Immunolocalisation of cathepsin D in normal and neoplastic human tissues

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SUMMARY The aspartic proteinase cathepsin D was purified from human spleen and localised in various formalin fixed paraffin embedded human tissues using the peroxidase-antiperoxidase (PAP) technique. Cathepsin D was shown not only in macrophages but also in other connective tissue cells, and in epithelium. It was present in spleen (littoral cells and cells within Malpighian bodies), liver (hepatocytes and Kupffer cells), lung (alveolar macrophages and bronchial epithelium), brain (neurones), lymph nodes (histiocytes in germinal centres, sinusoid lining cells) and stomach (parietal and mucous neck cells). Cathepsin D was also found in carcinomas of bronchus, stomach, colon, kidney, breast, ovary, bladder and pancreas, both in neoplastic epithelium and in stromal cells, but was seldom present in connective tissue neoplasms. A group of malignant lymphomas also contained the enzyme within scattered cells. The distribution of cathepsin D seems to be much wider than that of the structurally related aspartic proteinases pepsin, gastricsin, and renin.

Cathepsin D is a proteolytic enzyme that belongs to a family known as aspartic proteinases.¹ Many homologies in amino acid sequence have been shown to exist among the members of this group of enzymes,^{2,3} which includes pepsin, gastricsin, and renin. Like the other enzymes, cathepsin D has been found to be synthesised in the form of a precursor.^{2,4} The enzyme itself is a glycoprotein of approximate molecular weight 42 000 k, and it has an optimum pH of about 3.5.

The other aspartic proteinases have been shown to originate in precursor form in only a few specific sites in the human body: pepsinogen in the chief and mucous neck cells of the gastric body⁵; progastricsin in the same cells and in the deep glands of the gastric antrum,⁶ the duodenal glands, and the prostatic acinar epithelium^{7,8}; and renin in the juxtaglomerular apparatus of the kidney, as well as in the brain,^{9,10} and submaxillary glands.^{11,12} Moreover, malignant tumours of at least some of these sites produce the same enzymes as the normal tissue from which they have arisen.^{8,13–15} Cathepsin D remains the one mammalian aspartic proteinase whose sites of localisation have not been the subject of detailed investigation: it has been shown, however, within lysosomes in cultured human synovial cells (with biotin labelled pepstatin),¹⁶ in rabbit synovial cells (by immunohistochemistry),¹⁷ in rat skeletal muscle

(by electron microscopic detection of enzyme reaction product¹⁸), and in human skeletal muscle and phagocytes (by electron microscopic immunocytochemistry¹⁹). Cathepsin D has long been known to be present in human gastric mucosa²⁰ and, in biochemical terms, has been purified not only from this source^{21,22} but also from rat gastric mucosa,²³ pig myometrium,²⁴ calf thymus²⁵ and several other tissues.²⁶ An enzyme with similar properties has been extracted from human gastric adenocarcinomas.²⁷

In an attempt to characterise the sites of origin of this enzyme more thoroughly and to make a comparison with those of the other human enzymes we studied various normal and neoplastic human tissues using an immunoperoxidase (PAP) technique. We also compared the distribution of cathepsin D with that of certain other lysosomal enzymes.

Material and methods

PURIFICATION OF CATHEPSIN D

Cathepsin D was purified from normal human spleen by a three step procedure entailing affinity chromatography, as described by Afting and Becker.²⁴ This entailed an initial purification on a column of concanavalin A-agarose, followed by fractionation on a pepstatin-agarose column, and finally, gel filtration on Sephadex G-75. The enzyme thus obtained was composed of two subunits (molecular weights 28 000 and 14 000, respectively), as observed

on SDS-polyacrylamide gel electrophoresis.²⁸ The human cathepsin D molecule has previously been shown to comprise two chains.²⁹

PREPARATION OF ANTISERUM

A polyclonal antiserum was raised by repeated immunisation of three rabbits with cathepsin D emulsified in complete Freund's adjuvant by sonication. Aliquots of 50 µg were injected intradermally and intramuscularly, followed one month later by a similar dose given intramuscularly. The rabbits were boosted with 37 µg intramuscularly at two week intervals. The sera were tested for immunoreactivity on formalin fixed paraffin embedded sections of normal human spleen using the PAP method. Two of three rabbits showed satisfactory titres. One of these died and the other was bled out one week after the fourth injection.

