*Alimentary tract and pancreas***Demonstration of pepsinogen C in human pancreatic islets**

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SUMMARY Pancreatic tissue from 16 post mortem kidney donors have been examined for the content of pepsinogens. A zymogen with electrophoretic mobility, isoelectric point and molecular weight equal to that of pepsinogen C of gastric origin was found in all specimens. A comparison between pepsinogen C extracted from pancreatic tissue and gastric mucosa demonstrated immunological identity. Quantitative measurements with a radioimmunoassay showed pepsinogen C concentrations in pancreatic tissue three to 80 times higher than those of blood serum. Immunohistochemical staining gave positive reaction for pepsinogen C only in the alpha cells of the pancreatic islets.

Gastric proteases belong to the superfamily of aspartic proteases (EC 3.4.23). The predominant gastric proteases of adult mammals are designated pepsin A (EC 3.4.23.1) and pepsin C (EC 3.4.23.3). For both proteases several isoenzymes and isozymogens may be observed. In native state the two types show no immunochemical cross reactions. Within each type the isozymogens cannot be distinguished with polyclonal antisera.^{1,2}

The proenzyme of pepsin A (pepsinogen A) is synthesised in the chief and mucous neck cells of the fundic mucosa,³ whereas the proenzyme of pepsin C (pepsinogen C) is found in the same cells as well as in cells of cardiac, pyloric and Brunner's glands.⁴ Furthermore, both pepsinogen A and C have been shown in serum,⁵⁻⁸ in gastric gland heterotopia and metaplasia anywhere in the gastrointestinal tract³ and in a Meckel's diverticulum.⁹ Pepsinogen A has been demonstrated in normal urine^{8,10-12} and pepsinogen C in seminal fluid,¹³⁻¹⁶ the seminal vesicles² and the prostate gland.^{2,17}

Apart from renin (EC 3.4.23.16) members of the

superfamily of aspartic proteases localised outside the gastrointestinal tract have traditionally been designated cathepsin D or E.¹⁸ During a series of investigations on the general distribution of aspartic proteases, we observed that several tissues contained zymogens and proteases with electrophoretic and immunochemical properties that were indistinguishable from gastric pepsinogen A and C.^{2,19}

This study deals with our findings of a proenzyme in the human endocrine pancreas that is indistinguishable from gastric pepsinogen C.

Methods**REAGENTS**

Agar Noble (Difco, Detroit, USA); Agarose type II (Sigma, St Louis, USA); Agarose HSA (Litex, Glostrup, Denmark); Aprotinin (Bayer, Leverkusen, FRG); Acrylamide (BioRad, Richmond, USA); N,N'-methylene-bis-acrylamide (Sigma, St Louis, USA); Benzamidiniumchloride (Merck, Darmstadt, FRG); Human albumin (Behringwerke, Marburg, FRG); ¹²⁵I-Nal (Hoechst, Frankfurt am Main, FRG); Lactoperoxidase (Sigma, St Louis, USA); Phenylmethylsulfonylfluoride (Sigma, St Louis, USA); Sephadex G-25 (Pharmacia, Uppsala, Sweden); Triton X-100 (Serva, Heidelberg, FRG);

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3-3' diaminobenzidine tetrahydrochloride (Sigma, St Louis, USA); HAWP nitrocellulose (Millipore, Molsheim, France); Sodium dodecylsulfate (Merck, Schuchardt, FRG); Donkey antirabbit antibody coated cellulose (Wellcome, Dartford, UK).

SAMPLES OF TISSUES

Pancreatic tissue was obtained from post mortem kidney donors. The donors were victims of accidents and without systemic disease. The time of ischaemia was kept as short as possible (15–120 min). The pancreas were dissected free from fat and connective tissue, washed with ice cold 0.05 M sodium phosphate pH 6.0 and cut into pieces of 0.5 cm.³

Extraction of 1 g wet weight tissue took place in a Potter-Elvehjem homogeniser with 3 ml of 0.05 M sodium phosphate pH 6.0 made 5 mM with phenylmethylsulfonylfluoride, 20 mM with benzamidine and 40 μ M with aprotinin. Triton X-100 was then added to the homogenate at a final concentration of 2% (v/v) and sonication took place for three times 10 s in an ice bath. Cell debris were removed by centrifugation at 20 000 g at 5°C for 30 min. The supernatant was stored at –20°C until assayed.

