An Immunochemical and immunohistochemical study on pancreastatin-like immunoreactivity using synthetic peptides

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ABSTRACT

Chemical synthesis of pancreastatin fragments (33-49), (39-49), (42-49) and (45-49) was carried out using a solid-phase synthesizer, and antisera were characterized. The synthetic pancreastatin (33-49) coupled with keyhole limpet hemocyanine (KLH) was used as immunogen and antibodies were produced in New Zealand white rabbits. One of the antisera, T-2602, which showed the highest titer, recognized the C-terminal region of pancreastatin and cross-reacted equally with both pancreastatin (33-49) and native pancreastatin, but not with other known peptide hormones. Gel-filtration analysis of aliquots of tissue homogenates revealed the existence of four major components of pancreastatin immunoreactivity which corresponded to the elution position for pancreastatin precursor (void volume), native pancreastatin, pancreastatin (33-49), and related small peptide fragment in porcine pancreas. Analysis of tissue homogenates from the porcine adrenal gland revealed a predominant large-molecular form and a small amount of native pancreastatin. The immunohistochemical study using the antiserum T-2602 showed widespread immunoreactivity in amine/peptide producing endocrine cells. Not only pancreatic islet cells but also gut endocrine cells and adrenal chromaffin cells showed intense immunoreactivity in their secretory granules. As this immunoreactivity precisely coincided with that for chromogranin A, it is suggested that pancreastatin is a peptide closely correlated with chromogranin A.

Pancreastatin was recently isolated from porcine pancreas and its primary structure was determined (9). This peptide consists of 49 amino acid residues and has a striking sequence homology with residues 243-294 of bovine chromogranin A (1, 4). Whether or not this peptide is processed from chromogranin A must await the verification of the amino acid sequence of the porcine chromogranin A. Pancreastatin has been reported to suppress insulin secretion upon glucose stimulation (9). Chemical synthesis of peptides corresponding to pancreastatin (33-49), (39-49), (42-49) and (45-49) followed the stepwise solid-phase method (6). The N'-amino function was protected by the Boc(tert-butyloxy carbonyl) group except in the case of arginine, which was protected by the Aoc(amyloxy carbonyl) group for arginine. Side chain functional groups were protected as follows: tosyl for arginine and benzyl for threonine and glutamic acid.

The coupling reaction was monitored by the ninhydrin test of Kaiser et al. (5). The crude material was purified by semipreparative HPLC using a gradient of acetonitrile in 0.05% aqueous trifluoroacetic acid. N'-Tyrosyl pancreastatin (33-49) was also prepared and
used for radioiodination.

Five New Zealand white rabbits were immunized at 2-week intervals by multiple subcutaneous injections of pancreastatin (33-49)-KLH conjugate emulsified in complete Freund's adjuvant. Ten days after the 8th immunization, the animals were bled and serum was collected by centrifugation. Titers of the antisera obtained were assessed by their abilities to bind with $^{125}$I-tyrosyl pancreastatin (33-49).

N$^\circ$-Tyrosyl pancreastatin (33-49) was iodinated by the chloramine-T method (3). The separation of the labeled peptide from the free iodide was conducted by gel filtration on a Sephadex G-25. The specific activity of the labeled peptide was about 350 $\mu$Ci/μg.

The standard diluent was 10 mM phosphate buffer, pH 7.4, containing 0.14 M NaCl, 0.5% BSA, 0.01% NaN$_3$, 25 mM EDTA-2Na and 0.1% Triton X-100. A mixture containing 0.1 ml of standard pancreastatin (33-49) or unknown sample, 0.1 ml of appropriate diluted antiserum and 0.4 ml of standard diluent was incubated at 4°C for 48 h. To the above solution was added 0.1 ml of $^{125}$I-tyrosyl pancreastatin (33-49) (about 5,000 cpmp) and the mixture was incubated at 4°C for an additional 48 h. Then diluted normal rabbit serum (0.1 ml), anti-rabbit IgG goat serum (0.1 ml) and 6% (w/v) polyethylene glycol 6,000 (1.0 ml) were added to each tube. After incubation at 25°C for 30 min, the precipitate was separated by centrifugation at 3,000 rpm for 30 min at 4°C. Then the supernatant was decanted into another tube and both bound and free labeled peptides were counted in a gamma counter.

Porcine pancreas or adrenal gland was homogenized and extracted with a five-fold excess of boiling water for 5 min before adding acetic acid to a final concentration of 1.0 M. After having been left overnight at room temperature, the extract was filtered on cheese-cloth, the filtrate was centrifuged at 3,000 rpm for 20 min at 4°C and the supernatant was lyophilized.

A part of the lyophilized crude extract was applied to a gel filtration on a Sephadex G-50 superfine column (2.2×85 cm) equilibrated with 3 M acetic acid. The column was eluted with the same solvent, allowed to run at 3 ml/h and 2.0 ml fractions were collected. Fractions were lyophilized and reconstituted with standard diluent. All other fractions were assayed in duplicate for immunoreactive pancreastatin as described above.

