-horseradish peroxidase complex (K0355; Dakopatts; used according to manufacturer's instructions) for 40 min and then DAB (diaminobenzidine, Sigma) substrate. Slides were counterstained with Mayer's hemalum.

Results

Initial Detection of the Proteinase Activity

Extracts from the *E. granulosus* cyst were prepared with the initial aim of looking at interfer-

ence with host complement; a cocktail of proteinase inhibitors was added at all steps during the extraction procedure. Surprisingly, several extracts showed a potent proteolytic activity against bovine ^{125}I-C3(NH_3). This activity was highest in several extracts from the HCW obtained after the liquid nitrogen pulverization step; it was absent from HCF and bovine plasma, included as a control (data not shown). The presence of the activity was reproducible over three independent batches of extracts prepared (data not shown). The proteolytic event is illustrated in Fig. 1 for the 2 M NaCl extract, which was the richest in the activity. The proteolysis event resulted in the cleavage of the α chain of C3(NH_3) into a highly labeled 44-kDa fragment and a weakly labeled complementary fragment of approximately 80 kDa (Fig. 1). A

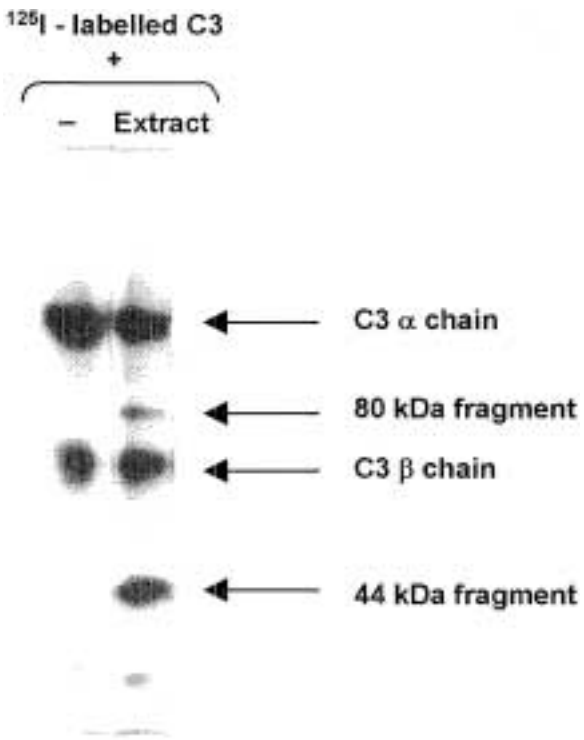

Fig. 1. Proteolysis of radiolabeled amidated bovine complement C3 by an activity found in preparations of the *E. granulosus* HCW. ^{125}I-C3(NH_3) (11 ng/track) was incubated (1 min at 37°C, 50 μl total volume) with or without 30 μl of the 2 M NaCl extract from HCW. Reaction mixtures were added to SDS-PAGE sample buffer, further incubated for 20 min at 37°C, run on SDS-PAGE (7.5% w/v acrylamide, reducing conditions) and autoradiographed. The 44-kDa (highly labeled) and 80-kDa (slightly labeled) fragments from the α chain of C3(NH_3) generated by the proteolytic activity under study are indicated.

series of experiments (not shown) indicated that the 44- and 80-kDa fragments arose from the N- and C-termini of the α chain of C3, respectively. Further experiments revealed that the observed cleavage event took place during the process of denaturation, after addition of the SDS-PAGE sample buffer.

Purification of the Proteolytic Activity and Identification of the Proteinase Involved as the Bovine Homologue of the Known Mammalian Cathepsin K Cysteine Proteinases

The PBS extract of the HCW was used as source of material to purify the proteolytic activity. The activity was followed on the basis of the cleavage of the α chain of ^{125}I-labeled C3(NH_3) taking place during denaturation, as noted above. The activity eluted from a resource Q anion exchange column at a pH of 8.0 at around 240 mM NaCl (Fig. 2A; fraction eluting at 20 ml). SDS-PAGE analysis of the fractions showed that, both under nonreducing and reducing conditions, a band of 29 kDa and a closely spaced group of bands of around 40 kDa peaked with the proteolytic activity under study (Fig. 3A). The pooled active fractions were refractionated on Superose 12 FPLC; the activity, eluting at 14 ml (Fig. 2B), was associated with the group of bands of around 40 kDa (Fig. 3A).

N-terminal sequences of both the 40-kDa complex band and the 29-kDa band from the fraction eluting at 20 ml from the anion exchange step of the purification (Fig. 3A) are shown in Fig. 4. Both sequences showed a high degree of identity to the known cathepsin K cysteine proteinases of mammalian origin. In addition, the sequence for the 29 kDa band was found to be almost identical (one conservative change in 15 residues; Fig. 4) to the published N-terminal sequence of a 28-kDa protein from bovine pituitary glands that binds to heparin. This latter protein had been reported, before the cloning of the cathepsins K, as a cysteine proteinase related to cathepsins L and H (25).

