A cysteine proteinase secreted from human breast tumours is immunologically related to cathepsin B

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The stable cathepsin B-like cysteine (thiol) proteinase secreted from human breast tumours in culture was shown to be destabilized by mercurial compounds. After such treatment the enzyme cross-reacts in a radioimmunoassay with a monospecific antiserum to human liver cathepsin B. It is suggested that the secreted enzyme may be a precursor form of lysosomal cathepsin B.

Proteolytic enzymes have long been suspected to play a role in tumour invasiveness and metastasis. The secretion of several proteinases such as collagenases, plasminogen activators and lysosomal proteinases from tumour tissues and transformed cells has been reported (for review see Sylvén, 1974; Quigley, 1979; Recklies & Poole, 1982). Human breast-tumour explants maintained in organ culture have been shown to secrete selectively a cysteine proteinase that is enzymically similar to lysosomal cathepsin B (Poole *et al.*, 1978; Recklies *et al.*, 1980). The enzyme shows essentially identical affinities for synthetic substrates and is sensitive to the same range of inhibitors (Mort *et al.*, 1980). However, the secreted enzyme is stable in that it retains its activity when subjected to neutral or alkaline pH. Lysosomal cathepsin B, on the other hand, is irreversibly denatured under these conditions (Barrett, 1973; Mort *et al.*, 1980). The secreted enzyme also has a higher molecular weight than that of lysosomal cathepsin B and a different isoelectric-focusing pattern (Mort *et al.*, 1980). Because of these differences in physical properties between cathepsin B and the secreted thiol proteinase, the question arose as to whether the two proteinases were related or whether the secreted enzyme was a different species. This problem could be approached immunochemically once it was found that the tumour proteinase could be rendered similar to cathepsin B with respect to alkaline stability after treatment with mercurials.

Abbreviations used: Cbz-, α -N-benzyloxycarbonyl-; Mes, 4-morpholine-ethanesulphonic acid.

Experimental

Materials

Cbz-Arg-Arg 4-methoxynaphthylamide was purchased from Enzyme Systems Products (Livermore, CA, U.S.A.). Ultrogel AcA-54 was from LKB (Fisher Scientific, Montreal, Que., Canada). *Staphylococcus aureus* was obtained from The Enzyme Center, Boston, MA, U.S.A. Human liver cathepsin B was purified essentially by the procedure of Barrett (1973), and a monospecific antiserum was raised with this preparation in a sheep (Mort *et al.*, 1981). Tumour medium containing cysteine proteinase activity was collected from explants of human adenocarcinomas of the breast as described previously (Recklies *et al.*, 1980) and stored frozen until use.

Determination of alkaline stability

Pooled tumour medium was dialysed against 200 vol. of 200 mM-NaCl/20 mM-Mes/NaOH buffer, pH 7.0. For partial purification pooled tumour medium was chromatographed on organomercurial-Sepharose as described previously (Recklies *et al.*, 1982). Bound material was eluted with 10 mM-cysteine in 200 mM-NaCl/1 mM-EDTA/50 mM-sodium acetate buffer, pH 5.5. Cysteine was removed from the combined active fractions by dialysis against the elution buffer without cysteine. All proteinase samples were diluted with 0.1% bovine serum albumin in water to give approximately the same amount of activity when assayed with Cbz-Arg-Arg 4-methoxynaphthylamide as substrate (25–30 nmol of 4-methoxynaphthylamine released/h per 50 μ l sample).

Alkaline stability was determined by incubating 50 μ l portions of the diluted enzyme preparations in 25 mM-Tris/HCl (final concentration) buffer, pH 8.0, in a sample volume of 200 μ l. After a 30 min incubation at 40°C, 600 μ l of assay buffer (1.33 mM-EDTA/2.67 mM-cysteine/67 mM-Mes/NaOH buffer, pH 5.5) was added, followed by 20 μ l of Cbz-Arg-Arg 4-methoxynaphthylamide (20.5 mM in 5% dimethylformamide). Incubation with substrate was for 60 min at 40°C, whereupon 4-methoxynaphthylamine released was determined as described by Barrett (1976). To destabilize the cysteine proteinase activity, 25 μ l of dialysed tumour medium was incubated with 25 μ l of either water or 1% dimethyl sulphoxide (controls) or with 25 μ l of 1 mM-4-aminophenylmercuric acetate in 1% dimethyl sulphoxide or in 1 mM-HgCl₂ in water. After preincubation for 15 min at 22°C, samples were adjusted to pH 8.0 by addition of 50 μ l of 0.1 M-Tris/HCl buffer, pH 8.0, and 100 μ l of water. Total activity was determined by adding the pH 8.0 buffer after addition of the assay buffer such that samples were not exposed to pH 8.0.

