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Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues

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Our laboratory has previously demonstrated that increased malignancy of several histological types of human and animal tumours is associated with increases in their cathepsin B activity, particularly cathepsin B activity associated with plasma-membrane/endosomal vesicles or shed vesicles. Here we report that cathepsin B from normal or tumour tissues degrades purified extracellular-matrix components, type IV collagen, laminin and fibronectin, at both acid pH and neutral pH. The number and sizes of degradation products were analysed by SDS/PAGE. Cathepsin B from both sources exhibited similar activities towards, and similar patterns of cleavage of, the extracellular-matrix proteins. At neutral pH, cathepsin B from both sources appeared to undergo autodegradation, a process that was decreased in the presence of alternative substrates such as the extracellular-matrix proteins. Cathepsin B readily degraded type IV collagen at 25 °C, indicating activity towards native type IV collagen. Fibronectin degradation products of 100–200 kDa and of 18 and 22 kDa were observed. A single 70 kDa fragment was released from laminin under non-reducing conditions and multiple fragments ranging from 45 to 200 kDa under reducing conditions. These results suggest that cathepsin B at or near the surface of malignant tumour cells may play a functional role in the focal dissolution of extracellular matrices.

INTRODUCTION

Proteolytic enzymes of several classes have been implicated in the malignant progression of tumours (reviewed in refs. [1] and [2]), and endogenous inhibitors of metalloproteinases such as tissue inhibitors of metalloproteinases (TIMP) [3] and of cysteine proteinases such as stefin A [4] have been postulated to act as tumour suppressors or anti-oncogenes. Our laboratory among others has provided correlative evidence that the cysteine proteinase cathepsin B may be one component of a multiproteinase cascade associated with malignancy of both human and rodent tumours (reviewed in refs. [5] and [6]). Among the correlative evidence obtained in animal and human tumours of several histological types are the following observations: (1) parallel increases in expression of the cathepsin B gene and malignancy; (2) parallel increases in activity of cathepsin B and malignancy; (3) parallel increases in surface-association of cathepsin B and malignancy. The enhanced surface-association of cathepsin B in malignant tumours suggests that cathepsin B could play a degradative function as metastatic tumour cells move from one site in the body to another.

Cathepsin B, like other cysteine proteinases, is often termed a promiscuous proteinase or one with broad substrate-specificity (for a review see ref. [7]). Physiological substrates for cathepsin B are a matter of speculation. However, degradation of collagens and proteoglycans by cathepsin B has been shown to occur at acid pH [8,9]. The purpose of the present study was to determine the action of human cathepsin B at both acid and neutral pH on the major constituent of the basement membrane, type IV collagen, and on two adhesive glycoproteins, laminin and fibronectin, that have been implicated in attachment of metastatic tumour cells to extracellular matrices [10,11]. In an earlier study, we had demonstrated that cathepsin B from two human tumours degrades laminin to specific limited products [12]. In the present study we determine which of these products were released under non-reducing conditions and extend the studies to include degradation of laminin by cathepsin B from a normal human tissue, i.e. normal human liver. Degradation products were analysed by SDS/PAGE, immunoblotting and h.p.l.c. gel-filtration chromatography. The results indicate that human cathepsin B can degrade all three matrix proteins at both acid and neutral pH.

EXPERIMENTAL

Materials

L-3-Carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) and Tween-20 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Z-Arg-Arg-NH-Mec was purchased from Enzyme Systems Products (Dublin, CA, U.S.A.). Nitrocellulose membranes, peroxidase-conjugated goat anti-(rabbit IgG) IgG and goat anti-(mouse IgG) IgG, the Protein-A-based affinity kit, the Bio-Sil TSK-250 column and chromatographic supplies were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were of reagent grade and were obtained from commercial sources.

Tissues

Normal human liver tissue was obtained from cadavers less than 6 h *post mortem* and transported on ice to our laboratory, where it was frozen and stored at −20 °C before use. Tumour samples (human breast carcinoma) obtained from surgical resections were transported immediately to the Department of Pathology, where sections were frozen at −70 °C. Tumour sections were transferred to our laboratory where they were stored at −20 °C before use. If necessary, tumour specimens were pooled to provide an adequate amount of starting material for purification.