TISSUE STUDIED

Comparison of different fixatives showed that labelling of tissues using the PAP method produced the best results with either Bouin's fixative or buffered formalin. Buffered formalin fixed, paraffin embedded tissues from the routine surgical and necropsy files of the pathology department, Leeds General Infirmary, were therefore used.

Tissues examined for cathepsin D were as follows: spleen (n = 7), liver (n = 5), gastric body (n = 26) and antrum (n = 14), duodenum (n = 10), small bowel (n = 5), large bowel (n = 6), lung (n = 6), prostate (n = 7), lymph nodes (n = 6), brain (n = 5), joint synovium (n = 2), placenta (n = 7), kidney (n = 5). Neoplasms studied included adenocarcinomas of stomach (n = 19), colon (n = 6), pancreas (n = 2), breast (n = 6), ovary (n = 6), carcinomas of

Fig 1 Normal spleen labelled for: (a) cathepsin D. Note labelling of cells (S) lining sinusoids and of cells (M) in white pulp; (b) lysozyme. Note widespread labelling of cells in red pulp but not of those (S) lining sinusoids or in white pulp (W). $\times 340$.

kidney (n = 5) and bladder (n = 8), and squamous carcinomas of bronchus (n = 8) and skin (n = 5). A selection of soft tissue sarcomas (n = 5) and some benign tumours—namely, dermatofibromas (n = 2) and breast fibroadenomas (n = 5)—and a group of Hodgkin's (n = 5) and non-Hodgkin's lymphomas (n = 9) were also included. Selected sections of normal spleen (n = 7), liver (n = 5), stomach (n = 8) and colon (n = 7) and carcinomas of stomach (n = 8) and colon (n = 6) were also labelled for lysozyme.

IMMUNOLocalISATION PROCEDURE

The peroxidase-antiperoxidase (PAP) method was used. Sections (6 μ m) were incubated for 30 minutes with rabbit anticathepsin D or rabbit antilysozyme (Dakopatts A/S, Denmark) diluted 1/100 or 1/1000, respectively, in 0.05M Tris buffered saline (TBS), pH 7.6. Sections were then overlain with swine antirabbit immunoglobulin (Dakopatts), diluted 1/40 in TBS for

30 minutes, followed by rabbit PAP (Dakopatts) at 1/100 in TBS for 15 minutes. Sections were developed in diaminobenzidine (DAB) and counterstained with either methyl green or haematoxylin, although on the lung sections the Prussian blue reaction was performed. Trypsinisation was found to decrease labelling considerably and was not performed.

CONTROLS

Controls were performed on selected strongly positive cases (one spleen, five malignant lymphomas, two colonic carcinomas) by incubating sections with a 1/200 dilution of anticathepsin D, which had been preincubated for one hour at room temperature with a different preparation of cathepsin D (1 mg/ml) kindly supplied by Dr AJ Barrett, Strangeways Laboratory, Cambridge). The dilution of antiserum was higher than in the rest of the study to economise on antigen.

Fig 2 Normal liver labelled for: (a) cathepsin D. Note labelling of hepatocytes and few Kupffer cells; (b) lysozyme. Note labelling of Kupffer cells. $\times 340$.

Fig 3 Cathepsin D in gastric body mucosa. Note labelling of parietal cells (P). Chief cells (C) are negative. $\times 520$.

Fig 4 Cathepsin D in granules in mucous neck cells of gastric body. $\times 520$.

Results

NORMAL TISSUES

Spleen

In the spleen cathepsin D was shown in the littoral cells lining the sinusoids (fig 1a). There was also intense reactivity with single large cells in the Malpighian bodies; these had abundant cytoplasm, often containing vacuoles, and appeared morphologically to be macrophages (fig 1a). The pattern observed with antikathepsin D in spleen was different from that obtained with antilysozyme, which reacted strongly with numerous cells in the red pulp (fig 1b) but with only occasional littoral cells and with very few cells in the white pulp.