The sequence information indicated that the enzyme in our extracts was the bovine counterpart of the known cathepsin K enzymes, rather than a parasite-derived enzyme. Over a total of 32 residues, our sequence differed in only 1–4 positions from the mammalian cathepsins K, while differing in 10 positions from their chicken counterpart (Fig. 4). Cathepsin K, like other members of the papain

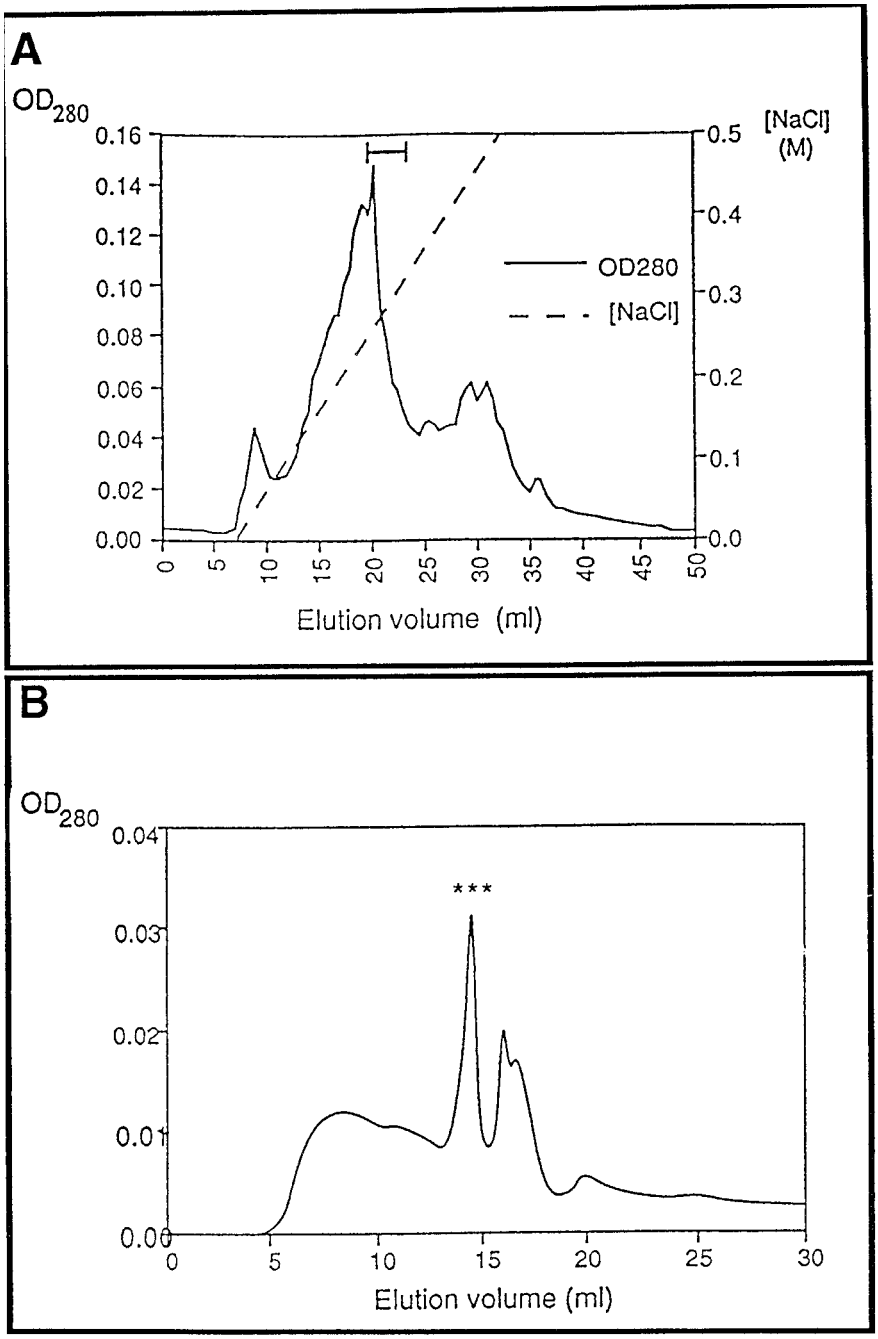

Fig. 2. Purification of the proteolytic activity. The PBS extract of the HCW was fractionated on resource Q anion exchange (A) and the active fractions (bar) rechromatographed on superose 12 gel filtration (B). Activity was followed on the basis of the cleavage of ^{125}I-C3(NH_3) by SDS-PAGE and autoradiography. Active fractions in (B) are indicated by asterisks.

family, is synthesized as a pre-proenzyme. Cleavage of the hydrophobic leader peptide releases the proenzyme, in which the proregion covers the active site cleft. Proteolytic degradation of the proregion gives rise to the mature, active enzyme. The alignments indicated that the 40-kDa bands and the 29-kDa band in the HCW extracts corresponded to the proenzyme and mature enzyme forms of cathepsin K, respectively. The molecular weight values of the bands agreed well with the those reported for the proenzyme and active enzyme forms of the known mammalian cathepsins K, including both values predicted from cDNA