Abbreviations used: E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; EHS tumour, Englebreth–Holm–Swarm tumour; TIMP, tissue inhibitors of metalloproteinases; Z-Arg-Arg-NH-Mec, benzyloxycarbonylarginylarginine 4-methylcoumarin-7-ylamide.

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Cathepsin B

Cathepsin B from normal human liver or human breast carcinoma was purified to homogeneity by a described [12] modification of the Willenbrock & Brocklehurst procedure [13]. Cathepsin B was stored at −20 °C in concentrated portions (1 mg/ml) in 20 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA. Alternatively, normal human liver cathepsin B (lot 801255) purified by the method of Rich *et al.* [14] was obtained from Calbiochem (La Jolla, CA, U.S.A.) and stored at −20 °C in concentrated portions (25 μg/100 μl) in 50 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA, 0.1 mM-2,2-dipyridyl disulphide and 50% (v/v) glycerol. The commercial cathepsin B was dialysed before use in buffer appropriate to the experimental protocol for which it was being used. Purity of both preparations of cathepsin B was verified before use on silver-stained SDS/PAGE gels and by immunoblotting analysis with a monospecific rabbit antibody to human liver cathepsin B. No differences were observed in degradative activities between the commercially obtained cathepsin B and the cathepsin B purified in our own laboratory.

Cathepsin B assay

Before use in degradation assays, the activity of cathepsin B (EC 3.4.22.1) was measured against a selective substrate, Z-Arg-Arg-NH-Mec, by a stopped assay under the conditions previously described [15]. The molar concentration of cathepsin B was determined by active-site titration with E-64 [16]. The titrations were performed by preincubating the enzyme with activator (10 mM-dithiothreitol/5 mM-EDTA, pH 5.2) for 10 min and then adding E-64 for 30 min at 37 °C before adding the Z-Arg-Arg-NH-Mec substrate.

Extracellular matrix proteins

Laminin was purified by neutral salt extraction and size exclusion chromatography from the Englebreth–Holm–Swarm (EHS) tumour as described previously [11]. Type IV collagen was extracted in guanidine hydrochloride/dithiothreitol from EHS tumour grown in lathyritic mice according to a modification of the method by Kleinman *et al.* [17] as previously reported [18]. Type IV collagen purified by DEAE-cellulose anion-exchange chromatography was subjected to centrifugation at 110000 *g* for 90 min to clear aggregates greater than 50 S. The supernatant was decanted and stored at 4 °C until use. The concentration of type IV collagen was determined spectrophotometrically by using absorbance values at wavelengths of 215 nm and 225 nm for calculation [19]. Human plasma fibronectin was purified by sequential ion-exchange and gelatin affinity chromatography as described previously [11]. The purity and subunit integrity of all three matrix proteins was assessed by SDS/PAGE and staining with Coomassie Blue.

Rabbit polyclonal anti-fibronectin was prepared from pooled immune sera by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography and affinity chromatography over fibronectin affinity columns as previously described [11]. Monoclonal antibodies were generated in Balb/C mice against a 33 kDa *C*-terminal A-chain-derived heparin-binding fragment purified from tryptic/catheptic digests of intact human plasma fibronectin as described previously [20]. Immunoblot analysis [20,21] of these antibodies against A-chain- and B-chain-derived *C*-terminal heparin-binding fragments demonstrated that antibody AHB-3 recognizes only the A-chain-derived fragment. AHB-3 was purified from ascites by using a Protein-A-based affinity kit and the protocol supplied by the manufacturer. Both polyclonal and monoclonal antibodies were divided into small portions and stored in phosphate-buffered saline at −80 °C before use.

Digestion of extracellular matrix proteins

Before use, cathepsin B was activated by incubation in the presence of 10 mM-dithiothreitol/5 mM-EDTA, pH 5.2, for 10 min at 37 °C. Laminin, fibronectin or type IV collagen was dialysed against either 50 mM-sodium phosphate buffer, pH 6.5 or 7.4, containing 1 mM-EDTA or 100 mM-sodium phosphate buffer, pH 5.0, containing 1 mM-EDTA and then incubated with cathepsin B at an enzyme/substrate ratio of 1:10 or 1:100 (w/w). The time course of degradation was monitored for periods up to 12 h at 37 °C for laminin and fibronectin. The incubation with type IV collagen was carried out at 25 °C in order to assess degradation of native type IV collagen. The pH was monitored to ensure that it was maintained during the course of the incubation. The reactions were stopped by adding either a 100-fold molar excess of E-64 or SDS/PAGE sample buffer, depending on the assay system. Controls were run in the absence of cathepsin B or with cathepsin B irreversibly inactivated by preincubation with a 100-fold molar excess of E-64 for 30 min at 37 °C before the addition of substrate.