Extraction of samples of liver and spleen tissues for control experiments took place as described for pancreatic tissues. Fixation for immunohistochemistry took place in Bouins fixative for six hours at 4°C. The tissues were kept in 70% (v/v) alcohol until final embedding in paraffin.

ENZYMES AND HORMONES

Pepsinogen A was prepared from human gastric mucosa¹ or human urine¹⁰; pepsinogen C was prepared from human gastric mucosa²⁰; human renin was a gift from Dr I Rubin, Copenhagen, Denmark; insulin and glucagon was from NOVO, Bagsværd, Denmark; somatostatin was from Cambridge Research Biochemicals, Harston, UK.

ANTISERA

Antisera against pepsinogens were raised in rabbits²¹ at Statens Seruminstitut, Copenhagen, Denmark by the courtesy of Dr N H Axelsen. The immunoglobulin fractions of the antisera were purified by salt precipitation and ion exchange chromatography.²¹ The following antibodies were obtained from Dakopatts, Copenhagen, Denmark: horseradish peroxidase conjugated porcine antirabbit IgG (P 217), porcine antirabbit IgG (Z 196), peroxidase antiperoxidase complex (Z 113). Rabbit antiinsulin and rabbit antiglucagon was a gift from Dr J J Holst, Copenhagen, Denmark. Rabbit antisomatostatin was from Cambridge Research Biochemicals, Harston, UK. Rabbit antirenin was a gift from Dr K Poulsen, Copenhagen, Denmark.

ELECTROPHORESIS

Agar gel zone electrophoresis was carried out in 1% (w/v) agar gels in 0.05 M sodium phosphate pH 6.0 for two hours at a gradient of 15 V/cm at constant voltage as described previously.^{21,19} Crossed tandem immunoelectrophoresis was carried out in 1% (w/v) agarose gels.²¹

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis was carried out in vertical slab gels (T=15%, C=0.5%).²² The proteases were detected by indirect peroxidase staining after transferring of proteins to nitrocellulose.²³

Isoelectric focusing was performed in 1 mm thick polyacrylamide gels (T=7.5%, C=3%) containing 5% (v/v) Pharmalyte (pH 2.5–5) for 3 h at 1500 V (constant voltage) after one hour of prefocusing.

DETECTION OF ACTIVITY AFTER ELECTROPHORESIS

After electrophoresis the protease containing zones were visualised as caseograms – that is, overlaying a skim milk containing gel on top of the electrophoresis gel and observation of clotted casein.^{21,19}

QUANTIFICATION OF PEPSINOGENS

Pepsinogen C was measured by a double antibody solid phase radioimmunoassay (RIA) with standards purified from human gastric mucosa.²⁰

Pepsinogen C was labelled with ¹²⁵I by a lactoperoxidase method.²⁴ In brief, 1.63 μ g pepsinogen C dissolved in 115 μ l 0.5 M sodium phosphate pH 7.5 was mixed with 18.5 MBq ¹²⁵I in 100 μ l water, 5 μ g lactoperoxidase in 10 μ l 0.5 M sodium phosphate pH 7.4 and 10 μ l 352 μ M solution of hydrogen peroxide. Iodination was terminated after 30 min by addition of 1 ml water. ¹²⁵I-pepsinogen C was obtained by gel filtration on Sephadex G-25 eluted with 0.1 M sodium phosphate pH 7.4. Human albumin (200 μ l 2% (v/v)) was added to 6 ml of tracer and aliquots of 500 μ l were stored frozen.

Immediately before use the tracer was diluted with the RIA buffer: 0.04 M sodium phosphate pH 7.4 with 0.15 M sodium chloride, 0.01 M EDTA, 5 g/l human albumin and 5 mg/l phenylmercuric acetate. With freshly prepared tracer the dilution factor was determined allowing a radioactive concentration of 1.1 MBq/l of diluted tracer solution. The radioimmunoassay procedure was as follows: 50 μ l pepsinogen C standard (2.6–191 μ g/l) or test substance were mixed with 200 μ l ¹²⁵I-pepsinogen C solution, 200 μ l rabbit anti-pepsinogen C (1:30 000) and 300 μ l RIA-buffer. The mixture was incubated for 48 h at room temperature. Counting of bound pepsinogen C was performed after precipitating with 50 μ l of a suspension of donkey antirabbit antibody coated cellulose. Detection limit was 2.6 μ g/l. The intra-

Fig. 1 Caseogram[™] after agar gel electrophoresis in 0.05 M sodium phosphate, pH 6.0 and 15 V/cm for two hours. Lane M: partly activated extract of gastric mucosa; lane P: extract of human pancreas. Application volume 20 μ l per slit. Pg=pepsinogen, Pn=pepsin. Appl. = application point. Anode at top.

assay and the interassay precision were 6.1% and 10%, respectively. Cross reaction with pepsinogen A was less than 0.3%.

