IMMUNOREACTIVITY AGAINST ANTI-GROWTH HORMONE-RELEASING HORMONE (GHRH) SERA IN HUMAN PANCREAS AND PANCREATIC ENDOCRINE TUMORS: EVIDENCE OF PITFALL IN IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Localization of growth hormone-releasing hormone (GHRH) in normal human pancreas was examined immunohistochemically with four different anti-GHRH sera. Antisera raised against synthetic human GHRH(1-40)OH and GHRH(1-29)-Gly₄-Cys-NH₂ gave no positive immunoreaction. Two other antisera (RG107 and #4676) raised against synthetic human GHRH(1-44)NH₂ reveal GHRH-like immunoreactivity in pancreatic polypeptide (PP) cells, but the immunoreactivity was abolished when RG107 or #4676 was absorbed by synthetic human PP(1-36)NH₂. Among sixteen pancreatic endocrine tumors, only one tumor from an acromegalic patient contained many cells that reacted with all four anti-GHRH sera; this immunoreactivity disappeared by the addition of synthetic GHRH(1-44)NH₂ but not of PP(1-36)NH₂. The remaining fifteen tumors, which included eleven PP-positive tumors, did not show any immunoreactivity against the four anti-GHRH sera. The findings suggest that the anti-GHRH-44 sera (RG107 and #4676) contain not only an antibody for GHRH but also antibody that recognizes PP or an antibody that recognizes both GHRH and PP, and also that cells with genuine GHRH immunoreactivity are absent in normal human pancreas. In addition, it is suggested that the molecular structure of PP in normal pancreas differs from that in pancreatic endocrine tumors.

Since growth hormone-releasing hormone (GHRH) was first isolated from human pancreatic tumor (6, 11), localization of GHRH in normal human tissues has been investigated. Although the pancreas has received special attention by many investigators, results are conflicting with respect to the presence of GHRH in this organ. By radioimmunoassay,

Yamaguchi *et al.* (18) could not detect any immunoreactive GHRH in normal pancreas, whereas Shibasaki *et al.* (16) found it in a large amount. Immunohistochemically, Asa *et al.* (1), Bostwick *et al.* (3) and Dayal *et al.* (4) did not find any GHRH-immunoreactive cells, but Bosman *et al.* (2) observed GHRH-immunoreactivity in pancreatic polypeptide (PP) cells.

Fig. 1 Normal human pancreas immunostained with RG107. Many immunoreactive cells are seen in the lower half of the photograph (posterior-inferior region of the head). $\times 40$

These inconsistent results seem to be due to differences in immunological properties of the anti-GHRH sera used by various workers. In this work, we examined immunohistochemically the localization of GHRH-immunoreactivity in normal human pancreas and pancreatic endocrine tumors using four different anti-GHRH sera and one anti-PP serum, and found that some anti-GHRH sera contained an antibody that recognized PP.

MATERIALS AND METHODS

Antisera

Four antisera were raised in rabbits against synthetic human GHRH(1-44)NH₂, GHRH(1-40)OH and GHRH(1-29)-Gly₄-Cys-NH₂ (Table 1). Anti-PP serum against synthetic human PP was purchased from Miles Laboratories, Elkhart, IN, U.S.A. Synthetic human GHRH(1-44)NH₂ and GHRH(1-40)OH were from Peninsula Laboratories, San Carlos, CA, U.S.A. Synthetic human GHRH(1-29)-Gly₄-Cys-NH₂ was a kind gift from Dr Arthur M. Felix, Hoffmann-La Roche Inc., Nutley, NJ, U.S.A. Two anti-GHRH-44 sera (RG107 and #4676) are highly specific for the C-terminal portion of GHRH(1-44)NH₂ and did not recognize GHRH(1-40)OH and GHRH(1-37)OH as described elsewhere (13, 15). Cross-reactivities of RG107 with other peptides including synthetic human PP were not detected

by radioimmunoassay (13). Anti-GHRH-40 serum (#4274) recognizes GHRH(1-44)NH₂, GHRH(1-40)OH and GHRH(1-37)OH (15).