At timed intervals, samples from incubation mixtures of laminin and cathepsin B were subjected to gel-filtration chromatography on a Bio-Sil TSK-250 column (300 mm × 7.5 mm) under non-reducing and non-denaturing conditions. These samples were run at a flow rate of 1 ml/min in 50 mM-sodium phosphate/150 mM-sodium sulphate buffer, pH 6.8.

SDS/PAGE, immunoblotting and *N*-terminal sequence analysis

Slab-gel electrophoresis was carried out in 4–18% linear gradient polyacrylamide gels in the presence of SDS as described by Laemmli [22]. Gels were silver-stained according to the method of Merril *et al.* [23]. For immunoblotting, proteins separated by SDS/PAGE were transferred electrophoretically from unstained gels to nitrocellulose membranes by using a Bio-Rad Trans-Blot apparatus. Incubation of primary and secondary antibodies with the nitrocellulose membranes was essentially as recommended by Bio-Rad Laboratories. Antibody–antigen complexes were detected with peroxidase-conjugated goat anti-(mouse IgG) IgG or goat anti-(rabbit IgG) IgG. For *N*-terminal sequencing studies, proteins separated by SDS/PAGE were transferred electrophoretically from unstained gels to Immobilon-P nylon membrane (Millipore, Bedford, MA, U.S.A.) and the membrane was stained with Coomassie Brilliant Blue according to Millipore's procedure. *N*-Terminal sequencing of the stained proteins on the membrane was performed by Dr. J. Leykam of the Macromolecular Structure Facility, Michigan State University, East Lansing, MI, U.S.A.

Determination of protein

Protein concentrations were determined by using a modified Bradford procedure [24], with BSA as standard. During the course of purification of either enzyme or degradation products, protein concentrations were determined by measurement of the absorbance at 280 nm.

RESULTS

Degradation of fibronectin

Human liver cathepsin B (Figs. 1 and 2) and human breast carcinoma cathepsin B (Fig. 2) degraded both bands of human plasma fibronectin readily at an enzyme/substrate ratio of 1:10. This degradation was both pH- and time-dependent, occurring almost to completion within 1 h at pH 5.0 (Fig. 1, lane B) and to lesser degrees within 1 h at pH 6.5 (results not shown) or pH 7.4 (Fig. 1, lane D). The degradation of fibronectin by cathepsin B resulted in a series of distinct products ranging from 100 to

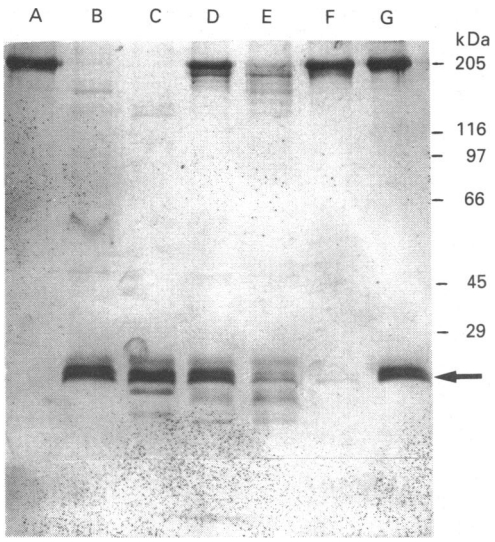

Fig. 1. Digestion of fibronectin by human liver cathepsin B

Lane A, control, 20 μg of fibronectin incubated for 12 h at 37 °C, pH 7.4; lane B, 20 μg of fibronectin incubated with 2 μg of cathepsin B for 1 h at 37 °C, pH 5.0; lane C, same conditions as in lane B incubated for 12 h; lane D, 20 μg of fibronectin incubated with 2 μg of cathepsin B for 1 h at 37 °C, pH 7.4; lane E, same conditions as in lane D incubated for 12 h; lane F, 20 μg of fibronectin incubated with 0.2 μg of cathepsin B for 12 h at 37 °C, pH 6.5; lane G, control, 20 μg of fibronectin incubated for 12 h at 37 °C, pH 7.4, with 2 μg of cathepsin B that had been pretreated with a 100-fold excess (mol/mol) of E-64 inhibitor. Protein molecular-mass standards migrated as indicated. The digestion products were analysed by electrophoresis on 4–18% gradient SDS/polyacrylamide gels under reducing conditions. Under these conditions, the heavy chain (25 kDa) of cathepsin B migrated as a doublet visible at the arrow.