IMMUNOHISTOCHEMISTRY

Series of paraffin sections (approx 6 μ m) from pancreas were immunostained by the peroxidase antiperoxidase method.²⁵ The primary antibodies were diluted in 0.005 M TRIS buffer pH 7.6 containing 0.15 M sodium chloride, 0.3% (v/v) Triton X-100 and 5% (v/v) normal porcine serum: anti-pepsinogen C 1:400; anti-insulin 1:3200; antiglucagon 1:1600; antisomatostatin 1:1600 and antirenin 1:1600. Sections were incubated overnight at 4°C. The following controls were undertaken: (a) omission of incubation with primary antibody; (b) incubation with homologous non-immune serum; (c) incubation with

Fig. 2 Tandem crossed immunoelectrophoresis of pancreatic extract and pepsinogen C. The following samples were applied: P well (extended), 25 μ l of human pancreatic extract; G well, 5 μ l chromatographically purified gastric pepsinogen C (0.9 g/l). First dimension electrophoresis (anode to the right) was run at 10 V/cm for 60 min at 12°C in agarose gel (1%) with Tris/Barbital buffer pH 8.6. Second dimension electrophoresis (anode at the top) was run at 2 V/cm at 12°C overnight, with the same agarose gel and buffer as in the first-dimension, but with antibodies against pepsinogen C (4 μ l/cm²). Stained with Coomassie Brilliant Blue R 250.

anti-pepsinogen C preabsorbed with pepsinogen C, pepsinogen A, glucagon, somatostatin or human renin; (d) sections of human gastric mucosa served as positive control for the pepsinogen C staining.

Results

By agar gel zone electrophoresis and detection of proteolytic activity by the caseogram technique,¹⁹ a zymogen with electrophoretic mobility corresponding to that of gastric pepsinogen C was observed in all of 16 examined extracts of human pancreas. The results from agar gel electrophoresis are exemplified in Figure 1, which shows a reference of partly activated mucosal extract run in parallel with a pancreatic extract. The zymogen present in pancreatic extract has the mobility of pepsinogen C1.

Fig. 3 Immunoblot of SDS polyacrylamide gel electrophoresis ($T=15\%$, $C=0.5\%$). Detection by indirect peroxidase staining. 1: incubation with rabbit anti-pepsinogen C (1:1000) for 16 hours at room temperature. 2: incubation with porcine antirabbit IgG conjugated with horseradish peroxidase (1:1000) for two hours at room temperature. 3: peroxidase staining with 3-3' diaminobenzidine tetrahydrochloride. Samples: lane P, 15 μ l (1:2) extract of human pancreatic tissue; lane G, 10 μ l chromatographically purified gastric pepsinogen C (containing 90 ng). Both samples were reduced with 5% dithioerythritol for 5 min at 100°C .

This isozymogen is only a minor component in gastric mucosa but is the predominant isozymogen of the seminal pepsinogen C. The isozymogens are not well characterised, but upon activation the three isozymogens give rise to a pepsin C with the same mobility (data not shown). It should be noted that at pH 6.0, pepsin C and pepsinogen A3 have about the same electrophoretic mobilities. Zymogens corresponding to pepsinogen A was present in seven of the extracts.

Fig. 4 Caseogram of isoelectric focusing in 1 mm thick polyacrylamide gels ($T=7.5\%$, $C=3\%$) containing 5% Pharmalyte (pH 2.5-5). The samples were activated one hour with equal volume of 0.1 M HCl prior to application: lane P, 15 μ l extract of human pancreatic tissue; lane G 10 μ l chromatographically purified gastric pepsin C (containing 110 ng).

Comparison of the pancreatic zymogen with pepsinogen C of gastric origin, showed immunological identity when tested by crossed tandem immunoelectrophoresis; the precipitates fused without spurs (Fig. 2).

Determined by SDS-polyacrylamide gel electrophoresis the molecular weight of the pancreatic zymogen was equal to that of gastric pepsinogen C (M_r 40 000) (Fig. 3).

By isoelectric focusing of activated gastric pepsinogen C, pI is 3.5 and activated pancreatic extract gave three proteolytic bands pI 4.4, 4.1 and 3.5 (Fig. 4) of which only the last had immunological homology with pepsinogen C.