Liver

Liver showed widespread labelling of cathepsin D in hepatocytes but in only a small number of Kupffer cells (fig 2a). In contrast, lysozyme was present in

most Kupffer cells and a few hepatocytes (fig 2b).

Stomach

In the body of the stomach there was strong labelling of almost all of the parietal cells (fig 3). In most specimens the chief cells were negative, although in occasional cases there was a diffuse positivity, which was very weak compared with that in the parietal cells. There was also a granular reaction in the mucous neck cells, particularly on the luminal side of the nucleus, deep to the mucus globule (fig 4). A few mononuclear cells, probably macrophages, in the lamina propria were also positive. In contrast, lysozyme was strongly labelled in large numbers of cells in the lamina propria in most cases and less strongly in the mucous neck cells in fewer cases. The ganglion cells of the myenteric plexus were also consistently strongly positive for cathepsin D but not for lysozyme.

Fig 5 Cathepsin D in granules in cells of gastric antral glands. $\times 520$.

In the gastric antrum the epithelial cells lining the deep glands were positive (fig 5). Labelling of mucous neck cells, parietal cells, and mononuclear cells in the lamina propria was similar to that in the body of the stomach.

Bowel

In the duodenum and small and large bowel cathepsin D was most strongly labelled in the cytoplasm of mononuclear cells, probably macrophages, of the lamina propria. In the colon these cells containing cathepsin D tended to lie just below the surface epithelium, whereas those containing lysozyme were widely distributed throughout the lamina propria. There was weak labelling of the surface epithelium for cathepsin D in some cases, but the cells in the crypts were negative, although in three cases the duodenal Brunner's glands showed weak positivity.

Lymph nodes

Tingible body macrophages within germinal centres

Fig 6 Cathepsin D in germinal centre of a reactive lymph node. Note labelling of tingible body macrophages. $\times 340$.

were consistently strongly positive for cathepsin D (fig 6), as were occasional stellate cells, which seemed to be dendritic reticulum cells. Sinus lining cells showed a similar but weaker reaction pattern to those in the spleen. Most sinus histiocytes were weakly positive.

Lung

Of the sections from the six lungs studied, five contained abundant alveolar macrophages, all of which were rich in cathepsin D. Counterstaining of PAP labelled sections with Prussian blue showed haemosiderin in the cells containing cathepsin D; at least some of the haemosiderin was in different granules, although the intense brown reaction of the DAB may have obscured some of the blue staining haemosiderin. Less commonly, alveolar lining cells were positive. Where present, the respiratory epithelium lining the bronchioles showed granular cytoplasmic labelling near the luminal aspect.

Fig 7 Cathepsin D in neurones of cerebral cortex. $\times 210$.

Brain

The mononuclear phagocytes of the brain (the microglia) were negative, as were the astroglia. Neurones of the cerebral and cerebellar cortex, however, showed granular cytoplasmic reactivity for cathepsin D (fig 7). Two cases of brain included choroid plexus, the surface epithelium of which gave a positive reaction for cathepsin D.

Other tissues

In the four cases of normal kidney cathepsin D was mainly confined to proximal and distal tubular epithelium, although in two cases there was also focal glomerular reactivity. Prostate has already been shown to contain gastricsin, an aspartic proteinase related to cathepsin D.⁷ Although cathepsin D was present in several cases, however, labelling was weak and was shown in only a small proportion of acinar lining cells and occasional stromal cells. The syncytiotrophoblast of the placental chorionic villi

Fig 8 Cathepsin D in gastric adenocarcinoma. Note focal labelling of neoplastic cells (N) and of some stromal cells (S). $\times 340$.

exhibited granular cytoplasmic reactivity, but the Hofbauer cells, which are the mononuclear phagocytes of the placenta, were negative. Both specimens of joint synovium were negative for cathepsin D. In the bone marrow labelling was confined to non-haemopoietic cells, which seemed to be histiocytes, with no cathepsin D in megakaryocytes, or in erythroid, or myeloid cells. In particular, neutrophils were negative.