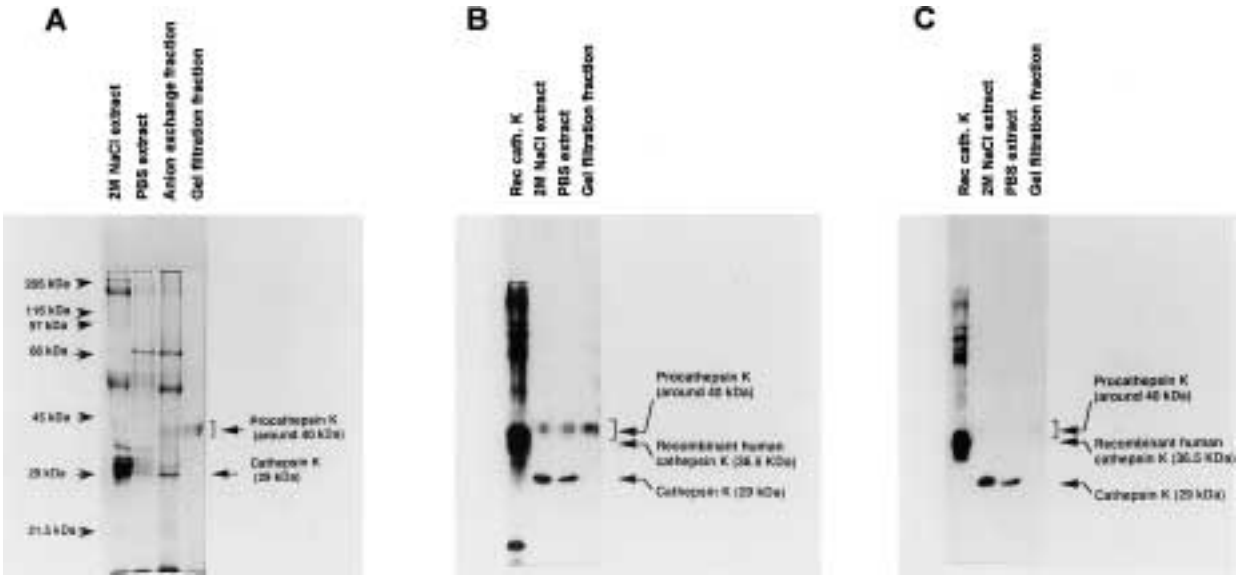

Fig. 3. Bovine cathepsin K from the *E. granulosus* HCW, visualized on SDS-PAGE and by Western blot. The crude extracts and the peak active fractions from the resource Q anion exchange step and from the subsequent gel filtration step were run on SDS-PAGE (10% w/v acrylamide, reducing conditions) and either Coomassie-stained (A) or transferred to nitrocellulose and probed with antibodies to recombinant human cathepsin K (B), with antibodies to a C-terminal peptide of the enzyme (C), or with normal rabbit serum (not shown). For Coommassie staining, 20 μg (extracts and anion exchange fraction) or 6 μg of protein (gel filtration fraction) were loaded per track. For Western blotting, 7 μg (extracts) or 1 μg (gel filtration fraction) of protein were loaded per track; recombinant human cathepsin K (rec cath K; 1 μg) was included as a positive control. The 29-kDa and 40-kDa bands from the anion exchange peak fraction, which were subjected to N-terminal sequencing, are indicated in (A). Note that these correspond to the procathepsin K and cathepsin K bands detected by Western blotting (B). The control blot probed with normal rabbit serum gave no staining at all.

sequences (1–4,26) and values observed for the native (5,6) or recombinant enzymes (11,27–31).

The observations were reinforced by Western blot analysis using antisera raised against the recombinant human enzyme or a C-terminal peptide thereof. Both antisera recognized the mature enzyme form, and the antiserum raised against the recombinant enzyme recognized in addition the complex band corresponding to the procathepsin form (Figs. 3B and 3C). No other bands were recognized in the HCW extracts.

It should be noted that the fraction active in proteolysis of C3(NH_3) obtained after the gel filtration step contained the procathepsin but not the mature cathepsin (Fig. 3B). In all likelihood, this was due to only the procathepsin form escaping the iodoacetamide (inhibitor) treatments used during extraction, and then becoming autoactivated during the preparation of samples for SDS-PAGE. A rough estimation from OD_{280} readings indicated that procathepsin K constituted at least 0.5% of the total protein in the extract used for its purification (PBS extract), which was not the richest in this molecule (see Figs. 3A and 3B, tracks marked "2M NaCl extract" and "PBS extract").

Immunohistochemical Localization of Cathepsin K in the Hydatid Cyst Wall and Host Inflammatory Cells

The antiserum against recombinant human cathepsin K, shown by Western blot to cross-react with the enzyme in its two forms in our extracts (Fig. 3B), was used in immunohistochemical studies. The enzyme was shown to be associated with the structures present on both sides of the host–parasite interface (Fig. 5A–H). This is consistent with results discussed, which indicate both a host origin and an association with parasite structures for the enzyme.