Fig. 5 Serial sections of human pancreas stained with (A) anti-pepsinogen C (1:400) and (B) anti-pepsinogen C (1:400) pre-incubated with pepsinogen C (final concentration 0.25 mg/l) for two hours at room temperature before staining. Serial sections of human pancreas stained with (C) anti-pepsinogen C (1:400) and (D) anti-glucagon (1:1600). Counterstained with Meyers haematoxylin. Arrows indicate identical cells.

Quantitative determination of pepsinogen C in 16 extracts from different pancreatic glands showed that the concentration range of pepsinogen C was 32–816 ng (median 297) per gram wet weight of tissue. An additional pancreas was cut into 11 sections from caput to cauda. This experiment showed that the middle part of the pancreatic body contained most pepsinogen C: 1078–2004 ng per gram tissue, the caput contained 368–512 ng per gram tissue and the cauda 176–311 ng per gram tissue. Extracts of spleen and liver from four individuals contained pepsinogen C in concentrations not exceeding 5 ng/g weight of tissue. Determined by the present method, the con-

centration range of pepsinogen C in serum of normal human controls is 6–37 ng/ml (median 12 ng/ml).

In sections of pancreatic tissue from 11 individuals, staining for pepsinogen C was positive in all. Positive staining was observed in cells predominantly in the peripheral parts of the pancreatic islets (Fig. 5A). Staining of serial sections for glucagon showed positive reactions in the same cells as those stained with anti-pepsinogen C (Fig. 5C and 5D). The pepsinogen C positive staining was not inhibited by prior incubation with pepsinogen A (10 μ M), glucagon (10 μ M), somatostatin (10 μ M) or renin (38 000 Goldblatt units/l). By contrast, absorbtion

experiments with pepsinogen C (10 μ M) resulted in complete abolition of staining (Fig. 5B). The same antipepsinogen C stained the chief and mucous neck cells of gastric mucosa.

Discussion

Pepsinogens have until now been localised only in cells of exocrine glands. The major source of pepsinogens is the gastric mucosa, even though extra-gastric pepsinogens have been observed. The pepsinogens in serum and urine are assumed mainly to arise from the gastric glands due to reabsorption during cell turnover.¹ In spite of use of inhibitors of the pancreatic serine proteases small amount of non-pepsin proteases in Figure 1 are interpreted as serine proteases. In nearly all extracts small amounts of active pepsins were present.

In all examined extracts zymogens with the same properties as gastric pepsinogen C were observed. The concentration of pepsinogen C is three to 80 times higher in pancreatic tissue than in serum. Furthermore, organs rich in blood like the spleen and the liver did not show higher pepsinogen C levels than that of serum. From this we conclude that it is not trapped serum pepsinogen C that is observed.

As regards to the specificity of the immunochemical reactions one has to consider the possibility that the staining for pepsinogen C might be because of antigenic determinants that are common to other aspartic proteases. Dreyer *et al.*²⁶ have recently found that, although the primary structure of protease A from baker's yeast has only 37% of identity with calf chymosin, protease A may be detected with anticalf chymosin by immunoblotting. In spite of such uncertainty we must emphasise that the presence of pepsinogen C was demonstrated in pancreatic extracts with several techniques and that in the immunohistochemical experiments only the alpha cells gave positive reaction for pepsinogen C.

As mentioned above, pepsinogen A was present in about half of the pancreatic specimens that were examined. These investigations are still in progress.

The significance of the pancreatic pepsinogen C in endocrine cells is unknown. An acid environment is necessary for pepsinogen activation and for its proteolytic activity. In intracellular compartments, however, pH could be low enough for activation of pepsinogens and for pepsins to exert their proteolytic activity.²⁷ These enzymes are generally regarded as rather unspecific in sense that general and clear cut rules for their specificities have not been found. But these enzymes have an extended binding cleft that may accommodate a substrate peptide chain with at least eight amino acid residues.¹ Thus pepsin C may have a potential specificity that may

serve in regulatory functions. Whether the general and unspecific proteolytic activity is involved in degenerative diseases of the pancreatic gland remains to be investigated.

An aspartic protease zymogen have also been isolated from monkey lungs.²⁸ This enzyme was called procathepsin D-II, but in all analysed properties it was indistinguishable from pepsinogen C. These results support the view that the occurrence of extra gastric pepsinogens is more widespread than previously thought.

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