Radioimmunoassay of human cathepsin B

The radioimmunoassay method for human cathepsin B has been described previously (Recklies & Mort, 1982). The sheep antiserum used in this assay has been demonstrated to be monospecific for human cathepsin B (Mort *et al.*, 1981; Recklies & Mort, 1982). Samples to be assayed were incubated with appropriately diluted anti-(cathepsin B) serum (0.3 μ g of immunoglobulin G/tube) and ¹²⁵I-labelled cathepsin B (1–1.5 ng/tube) in a final assay volume of 220 μ l in 200 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0. Rabbit anti-(sheep Fc) serum was used as a second-step antibody to aid separation of bound from free antigen by adsorption on protein A-bearing *Staphylococcus aureus*. The range of the radioimmunoassay was between 1 and 50 ng of unlabelled cathepsin B standard/tube.

Results and discussion

Antisera raised to cathepsin B tend to react only with the alkali-denatured enzyme (Barrett, 1973; Mort *et al.*, 1981). Previously a monospecific antiserum to cathepsin B isolated from human liver, which also only reacts with the alkali-denatured enzyme, has been prepared in this laboratory (Mort *et al.*, 1981). For immunochemical characterization a radioimmunoassay method was developed (Recklies & Mort, 1982) with assay conditions that allow alkali denaturation of lysosomal cathepsin B. The secreted stable cysteine proteinase does not compete very effectively in this assay, nor does it bind to anti-(cathepsin B) antibody immobilized on Sepharose 4B (result not shown). However, since undenatured lysosomal cathepsin B does not react with the antiserum either, these observations did not rule out any immunological cross-reactivity between the two cysteine proteinases.

On attempting to purify the secreted tumour proteinase by chromatographic procedures similar to those used for the purification of human liver cathepsin B (Barrett, 1973), alkaline stability was lost. Thus, whereas the enzyme from untreated medium is stable to incubation at pH 8.0 and 40°C, enzyme partially purified by organomercurial-Sepharose affinity chromatography is substantially less stable (Table 1). The destabilization was also induced by either the ligand used in the affinity gel (4-aminophenylmercuric acetate) or HgCl₂, indicating that the Hg²⁺ ion is the destabilizing agent. Destabilization with HgCl₂ proceeded very rapidly at room temperature: about 50% of the initial stable activity was converted into the unstable form within 5 min in the presence of 0.5 mM-HgCl₂, and less than 10% remained stable after 15 min exposure to HgCl₂ (Table 1).

Since organomercurials are known to inhibit cathepsin B activity, concentrations were chosen such that in the presence of EDTA and cysteine no inhibition was detected in the final activity deter-

Table 1. Conversion of secreted cysteine proteinase from a stable into an unstable form

Alkaline stability was determined by incubating proteinase samples at pH 8.0 and 40°C for 30 min, followed by determination of residual activity as described in the text. For destabilization, tumour medium was incubated with the indicated reagents for 15 min at room temperature. Alkaline stability and residual cathepsin B activity were determined as indicated above.