On the parasite side, cathepsin K was associated mostly with the striations of the laminated layer (Figs. 5A and B). Determination of whether the enzyme was also associated with the germinal layer was difficult because of the

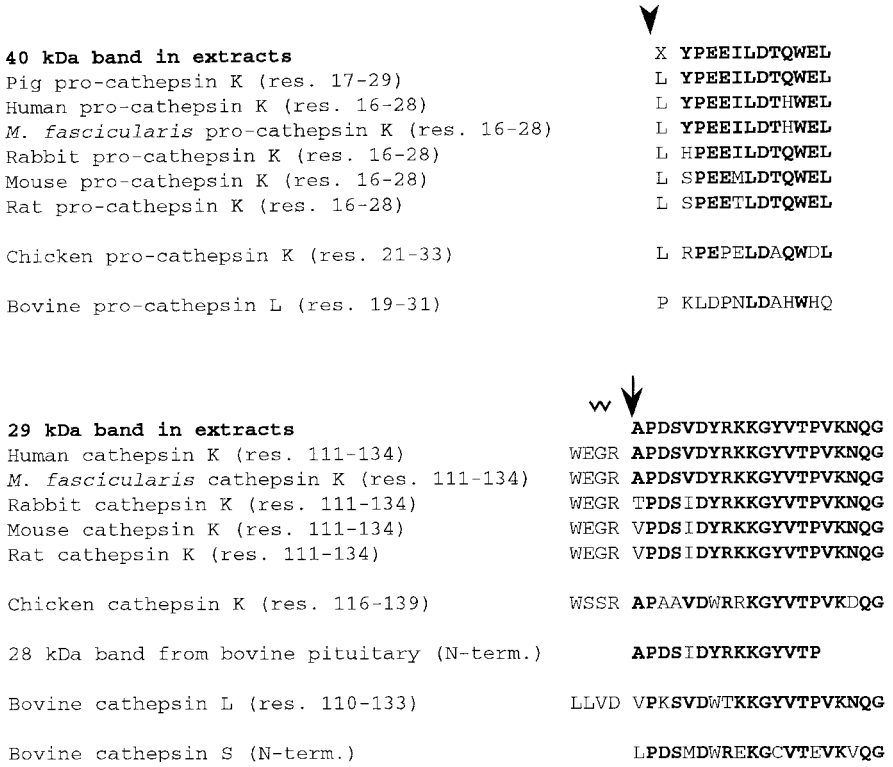

Fig. 4. Identification of the 40-kDa and 29-kDa bands in HCW extracts as pro- and mature bovine cathepsin K, from their N-terminal peptide sequences. The N-terminal peptide sequences derived from the bands in our extracts (X is an unidentified residue) have been aligned with matching sequences from the databases, with residues identical to those in our sequences given in bold type. Sequences shown correspond to the cathepsins K from pig (AF010306), human (U13665), *Macacca fascicularis* (AF070927), rabbit (D14036), mouse (X94444), rat (AF010306), and chicken (U37691) (all deduced from cDNA), plus the N-terminal amino acid sequence of a 28-kDa protein band from bovine pituitary, reported at the time as a cathepsin L–related molecule (25). Bovine cathepsin L (sequence deduced from cDNA, X91755) and bovine cathepsin S (P25326) have been included for comparison; this rules out the possibility that our protein bands could correspond to cathepsins L or S, the closest enzymes to cathepsin K. The starting site for the known procathepsins K, both predicted from sequence alignments (1–4,26) and experimentally determined for the recombinant enzymes (28,30,31) is indicated by an arrowhead. The starting site of the mature cathepsins K predicted from sequence alignments (1–4,26) and experimentally observed for the recombinant pepsin-activated enzymes (27,29) is indicated by an arrow; two additional starting sites, reported for the autoactivated recombinant enzymes (28,30,31) are shown (v).

strong, nonspecific reaction shown by this structure (data not shown).

On the host side, the enzyme was shown to be associated with the epithelioid and giant multinucleated cells in the layer immediately adjacent to the parasite (21–23) (Figs. 5C–H). No staining for the enzyme was detected in the connective tissue sheath or lung parenchyma (Figs. 5C–H, and results not shown).

Discussion

In this work, we have shown that the proteinase cathepsin K is expressed by the inflammatory cells elicited in the tissue response against a persistent pathogen. The enzyme was first purified from the outer wall of the *E. granulosus* cyst and then detected by immunohistochemistry in the host epithelioid and giant multinucleated cells surrounding the parasite in vivo.