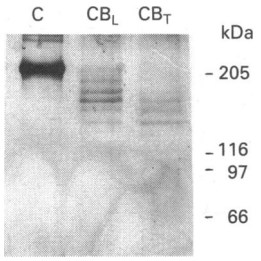

Fig. 2. Degradation of fibronectin by cathepsin B from normal and tumour tissues

Fibronectin (20 μg) was incubated with cathepsin B (2 μg) for 12 h at 37 °C, pH 7.4. Lanes from left to right represent fibronectin control (C), incubation with human liver cathepsin B (CB_L) and incubation with human breast carcinoma cathepsin B (CB_T). See the legend to Fig. 1 for details.

200 kDa and two smaller products of 18 and 22 kDa. The extent of cleavage was less at higher pH, yet the pattern of cleavage was not affected by the pH of the incubation buffer or the tissue source of cathepsin B. Similar patterns of cleavage were also observed after incubation of fibronectin with either the lysosomal or the plasma-membrane/endosomal-associated forms of cathepsin B that we have shown to be present in tumour cells (results not shown). At an enzyme/substrate ratio of 1:100, degradation of fibronectin was substantially slower than at an

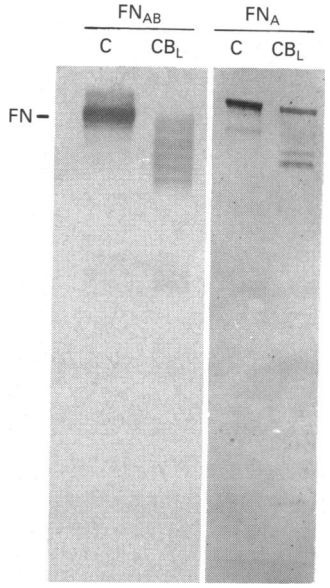

Fig. 3. Immunoblotting analysis of cathepsin B digests of fibronectin

The antibodies used were a polyclonal that recognizes both the A-chain and B-chain of fibronectin (FN_{AB}) and a monoclonal (AHB-3) that recognizes only the A-chain of fibronectin (FN_A). Fibronectin (20 μg) was incubated either alone (C) or with human liver cathepsin B (2 μg; CB_L) for 1 h at 37 °C, pH 5.0. See the Materials and methods section and the legend to Fig. 1 for details.

enzyme to substrate ratio of 1:10 (Fig. 1, lane F). However, under these conditions we were able to observe that the initial fibronectin product appears to run immediately below intact fibronectin (at approx. 200 kDa; Fig. 1, lane F). Two cysteine-proteinase inhibitors, E-64 and stefin A, were tested for their ability to inhibit degradation of fibronectin by cathepsin B. E-64 is a synthetic irreversible inhibitor used for active-site titration of cysteine proteinases [7,16], whereas stefin A, a member of the cystatin superfamily of endogenous low-molecular-mass protein inhibitors of the cysteine proteinases, has been classified as a pseudo-irreversible inhibitor (for a review see ref. [25]). Degradation of fibronectin by cathepsin B could be blocked by either cysteine-proteinase inhibitor (E-64, Fig. 1, lane G; stefin A, results not shown).

The fibronectin fragments produced by incubation with cathepsin B were analysed by immunoblotting with antibodies that recognize both the A-chain and B-chain of fibronectin or only the A-chain [20,21]. The initial cleavage product of 200 kDa could be observed with either the AHB-3 monoclonal antibody specific for the A-chain of fibronectin or the polyclonal antibody that recognizes both chains of fibronectin (Fig. 3). Two additional high-molecular-mass fragments also appear to come from digestion of the A-chain of fibronectin by cathepsin B. The remaining fragments ranging from 140 to 200 kDa may be either from the B-chain of fibronectin or fragments from the A-chain that do not contain the epitope recognized by the AHB-3 monoclonal antibody.