	Addition during preincubation	Activity stable to treatment
		at pH 8.0 (% of initial activity)
Tumour medium	—	92
Human liver cathepsin B	—	2.5
Tumour enzyme after organomercurial-Sepharose chromatography	—	32
Tumour medium	0.5% Me ₂ SO	90.8
Tumour medium	0.5 mM-H ₂ NPhHgOAc	23.0
Tumour medium	0.5 mM-HgCl ₂	8.5

mination. These compounds render the secreted proteinase unstable to alkaline pH without causing any loss of enzyme activity, provided that the pH is maintained below neutrality. Neither organomercurial compounds nor HgCl_2 affect the alkaline instability of lysosomal cathepsin B. Enzyme purified without the use of organomercurial-Sepharose affinity chromatography is as unstable to alkaline pH (Otto, 1971; Franklin & Mettrione, 1972) as are preparations obtained by using this purification step (Barrett, 1973; Mort *et al.*, 1980). Furthermore, cathepsin B activity in crude liver homogenates is equally unstable to alkaline pH. Under the conditions used for alkaline denaturation (pH 8.0, 40°C), cathepsin B activity in both crude liver homogenates and purified preparations decays with a half-life of 3–4 min.

The finding that alkaline stability of the secreted proteinase could be manipulated experimentally allowed the investigation of cross-reaction with the antiserum to lysosomal cathepsin B. The proteinase was partially purified from concentrated tumour medium and destabilized by organomercurial-Sepharose chromatography. Analysis of the eluted material by gel filtration revealed a peak of enzyme activity that cross-reacted with the anti-(human cathepsin B) serum, as shown in Fig. 1. This peak coincided with the cathepsin B-like activity measured with the synthetic substrate Cbz-Arg-Arg 4-methoxynaphthylamide (McDonald & Ellis, 1976). Human liver cathepsin B, which was chromatographed in a separate run and assayed enzymically, was eluted at a more retarded position compared with the tumour enzyme, demonstrating the larger molecular size of the secreted cysteine proteinase. A slight shoulder of the immunoreactive material in the tumour preparation is evident on the trailing edge of the peak. This is probably due to some smaller denatured lysosomal cathepsin B present in the original tumour medium, since there was no shoulder for enzyme activity.

Thus treatment of the secreted cysteine proteinase with Hg^{2+} ion or organomercurials renders it similar to lysosomal cathepsin B with respect to its stability at neutral or alkaline pH and immunoreactivity with antibody to denatured cathepsin B. These findings provide evidence that the thiol proteinase secreted from human malignant breast tumours is immunologically related to lysosomal cathepsin B. The rapid conversion from a stable into an unstable form by mercurials suggests that this transformation is achieved by a non-enzymic mechanism, perhaps a conformational change. Preliminary findings indicate that this destabilization of the secreted enzyme is not accompanied by a change in molecular weight.

The modification of proteinase properties by mercurials has been described for other systems. Mammalian collagenase, which is secreted mainly as

Fig. 1. Demonstration of cross-reaction of human tumour cysteine proteinase with antibody to cathepsin B

Culture medium partially purified by organomercurial-Sepharose chromatography was dialysed against 200 mM-NaCl/20 mM-Mes/NaOH buffer, pH 6.0, and applied to an Ultrogel AcA-54 gel-filtration column (85 cm $\times$ 1.5 cm). Elution was at 11 ml/h with the same buffer as above. Fractions were analysed by radioimmunoassay for cathepsin B (○) and for cathepsin B activity with Cbz-Arg-Arg 4-methoxynaphthylamide as substrate (●). The column was calibrated with purified human liver cathepsin B (□) and a mixture of ovalbumin (M_r 43 000), chymotrypsinogen (M_r 25 700) and ribonuclease A (M_r 13 700). Cross-reactive material is shown in cathepsin B equivalents (ng/ml sample); cathepsin B activity material is shown in cathepsin B equivalents (ng/ml of sample); cathepsin B activity is expressed as a percentage of the maximal activity to allow comparison of the elution positions of the tumour cysteine proteinase and human liver cathepsin B.

a latent enzyme, is activated by organomercurials (Lazarus *et al.*, 1972; Sellers *et al.*, 1977), and similar observations have been made with secreted neutral metallo-proteinases (Sellers *et al.*, 1978). The exact mechanism of action of mercurials in these systems is still controversial, and it remains to be determined whether susceptibility to mercurial compounds is a general property of secreted proteinases. The larger molecular weight of the abnormally secreted tumour enzyme compared with that of lysosomal cathepsin B suggests that the secreted form might be a precursor of the lysosomal enzyme.

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