The pancreas, duodenum and adrenal gland were removed from five pigs at a slaughter house. The specimens were fixed in Bouin's fluid for about 6 h, dehydrated by an ethanol-xylene series and embedded in paraffin. Paraffin sections were cut at 4-6 μm thick and some were cut serially at about 2.5 μm thick. Dewaxed paraffin sections were submitted to the peroxidase-antiperoxidase (PAP) method.
Antisera against pancreastatin (33-49), T-2602 and chromogranin (Immuno Nuclear Corporation, Minnesota, U.S.A.) were used at a dilution of 1:6,000 and 1:1,000, respectively. The chromogranin antiserum was generated in a rabbit against native bovine secretory protein I/chromogranin (Cat No. 63H2TB). The specificity of the immunoreaction was checked by preincubation of the antisera with synthetic pancreastatin (33-49) (20 μg per ml of diluted antiserum). Immunoreactivity to antiserum T-2602 was inhibited by preincubation of the antiserum with pancreastatin (33-49), while chromogranin immunoreactivity was not influenced by preincubation with pancreastatin (33-49) (Fig. 3).

Pancreastatin peptide fragments synthe-

Fig. 3 Four serial sections (2.5 μm thick) from porcine pancreas. Major population of islet cells was immunoreactive for both pancreastatin (b) and chromogranin (c). Pancreastatin-immunoreactivity was completely inhibited by preincubation of the antiserum with pancreastatin (33-49) (a), while the immunoreaction for chromogranin was not influenced by preincubation with the same C-terminal fragment of pancreastatin (d). ×410
sized by the solid-phase method were judged to be pure by thin layer chromatography and analytical high performance liquid chromatography using a gradient of acetonitrile in 0.05% trifluoroacetic acid. Amino acid analyses of the synthetic peptides gave the expected composition as shown by Tatemoto et al. (9). After the 8th immunization, antibodies generated in all rabbits were assessed by their binding abilities with $^{125}$I-tyrosyl pancreastatin

Fig. 4 (upper half) Two adjacent thin sections were stained with antisera against chromogranin (a) and pancreastatin (b). Many endocrine cells (arrowheads) in the crypts of the porcine duodenum were immunoreactive to both antisera. $\times 500$

Fig. 5 (lower half) Two adjacent thin sections from the porcine adrenal gland stained with antisera against chromogranin (a) and pancreastatin (b). Medullary cells showed a similar stainability to both antisera. $\times 185$
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(33-49) (Fig. 1). One antiserum, T-2602, which showed the highest titer, was examined by a competition radioimmunoassay. This system could detect synthetic pancreastatin (33–49) at a dose of 1.4 fmol/tube. The synthetic peptides, pancreastatin (33–49), (39–49), (42–49) and (45–49), cross-reacted almost equally with this antiserum, suggesting that it recognized the C-terminal region site of pancreastatin (Fig. 2). The inhibition curve of native pancreastatin was almost completely superimposed on that of the standard pancreastatin (33–49). On the other hand, the other known peptide hormones, i.e. human gastrin-1, (Arg⁸)-vasopressin, neurotensin, gastrin-releasing peptide, rat pancreatic polypeptide, cholecystokinin octapeptide, motilin, substance P or luteinizing hormone releasing hormone, did not cross-react in this assay system, even when added in large excess. The dose-response curve for the extract of the porcine pancreas or adrenal gland was parallel to that of standard synthetic pancreastatin (33–49). To characterize the relative size of tissue immunoreactive pancreastatin, aliquots of tissue homogenates of the porcine pancreas and adrenal gland were submitted to a Sephadex G-50 superfine column. There were four major components in the pancreas extract, while a predominant large-molecular form, a small amount of native pancreastatin and the trace of several small peptide fragments were found in the adrenal gland. The concentrations of immunoreactive pancreastatin in the pancreas and adrenal gland extracts were 6 μg and 14 μg per gram wet weight, respectively.

An immunohistochemical study was carried out by using the antiserum T-2602. A major population of pancreatic islet cells in the pig was intensely immunoreactive for pancreastatin (Fig. 3b). The immunoreactivity was clearly localized in secretory granules. Chromogranin A antiserum immunolabeled islet cell granules in the same manner as the antiserum against pancreastatin (Fig. 3c).

Observation of serial sections stained alternatively with antisera against pancreastatin and chromogranin showed that pancreastatin-immunoreactive cells were identical to chromogranin A-immunopositive cells (Fig. 3, b and c). The pancreastatin-immunoreactivity was found widely in the granules of amine/peptide producing endocrine cells. Immuno-reactive endocrine cells were distributed densely in both intestinal villi and crypts of the porcine duodenum (Fig. 4b). The pancreastatin cells found in adjacent sections were also reactive with chromogranin antiserum (Fig. 4, a and b). In the adrenal gland, all the medullary endocrine cells showed immunoreactivity for pancreastatin, although the intensity of the immunoreaction was different among the cells. The same staining pattern was observed again in the adjacent sections stained with chromogranin antiserum (Fig. 5, a and b). These findings suggest that pancreastatin is not specific for the pancreas but is widely distributed in amine/peptide containing endocrine granules coinciding in location with chromogranin A. We could confirm that the anti-pancreastatin antiserum stained also various endocrine granules in humans but not those in other animals, suggesting the difference of amino acid sequences in the C-terminus (data not shown). The anti-chromogranin A antiserum stains endocrine granules of a larger variety of animals as we have experienced and as literature indicates (2, 7, 8). This discrepancy seems to be due to possibly different antigenic sites in the molecules of pancreastatin and chromogranin A.

These findings provide a valid basis for future studies on the relationship between pancreastatin and chromogranin A.

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