Degradation of type IV collagen

Soluble native EHS-tumour type IV collagen was degraded by human liver (Fig. 4) and breast carcinoma cathepsin B (results not shown) at 25 °C and an enzyme/substrate ratio of 1:10. This degradation was pH-dependent, occurring more rapidly at pH 5.0 (Fig. 4, lane B) than at pH 6.5 (results not shown) or pH 7.4 (Fig. 4, lane C). Degradation of type IV collagen at all three pH values was concentration-dependent, occurring more rapidly at an

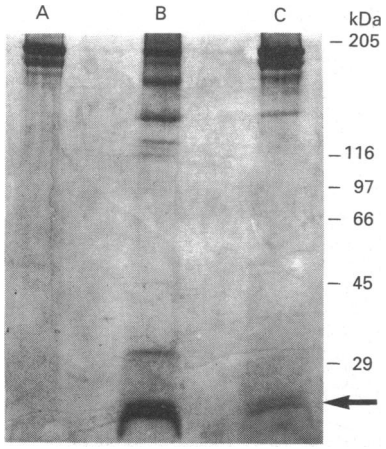

Fig. 4. Effect of pH on degradation of type IV collagen by human liver cathepsin B

Lane A, control, 20 μg of type IV collagen incubated for 12 h at 25 °C, pH 7.4; lane B, 20 μg of type IV collagen incubated with 2 μg of cathepsin B for 12 h at 25 °C, pH 5.0; lane C, 20 μg of type IV collagen incubated with 2 μg of cathepsin B for 12 h at 25 °C, pH 7.4. Protein molecular-mass standards migrated as indicated. Under these conditions, the heavy chain (25 kDa) of cathepsin B migrated as a doublet visible at the arrow. See the legend to Fig. 1 for details.

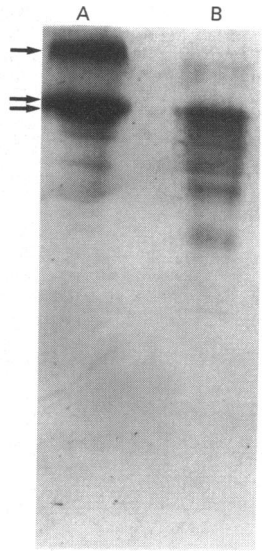

Fig. 6. Degradation of laminin by human breast carcinoma cathepsin B

Lane A, control, 25 μg of laminin incubated for 12 h at 37 °C, pH 6.5; lane B, 25 μg of laminin incubated with 2.5 μg of cathepsin B for 12 h at 37 °C, pH 6.5. Single and double arrows depict the positions of the A-chain and B-chain of laminin respectively.

enzyme/substrate ratio of 1:10 (Fig. 4) than of 1:100 (results not shown). The degradation of type IV collagen by cathepsin B resulted in major cleavage products of 160, 140, 120, 110 and 35 kDa. The amount of these products formed increased up to 24 h of incubation. Over the next 12 h (i.e. 36 h of incubation), these products underwent further degradation (results not shown). This pattern of cleavage was not affected by the tissue source of cathepsin B or the pH of the incubation buffer. The degradation of type IV collagen by cathepsin B at pH values of 5.0, 6.5 and 7.4 was blocked by the cysteine-proteinase inhibitor E-64 (results not shown).

Degradation of laminin

EHS-tumour laminin was degraded by both human breast carcinoma cathepsin B (Figs. 5 and 6) and human liver cathepsin B (Fig. 7) at an enzyme/substrate ratio of 1:10. Degradation products of laminin were analysed by h.p.l.c. gel-filtration chromatography (Figs. 5*a* and 5*b*), SDS/PAGE (Figs. 6 and 7), or by sequential h.p.l.c. gel-filtration chromatography and SDS/PAGE (Fig. 5*c*). The elution profiles from the gel-filtration column indicated that at pH 6.5 approximately 70% of the laminin was degraded over a 12 h incubation period (compare Fig. 5*a* with Fig. 5*b*). The elution profile consisted of a peak that was eluted at the same position as intact laminin (LM in Fig. 5*b*) and a second, single peak that was eluted later (LM-70 in Fig. 5*b*). Electrophoretic analysis (4–18% gradient SDS/polyacrylamide gel under reducing conditions) of the LM-70 peak demonstrated that it consisted of a single laminin fragment of 70 kDa (Fig. 5*c*), whereas analysis of the peak that was eluted at the same position as intact laminin (LM in Fig. 5*b*) demonstrated that this peak consisted of laminin fragments ranging in size