The proteolytic cleavage of bovine C3(NH_3) that constituted our initial observation was later shown to take place after addition of the SDS-PAGE sample buffer and was associated with chromatographic fractions containing the procathepsin K, rather than with the mature enzyme (which would have been inactivated by the iodoacetamide treatments used during

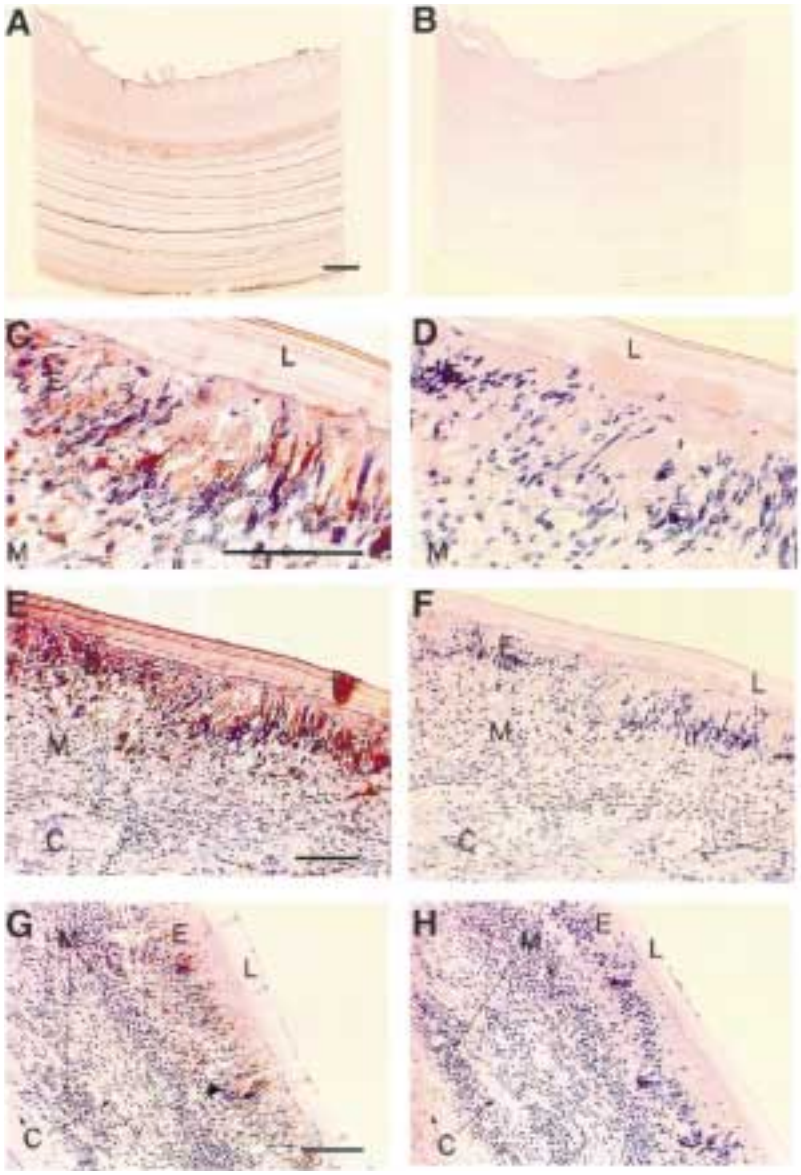

Fig. 5. Immunohistochemical localization of cathepsin K in the *E. granulosus* hydatid cyst and in the bovine tissue surrounding it. The laminated layer of the cyst wall from *E. granulosus*-infected bovine lungs, and in some cases the surrounding host tissue, were paraffin-embedded. Slides were probed with rabbit antiserum against human cathepsin K (A, C, E, G) or normal rabbit serum (B, D, F, H). Sera were used at 1/200 (A–F) or 1/300 (G, H). The intact laminated layer separated from the host tissue (not subjected to antigen retrieval) is shown in (A, B). The inner part of the laminated layer ("L") and the host tissue are shown in (C–H): the three layers of the host inflammatory infiltrate, namely epithelioid and giant cell layer ("E"), mononuclear and eosinophil infiltrate ("M"), and collagenous layer ("C") are indicated. A multinucleated giant cell morphology is indicated in (G) with an arrowhead.

extraction). Procathepsin K is known to become activated at low pH or high temperature (50°C) (28,30,31). This is an autocatalytic process thought to be triggered by a conformational change that unmasks the active site and makes the inhibitory propeptide more susceptible to proteolysis (30). The required conformational change is likely to have taken place in the presence of denaturants under our experimental conditions. It is not surprising that, once activated, the enzyme could act on the protein substrate in SDS-PAGE sample buffer. Proteolytic enzymes are often partially resistant to denaturation, and the addition of denaturants

facilitates their activity by unfolding their protein substrates. Additional experiments showed that the purified bovine cathepsin K from HCW (whether extracted as mature enzyme or extracted as proenzyme and autoactivated at low pH) can cleave ^{125}I-C3(NH_3) under nondenaturing conditions. The pattern of cleavage is then different from that seen under denaturing conditions (results not shown).

This is the first report of the purification of a native cathepsin K. The starting site of the mature bovine enzyme in our preparations coincides with the position previously predicted from sequence alignments, and reported for the recombinant human enzyme that was processed to the mature form by pepsin (27,29). In contrast, autocatalytic activation of the recombinant human or monkey enzymes is reported to give ragged N-termini (28,30,31) (Fig. 4).