NEOPLASMS

Carcinomas

In all cases of gastric carcinoma cytoplasmic labelling of the neoplastic epithelial cells or non-epithelial stromal cells, or both, occurred (fig 8). In some tumours labelling of the neoplastic cells predominated, whereas in others cathepsin D was mainly confined to the stromal cells. The pattern of reactivity with anti-

Fig 9 Focal labelling of cathepsin D in cells in granuloma in lymph node affected by lymphocyte dominant Hodgkin's disease. $\times 520$.

lysozyme was different: it labelled strongly many of the stromal cells. Lysozyme, however, was labelled in fewer neoplastic cells than cathepsin D. Carcinomas from colon, breast, ovary, kidney, bladder and pancreas all contained cathepsin D in neoplastic or in stromal cells, or both types, the predominance varying considerably from case to case. Antilysozyme labelled the stromal cells of colonic carcinoma, although the neoplastic epithelial cells were negative.

Squamous carcinomas of bronchus showed labelling of only occasional tumour cells. The connective tissue, however, showed strong labelling of macrophages as did the adjacent lung. Squamous carcinomas of skin showed labelling of only occasional tumour cells and some stromal cells. The malignant connective tissue tumours were generally negative with labelling of only focal cells in one case. One of the two dermatofibromas showed a strongly positive

reaction, apparently with histiocytes; the other was negative. Two of the breast fibroadenomas were positive with labelling of very occasional glands in each.

Malignant lymphomas

All lymphomas, except one case of non-Hodgkin's lymphoma, showed strong cytoplasmic reactivity, mainly in large cells scattered throughout the tumour. In one case of lymphocyte dominant Hodgkin's disease some of the cells in the granulomas seemed to contain cathepsin D, although the rest of the tumour tissue was negative (fig 9).

Controls

At a dilution of 1/200, higher than that used for the rest of study, the anticathepsin D still gave strong labelling in all the selected cases studied. The labelling of most cells was abolished, or in the most strongly positive cells, considerably reduced by preincubation of antiserum with cathepsin D.

Discussion

These results show that the localisation of cathepsin D in human renal tubules is similar to its distribution in the rat kidney,³⁰ but its reported presence in synovial cells^{16 17} has not been confirmed. Cathepsin D, however, is present within cells from a wide range of tissues, often in mononuclear phagocytes. Like lysozyme (muramidase),³¹ it seems to be more prominent in reactive histiocytes, such as those in lymph node and spleen, than in resting phagocytes in liver, brain, and placenta. The localisation, however, of cathepsin D in epithelium—for example, of bronchi and bowel and in neoplasms—shows that its presence is not indicative of phagocytic activity per se. It may be a sign of some other more general metabolic activity.

The wide distribution of cathepsin D is in strong contrast to that of the other aspartic proteinases pepsin, gastricsin, and renin. It is not clear why cathepsin D should have such a wide distribution. Because of the structural similarity between the aspartic proteinases, a common evolutionary origin has been suggested, and it could be that cathepsin D is the least highly evolved and that its distribution reflects its origin in unicellular organisms.²⁹

Cathepsin D is generally considered to be a lysosomal enzyme.²⁹ This would be consistent with its distribution in the body and its granular appearance in cells, although the possibility of its localisation within other subcellular structures—for example, in the gastric mucosa—cannot be excluded. Our limited experiments clearly show that the distribution of cathepsin D is different from that of lysozyme^{31 32} and, according to published accounts, from that of α_1

proteinase inhibitor (antitrypsin),^{33 34} although it seems to be similar to that of cathepsin B,^{35 36} a biochemically quite unrelated enzyme in the cysteine proteinase category.

Like the other aspartic proteinases, cathepsin D is found in neoplasms arising in those tissues where it occurs normally. Because of its wide distribution, it seems, unlike the others,^{8 13 14} to have no specificity as a tumour marker. Cathepsin D has been postulated to have a role in producing cachexia, possibly by enhancing proteolysis in muscle and other tissues.³⁷ This effect has been inhibited in animals by the aspartic proteinase inhibitor pepstatin, probably at the lysosomal level, although it is possible that increased production by the neoplasm itself could have a role.

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