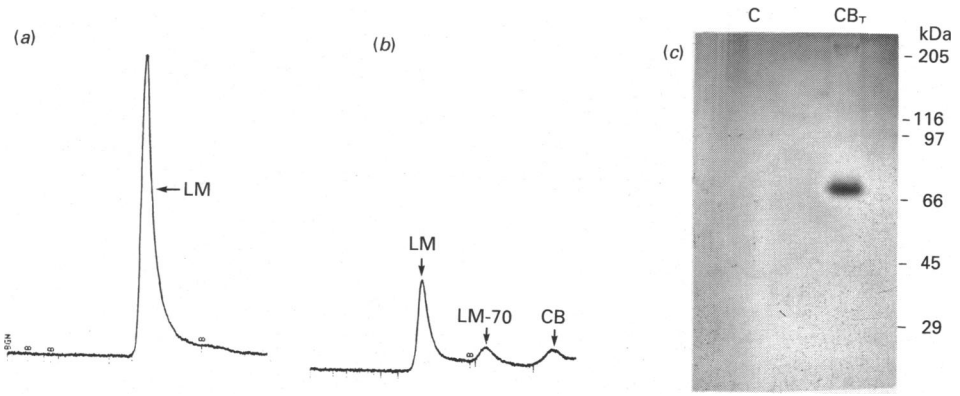

Fig. 5. Degradation of laminin by human breast carcinoma cathepsin B

(*a*) and (*b*) Elution profiles on a TSK-250 gel-filtration column of laminin incubated alone (*a*) or with human breast carcinoma cathepsin B at an enzyme/substrate ratio of 1:10 (*b*) for 12 h at 37 °C, pH 6.5. Laminin (LM), a laminin fragment (LM-70) and cathepsin B (CB) were eluted as indicated. (*c*) Electrophoretic analysis (4–18% gradient SDS/polyacrylamide gel under reducing conditions) of the fractions eluted from the TSK-250 gel-filtration column at 9 min. The lanes represent the 9 min fractions collected from the control incubation in the absence (C) and in the presence of cathepsin B (CB_T).