The host-derived enzyme seems to be strongly associated with the parasite cyst wall. The need for high ionic strength to extract the enzyme suggests that it is bound by interactions involving a strong charge component. It seems likely that cathepsin K is binding to anionic structures on the parasite cyst wall. Cathepsins K have considerable positive charge at neutral pH, unlike the rest of the members of this proteinase family. The crystal structure of human cathepsin K shows that it contains an area of positive surface charge, composed of three arginines and five lysines (32). As mentioned, a bovine cysteine proteinase that is probably cathepsin K was reported to bind to immobilized heparin (25). Interestingly, two other host proteins known to bind to heparin, namely complement factor H and annexin II, have been found to be concentrated in the *E. granulosus* cyst wall (24; Díaz A, Ibarguren S, Breijo M, Willis AC, Sim RB, submitted).

The local reaction to the *E. granulosus* cyst can display a continuum of morphologies, ranging from a granulomatous-type response to the presence of a collagenous sheath, a product of the resolution of the former. This depends partly on the host species, and in cattle (the host studied in this work) the inflammation generally does not resolve. Across the spectrum of host responses, cyst fertility correlates with resolution of inflammation, whereas intense responses are associated with infertility and, more extremely, degeneration of the cysts (20–23,33–35). Cathepsin K, a potent proteinase, could be instrumental in destruction of the parasite by the host inflammatory response. Survival of the parasite may well be associated with inhibition of host cathepsin K by an as yet unknown mechanism.

The question of the association of cathepsin K—a proteinase with a narrow, almost osteoclast-specific, cell distribution—with the inflammatory cells elicited by the cyst is of particular interest. Shi et al. (26) cloned human cathepsin K from tissue culture monocyte-derived macrophages; more recently, the human enzyme has been found to be expressed by synovial fibroblasts from rheumatoid joints (36), giant cells in osteoarthritic synovium (37), and by macrophages in atheroma plaques (38). However, our results constitute the first report of the association of this enzyme with the granulomatous response, a stereotyped, and thus controlled, form of inflammatory reaction.

Granulomas are elicited by persistent nonself bodies, and are thought to be directed both at walling off and at resorbing the unwanted material (reviewed in 14–19). We propose that cathepsin K may play a major role in extracellular digestion in granulomatous reactions, as it does in bone resorption. Many interesting connections and parallels can be drawn between these two types of process and the cells involved in them. Osteoclasts have, like epithelioid and giant cells, a monocytic origin. Inflammatory giant cells can be morphologically indistinguishable from osteoclasts (39), and share many surface markers with those (40). A functional relationship between osteoclasts and foreign-body giant cells was suggested two decades ago; Chambers (39) proposed that bone resorption involved the recognition of bone under particular circumstances as a foreign object—a process that does not differ from the resorption of foreign bodies in granulomata. More recently, giant cells in foreign-body reactions in bone (like those elicited by particles shed from prostheses) have been shown to be associated with pathological resorption of bone (41). Foreign-body giant cells and epithelioid cells can also resorb dead bone implants; this observation prompted the authors to propose a functional relationship, involving the secretion of common hydrolytic enzymes, between those cells and osteoclasts (42).

The information available on cathepsin K makes it a likely candidate for a proteinase specialized in the digestion of unwanted extracellular material. First, cathepsin K is a potent general digestive proteinase. It has been shown

so far to digest fibrinogen, osteonectin, type I collagen, elastin, and gelatin; its activity on these last three substrates is considerably higher than those of elastase, cathepsin L, or cathepsin S (20,27,28). Further, cathepsin K is the only cysteine proteinase capable of cleaving the type I collagen triple helix (43). Second, the enzyme is likely to act mostly extracellularly. Osteoclasts generate an acidified resorption pit on the surface of the bone tissue to which they are tightly apposed. This is made possible by an interesting adaptation of lysosomal function: the domain of the osteoclast cell membrane (known as the "ruffled border"), which is situated toward the bone surface, is analogous to a lysosomal membrane, and the resorption pit has been compared to a "giant extracellular lysosome" (reviewed in 44). An extracellular role for the enzyme is also supported by the observations that cathepsin K–deficient patients and mice display minimal (humans) or no (mice) accumulation of intracellular undegraded substrate in their osteoclasts (11,13). In addition, the protease is active on synthetic substrates over a wide range of pH values, up to approximately pH 8 (27). Third, cathepsin K seems to have a highly regulated expression, as would be expected for a potentially dangerous secreted effector. This is evident from its distinctive tissue distribution (1–6,11–13), and by the fact that it is expressed by monocyte-derived macrophages only after 15 days in culture, while the related cathepsins B, L, and S are expressed after just a few days (26). The production of cathepsin K may well be a specific feature of certain highly differentiated cells in the monocytic lineage specializing in extracellular degradation. It is likely that, similar to the case of osteoclasts (5), cathepsin K is the major cysteine proteinase in the inflammatory cells in our system. This is supported by (i) the striking abundance of cathepsin K in the HCW, and (ii) the observation that the ^{125}I-C3(NH_3)-cleaving activity purified as a single peak (corresponding to procathepsin K; Figs 2 and 3), in spite of the fact that the related (pro-) cathepsin L is known to cleave C3 efficiently (45). However, a comparative analysis of the expression of cathepsin K with the related cathepsins S and L in the granulomatous inflammatory cells—rather than in the HCW, which could well bind cathepsin K preferentially—is needed.