Tissue Specimens

The human pancreas was obtained at autopsy from 12 patients (1 day old-84 years old; 6 males and 6 females within 3 h after death. The specimens, including the posterior-inferior region of the pancreatic head without any particular lesions, were fixed in 10% formalin for 2 days to 2 years. Sixteen pancreatic endocrine tumors, some of which had the non-tumorous pancreatic tissue, were obtained from fourteen patients. Three patients had clinically overt insulinoma, and one represented the Zollinger-Ellison syndrome. There was one acromegalic patient with pancreatic tumor whose plasma level of GHRH was high (14). The remaining nine patients did not show any overt endocrine symptoms. These specimens were fixed in 10% formalin for 1-2 days.

Immunohistochemistry

Immunohistochemical studies on paraffin-embedded sections (3-4 μ m thick) were carried out by the avidin-biotin-complex (ABC) method (8) using an ABC Kit for rabbit IgG serum (Vector Laboratories, Burlingame, CA, U.S.A.). Sections were incubated with the primary antisera overnight at 4°C; procedural details are described elsewhere (12).

To examine the specificity of the immunostaining, absorption tests were performed for the primary antisera. The diluted antisera (1 ml) were incubated with 10 μ g of synthetic human GHRH(1-44)NH₂ (Peninsula Laboratories) or of synthetic human PP(1-36)NH₂ (Peninsula Laboratories) for 1 h at 37°C and then 24 h at 4°C. After centrifugation at 10,000 rpm, the supernatant was used for the tests.

RESULTS

Positive immunoreaction with the anti-GHRH-44 serum (RG107) was observed in normal human pancreas, most densely in the posterior-inferior region of the head (Fig. 1). The immunoreactivity was seen exclusively in PP-immunoreactive cells on serial sections

Fig. 2 Serial sections of pancreas stained with RG107 (a) and anti-PP serum (b). RG107 immunoreactivity is observed in PP-positive cells. Sections (1 μ m thick) were cut from the glutaraldehyde-fixed and Epon 812-embedded tissue. $\times 540$

(Fig. 2); about 70–80% of the PP-positive cells showed RG107-immunoreactivity. In the pancreatic tissue that had been fixed with formalin for more than nine months, no cells reacted with RG107, although many PP-positive cells were still seen. Immunostaining with another anti-GHRH-44 serum (#4676) gave the same results as those with RG107. With anti-GHRH-29 serum (RMB101) or anti-GHRH-40 serum (#4274), positive immunoreaction was never observed in normal pancreas.

On absorption tests (Table 1), the immunoreactivity against RG107 in normal pancreas disappeared after the absorption with synthetic human PP(1–36)NH₂, but not with synthetic human GHRH(1–44)NH₂ (Fig. 3). The immunoreactivity against #4676, however, was abolished after the absorption with GHRH(1–44)NH₂ or with PP(1–36)NH₂ (Fig. 4). Immunoreactivity against anti-PP serum was abolished by PP(1–36)NH₂, but not by GHRH(1–44)NH₂ (Fig. 5).

Many RG107-immunoreactive cells were observed in only one of sixteen pancreatic endocrine tumors (Fig. 6). This tumor resected from a patient with acromegaly proved to contain a large amount of immunoreactive GHRH by radioimmunoassay whose molecu-

Table 1 Profiles of the Antisera Used and Immunohistochemical Findings in the Normal Human Pancreas and GHRH-producing Pancreatic Tumor

Code No.	Raised against	Conjugated with	Mainly recognizes	Working dilution	Immunohistochemical findings using the antisera		
					Without absorption	Absorbed with GHRH	Absorbed with PP
RG107	Synthetic GHRH(1–44)NH ₂	Human α -globulin	C-terminus of GHRH-44NH ₂	1:500	+/+*	+/-	-/+
#4676	Synthetic GHRH(1–44)NH ₂	Bovine thyroglobulin	C-terminus of GHRH-44NH ₂	1:2,000	+/+	-/-	-/+
RMB101	Synthetic GHRH(1–29)-Gly ₄ -Cys-NH ₂	Bovine albumin serum	Not identified	1:2,400	-/+	nd/-**	nd/+
#4274	Synthetic GHRH(1–40)OH	Bovine thyroglobulin	GHRH-37OH, GHRH-40OH, GHRH-44NH ₂	1:2,000	-/+	nd/-	nd/+
64-711-1	Synthetic PP	Keyhole limpet hemocyanine	Not informed	1:4,000	-/-	nd/nd	nd/nd