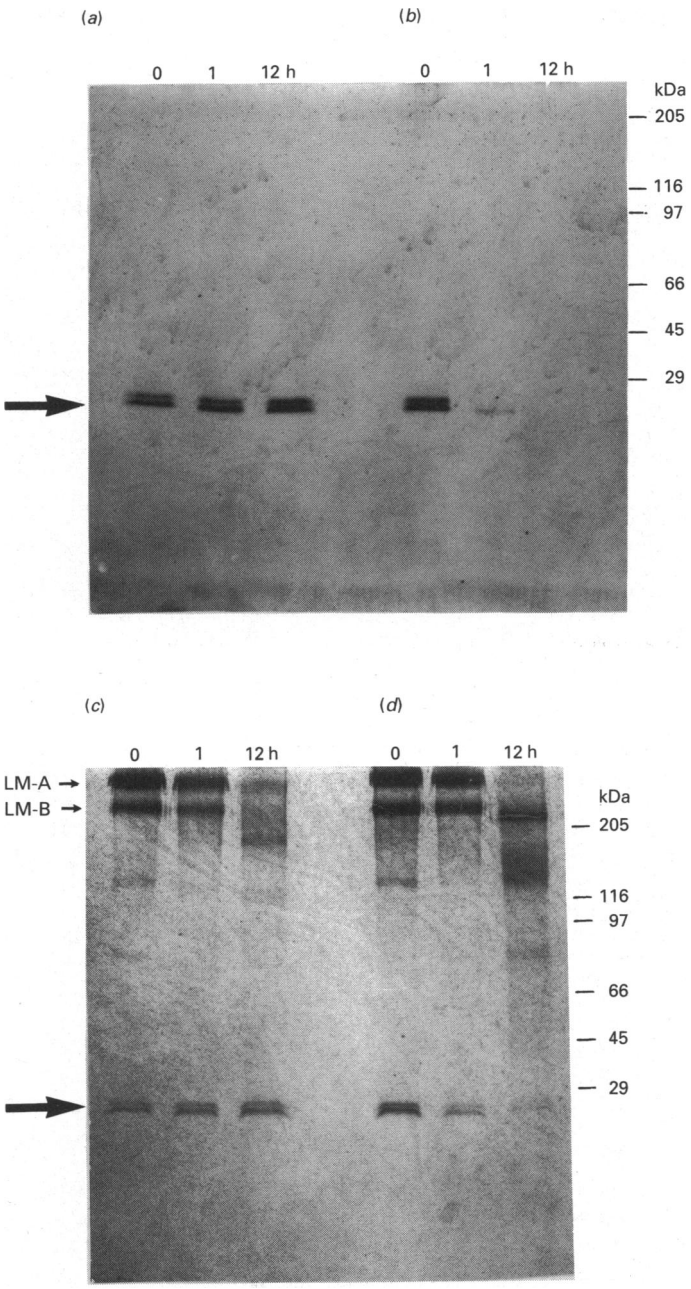

Fig. 7. Stabilization of cathepsin B in the presence of laminin

Human liver cathepsin B (1 μg) was incubated in the absence (*a* and *b*) or in the presence (*c* and *d*) of 20 μg of laminin at 37 °C for 0, 1 or 12 h at either pH 5.0 (*a* and *c*) or 7.4 (*b* and *d*). After incubation the samples were analysed by electrophoresis on 4–18% gradient SDS/polyacrylamide gels under reducing conditions. Under these conditions, laminin migrated as two chains, denoted by LM-A and LM-B, and the heavy chain (25 kDa) of cathepsin B migrated as a doublet visible at the arrow.

(*a*) Gln-Glu-Pro-Glu-Phe-Ser-Tyr-Gly-Cys-Ala-Glu-Gly-Ser-Cys-Tyr

(*b*) Ala-Glu-Gly-Ser-Cys-Tyr

Fig. 8. Comparison of (*a*) the predicted *N*-terminal sequence of the β1-chain of mouse laminin based on cDNA with (*b*) the *N*-terminal sequence of the 70 kDa fragment generated from mouse laminin by incubation with cathepsin B

from 45 to 200 kDa (results not shown; see also Figs. 6 and 7). Thus, under non-reducing conditions incubation of laminin with cathepsin B resulted in release of a single fragment of 70 kDa. *N*-Terminal sequence analysis suggests that the 70 kDa fragment was generated from the β1-chain of laminin (Fig. 8).

Although cathepsin B degraded both the 400 and 220/230 kDa bands of laminin, the A-chain (400 kDa band) appeared to be degraded more readily than the B-chains (Fig. 6). Degradation of laminin was both pH- and time-dependent, occurring more rapidly at pH 5.0 than at pH 7.4 (compare Fig. 7*c* with Fig. 7*d*). The degradation products formed from laminin by cathepsin B ran under reducing conditions as a diffuse smear over the range of 45–200 kDa with major bands at 140, 120, 100 and 70 kDa (Figs. 6 and 7). The pattern of cleavage was not affected by the tissue source of cathepsin B over time courses from 8 to 24 h (see ref. [12]). The extent of cleavage of laminin by cathepsin B was lower at higher pH. Thus in Fig. 7 the patterns of cleavage at the two pH values appear to differ. However, over an extended incubation at pH 7.4, the final cleavage products appeared to be identical with those present at pH 5.0. Degradation of laminin by cathepsin B at pH values of 5.0, 6.5 and 7.4 was blocked by the cysteine-proteinase inhibitors E-64 and stefin A (results not shown).

Autodegradation of cathepsin B

At neutral pH, cathepsin B underwent an apparent autodegradation (Fig. 7*b*). Autodegradation was not observed at pH 5.0 for incubation periods up to 12 h (Fig. 7*a*). In contrast, degradation of extracellular matrix proteins by cathepsin B occurred most rapidly at acid pH. Autodegradation like the degradation of extracellular-matrix proteins was time-dependent. The autodegradative process occurred more slowly if the incubation of cathepsin B at neutral pH was carried out in the presence of an alternative protein substrate such as laminin (Fig. 7*d*), fibronectin (Fig. 1, lane E) or type IV collagen (Fig. 4, lane C). Cathepsin B from both normal and tumour tissues underwent autodegradation at neutral pH. Autodegradation of cathepsin B was inhibited by the cysteine-proteinase inhibitor E-64 (Fig. 1, lane G). Cathepsin B products resulting from autodegradation ran as a smear below the heavy chain doublet. Their identity as cathepsin B products was confirmed by immunoblotting with a monospecific polyclonal antibody to human liver cathepsin B (results not shown).