It seems likely that the secretion of cathepsin K may be a major function of inflammatory epithelioid and giant multinucleated cells and that this enzyme may play an important role in the physiology of granulomas. Tissue necrosis often associated with granulomatous inflammation has been generally attributed to the release of free radicals and proteases (14–19). Cathepsin K could play an important role in granuloma-associated pathology observed in diseases such as tuberculosis and sarcoidosis. Experiments to assess the expression of cathepsin K in a range of granulomatous reactions are warranted. The contribution of cathepsin K to the overall proteolytic potential of granulomas should be evaluated using knock-out mice, now available (13).

Acknowledgments

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References

1. Tezuka K, Tezuka Y, Maejima A, et al. (1994) Molecular cloning of a possible cysteine proteinase

predominantly expressed in osteoclasts. *J. Biol. Chem.* **269:** 1106–1109.
2. Inaoka T, Bilbe G, Tezuka K-I, Kumegawa M, Kokubo T. (1995) Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. *Biochem. Biophys. Res. Commun.* **206:** 89–96.
3. Brömme D, Okamoto K. (1995) Human cathepsin O2, a novel cysteine proteinase highly expressed in osteoclastomas and ovary. Molecular cloning, sequencing and tissue distribution. *Biol. Chem. Hoppe-Seyler* **376:** 379–384.
4. Rantakokko J, Aro HT, Savontaus M, Vuorio E. (1996) Mouse cathepsin K: cDNA cloning and predominant expression of the gene in osteoclasts, and in some hypertrophying chondrocytes during mouse development. *FEBS Lett.* **393:** 307–313.
5. Drake FH, Dodds RA, James IE, et al. (1996) Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. *J. Biol. Chem.* **271:** 12511–12516.
6. Littlewood-Evans A, Kokubo T, Ishibashi O, et al. (1997) Localization of cathepsin K in human osteoclasts by *in situ* hybridization and immunohistochemistry. *Bone* **20:** 81–86.
7. Yamaza T, Goto T, Kamiya T, Kobayashi Y, Sakai H, Tanaka T. (1998) Study of immunoelectron microscopy localization of cathepsin K in osteoclasts and other bone cells in the mouse femur. *Bone* **23:** 499–509.
8. Inui T, Ishibashi O, Inaoka T, et al. (1997) Cathepsin K antisense oligodeoxynucleotide inhibits osteoclastic bone resorption. *J. Biol. Chem.* **272:** 8109–8112.
9. Votta BJ, Levy MA, Badger A, et al. (1997) Peptide aldehyde inhibitors of cathepsin K inhibit bone resorption both *in vitro* and *in vivo. J. Bone Miner. Res.* **12:** 1396–1406.
10. Thompson SK, Halbert SM, Bossard MJ, et al. (1997) Design of potent and selective human cathepsin K inhibitors that span the active site. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 14249–14254.
11. Gelb BD, Shi G-P, Chapman HA, Desnick RJ. (1996) Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* **273:** 1236–1238.
12. Johnson MR, Polymeropoulos MH, Vos HL, Isela Ortiz De Luna R, Francomano CA. (1996) A nonsense mutation in the cathepsin K gene observed in a family with pycnodysostosis. *Genome Res.* **6:** 1051–1055.
13. Saftig P, Hunzinker E, Whemeyer O, et al. (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin K-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 13453–13458.
14. Williams GT, Williams WJ. (1983) Granulomatous inflammation—a review. *J. Clin. Pathol.* **36:** 723–733.
15. Epstein WL, Fukuyama K. (1989) Mechanisms of granulomatous inflammation. *Immunol. Series* **46:** 687–721.
16. Turk JL. (1989) Current status review: a comparison of secretory epithelioid cells and phagocytosing macrophages in experimental mycobacterial granulomas. *Br. J. Exp. Pathol.* **70:** 589–596.
17. Soler P, Bernaudin J-F. (1993) Physiologie des granulomes. *Revue de Pneumologie Clinique* **49:** 257–261.
18. Robinson BWS. (1996) Sarcoidosis. In: Kradlin RL, Robison BWS (eds.) *Immunopathology of Lung Disease.* Butterworth-Heinemann, Boston, pp. 165–190.
19. Vermeulen MW, Fenton MJ. (1996) Immunopathology of tuberculosis. In: Kradlin RL, Robison BWS (eds.) *Immunopathology of Lung Disease*. Butterworth-Heinemann, Boston, pp. 231–266.
20. Thompson RCA. (1995) Biology and systematics of *Echinococcus.* In: Thompson RCA, Lymbery AJ. (eds.) *Echinococcus and Hydatid Disease.* CAB International, Wallingford, U.K., pp. 1–50.
21. Nieberle P, Cohrs P. (1967) *Textbook of the Special Pathological Anatomy of Domestic Animals.* Pergamon, Oxford, U.K., pp. 280, 546.
22. Rao DG, Mohiyuddin S. (1974) Incidence of hydatid cyst in bovines and histopathological changes of pulmonary tissue in hydatidosis. *Indian J. Anim. Sci.* **44:** 437–440.
23. Slais J, Vanek M. (1980) Tissue reaction to spherical and lobular hydatid cysts of *Echinococcus granulosus* (Batsch, 1786). *Folia Parasitologica* (*Praha*) **27:** 135–143.
24. Díaz A, Ferreira AM, Sim RB. (1997). Complement evasion by *Echinococcus granulosus:* sequestration of host factor H in the hydatid cyst wall. *J. Immunol.* **158:** 3779–3786.
25. Ho PL, Carpenter MR, Smillie LB, Gambarini AG. (1990) Co-purification of proteases with basic fibroblast growth factor (FGF). *Biochem. Biophys. Res. Commun.* **170:** 769–774.
26. Shi GP, Chapman HA, Bhairi SM, Deleeuw C, Reddy VY, Weiss SJ. (1995) Molecular cloning of human cathepsin O, a novel endoproteinase and homologue of rabbit OC2. *FEBS Lett.* **357:** 129–134.
27. Brömme D, Okamoto K, Wang B, Biroc S. (1996) Human cathepsin O2, a matrix-degrading cysteine protease expressed in osteoclasts. *J. Biol. Chem.* **271:** 2126–2132.
28. Bossard M, Tomaszek, TA, Thompson SK, et al. (1996) Proteolytic activity of human osteoclast cathepsin K. *J. Biol. Chem.* **271:** 12517–12524.
29. Linnevers CJ, McGrath ME, Armstrong R, et al. (1997) Expression of human cathepsin K in *Pichia pastoris* and preliminary crystallographic