DISCUSSION

Previous studies have established that cathepsin B from several normal tissues only degrades extracellular-matrix proteins at low pH [8,9,26]. Such observations suggested that cathepsin B might have activity against only the denatured forms of these proteins. We had shown that cathepsin B from human tumours (colon and breast) can degrade both the A- and B-chains of laminin at pH 7.0 [12]. This raised the possibility that cathepsin B from tumour tissues would have activity at a higher pH than cathepsin B from normal tissues. However, in the present study we have established that cathepsin B from both normal and tumour tissues can degrade three extracellular-matrix proteins, the adhesive glycoproteins laminin and fibronectin and the structural protein type IV collagen, at pH 7.4. Similar findings for the pH range of cathepsin B from normal tissue have been reported recently by Maciewicz *et al.* [27]. In that study human liver cathepsin B is shown to degrade the non-helical domains of several cartilage collagens (II, IX, XI) at pH 7.0.

The cleavage patterns for degradation of type IV collagen by cathepsin B indicate a specific, sequential proteolysis and the fragments produced differ from those produced by metallo-

proteinases [28,29]. Cathepsin B was able to degrade both the proteinase-resistant A-chain of laminin and the proteinase-labile B-chains of laminin [30,31]. Historically, cathepsin B has been classified as an endopeptidase and shown by many different investigators to possess activity against protein substrates (for a review see ref. [7]). Recently this has become an area of controversy as Tang and co-workers [32] have suggested that pig spleen cathepsin B has only exopeptidase activity. In contrast, Mason [33] established that cathepsin B from pig liver as well as from human or ox liver is an endopeptidase. The cleavage patterns that we observe after incubation of cathepsin B with fibronectin, type IV collagen and laminin indicate that in our hands cathepsin B has endopeptidase activity. At least two other recent studies also have found that cathepsin B has endopeptidase activity against extracellular-matrix proteins [26,27].

Cathepsin B had been shown previously to lose activity at or above neutral pH (see, e.g., refs. [7] and [34]). This loss of activity was presumed to be due to denaturation of the cathepsin B, although the published studies illustrating this loss in activity only establish that activity of cathepsin B against small synthetic substrates is decreased by incubation at pH values $\geqslant 7.0$. In the present study, we establish that on incubation at pH 7.4 a loss of activity of cathepsin B against a small synthetic substrate occurred concomitantly with an apparent autodegradation of cathepsin B. Thus our study suggests that autodegradation of cathepsin B at neutral pH may be responsible for the decrease in the activity of cathepsin B at neutral pH. Our study does not address the nature of the autodegradative process and it is possible that denaturation of a portion of the cathepsin B molecules may lead to the autodegradation of cathepsin B seen in the present study.

An extensive literature (for reviews see refs. [5], [6], [35] and [36]) is available illustrating that cathepsin B is released from both human and animal tumours and is present in small membrane vesicles shed by mouse 15091A mammary adenocarcinoma cells and in plasma-membrane/endosomal fractions isolated from rodent and human tumours. This literature suggests that cathepsin B is present at or near the tumour-cell surface and thus available to play a direct role in dissolution of the basement membrane by invading tumour cells. Findings in the present study that support a potential extracellular role for cathepsin B are (1) the ability of cathepsin B to degrade three extracellular-matrix proteins, i.e. type IV collagen, laminin and fibronectin, (2) the ability of cathepsin B to perform this degradation at neutral as well as acid pH, (3) the extended time course over which cathepsin B was able to degrade type IV collagen, laminin and fibronectin at neutral pH and (4) the ability of type IV collagen, laminin and fibronectin to extend this time course by decreasing the autodegradation of cathepsin B observed at neutral pH. These findings suggest that cathepsin B at or near the tumour-cell surface could participate in the proteolytic cascade thought to be responsible for the focal dissolution of extracellular matrices observed adjacent to invading tumour cells (for recent reviews see refs. [1] and [2]).

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