studies of an inhibitor complex. *Protein Sci.* **6**: 919–921.
30. McQueney MS, Amegadzic BY, D'Alessio K, et al. (1997) Autocatalytic activation of human cathepsin K. *J. Biol. Chem.* **272**: 13955–13960.
31. McQueney MS, Feild J, Hanning CR, et al. (1998) Cynomolgus monkey (*Macacca fascicularis*) cathepsin K: cloning, expression, purification and activation. *Protein Expr. Purif.* **14**: 387–394.
32. McGrath ME, Klaus JL, Barnes MG, Brömme D. (1997) Crystal structure of human cathepsin K complexed with a potent inhibitor. *Nat. Struct. Biol.* **4**: 105–109.
33. Yamashita J, Ohbayashi M, Konno S. (1957) Studies on echinonococcosis V. Experimental infection of the sheep. *Jpn. J. Vet. Res.* **5**: 43–50.
34. Thomas JA, Kothare SN. (1975) Tissue response in hydatidosis. *Indian J. Med. Res.* **63**: 1761–1766.
35. Smyth JD, Heath DD. (1970) Pathogenesis of larval cestodes in mammals. *Helminthol. Abstr.* **39**: 1–22.
36. Hummel KM, Petrow PK, Franz JK, et al. (1998) Cysteine proteinase cathepsin K mRNA is expressed in synovium of patients with rheumatoid arthritis and is detected at sites of synovial bone destruction. *J. Rheumatol.* **25**: 1887–1894.
37. Dodds RA, Connor JR, Drake FH, Gowen M. 1999. Expression of cathepsin K messenger RNA in giant cells and their precursors in human osteoarthritic synovial tissues. *Arthritis Rheum.* **42**: 1558–15593.
38. Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P. (1998) Expression of the elastinolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. *J. Clin. Invest.* **102**: 576–83.
39. Chambers TJ. (1978) Multinucleated giant cells. *J. Pathol.* **126**: 125–148.
40. Kadoya Y, al-Saffar N, Kobayashi A, Revell PA. (1994) The expression of osteoclast markers on foreign body giant cells. *Bone Miner.* **27**: 85–96.
41. Pandey R, Quinn J, Joyner C, Murray DW, Triffit JT, Athanasou NA. (1996) Arthroplasty implant biomaterial particle associated macrophages differentiate into lacunar bone resorbing cells. *Ann. Rheum. Dis.* **55**: 338–395.
42. Holtrop ME, Cox KA, Glowacky J. (1982) Cells of the mononuclear phagocytic system resorb implanted bone matrix: a histological and ultrastructural study. *Calcif. Tissue Int.* **34**: 488–494.
43. Garnero P, Borel O, Byrjalsen I, et al. (1998) The collagenolytic activity of cathepsin K is unique among mammalian proteinases. *J. Biol. Chem.* **272**: 32347–32352.
44. Mostov K, Werb Z. (1997) Journey across the osteoclast. *Science* **276**: 219–221.
45. Jean D, Hermann J, Rodrigues-Lima F, Barel M, Balbo M, Frade R. (1995) Identification on melanoma cells of p39, a cysteine proteinase that cleaves C3, the third component of complement: amino-acid-sequence identities with procathepsin L. *Biochem. J.* **312**: 961–969.