*+/-: positive in normal pancreas/positive in GHRH-producing pancreatic tumor. **nd/-: not done in normal pancreas/negative in GHRH-producing pancreatic tumor

Fig. 5

Fig. 4

Fig. 3

lar size was similar to GHRH(1-44)NH₂ when examined by gel filtration chromatography (14). This tumor also contained many cells that reacted with three other anti-GHRH sera (#4676, RMB101 and #4274), but did not contain PP-positive cells. In the remaining fifteen tumors, these anti-GHRH sera did not show any immunoreactivities. Eleven of fifteen tumors (73%) contained PP-positive cells. Immunoreactivity against RG107 or #4676 was occasionally observed in PP-positive cells in the normal pancreatic tissue adjacent to the tumor, where, in spite of the presence of many PP-positive tumor cells, RG107- or #4676-positive tumor cells were not found (Fig. 7).

Absorption tests demonstrated that the GHRH-immunoreactivities shown in the GHRH-producing tumor disappeared by the pretreatment of anti-GHRH sera with synthetic human GHRH(1-44)NH₂, but not with synthetic human PP(1-36)NH₂ (Table 1; Fig. 6). The PP-immunoreactivity in pancreatic tumors was abolished by PP(1-36)NH₂, but not by GHRH(1-44)NH₂.

DISCUSSION

The present immunohistochemical study with four anti-GHRH sera on normal human pancreas and a GHRH-producing pancreatic tumor strongly suggests that the anti-GHRH-44 sera (RG107 and #4676) contain not only a GHRH-specific antibody but also an antibody that recognizes PP. The immunoreactivity against these anti-GHRH sera seen in PP cells in normal pancreas proved to represent PP itself but not GHRH.

The antibodies directed against PP in the

anti-GHRH-44 sera had some peculiar properties compared with the genuine anti-PP serum raised against synthetic human PP. First, in the pancreatic tissue fixed in formalin for more than 9 months, the antibodies did not show any immunoreactivity in cells that still reacted with anti-PP serum, suggesting that

Fig. 3 Immunostaining of pancreas with RG107. Positive immunostaining against RG107 (a) is not abolished by the absorption with synthetic human GHRH(1-44)NH₂ (b), but abolished with synthetic human PP(1-36)NH₂ (c). $\times 150$

Fig. 4 Immunostaining of pancreas with #4676. Positive immunostaining against #4676 (a) is abolished by the absorption with either GHRH(1-44)NH₂ (b) or PP(1-36)NH₂ (c). $\times 150$

Fig. 5 Immunostaining of pancreas with anti-PP serum. Like in Fig. 3, positive immunoreaction (a) is not abolished by the absorption with GHRH(1-44)NH₂ (b), but abolished with PP(1-36)NH₂ (c). $\times 150$

Fig. 6 Immunostaining with RG107 of the pancreatic endocrine tumor that caused acromegaly. Many immunoreactive cells are seen (a). This immunostaining is abolished by the absorption with GHRH(1-44)NH₂ (b), but not with by PP(1-36)NH₂ (c). $\times 150$

Fig. 7 Serial sections of a pancreatic endocrine tumor stained with anti-PP serum (a) and RG107 (b). In non-tumorous pancreas, an islet cell (arrow) contains both immunoreactivities, but in the tumor tissue PP-positive cells are negative for RG107. Arrowheads indicate the margin of the tumor tissue. $\times 85$

little structural change might occur in the C-terminal portion of PP molecule. Second, immunoreactivity against these antibodies was never observed in pancreatic endocrine tumors, although they contained many tumor cells that reacted with anti-PP serum. This finding suggests that a structural difference in PP molecule may exist between pancreatic normal and tumor cells.

With regard to the origin of these antibodies directed against PP in the anti-GHRH sera, two possibilities are considered, *i.e.* contaminants in the synthetic immunogens and the molecular structure of GHRH(1-44)NH₂ itself. The first possibility is unlikely since, although the amount of contaminants would be very small (Quality Control Record, Peninsula Laboratories), intense immunoreactivities were observed in the normal pancreas at

dilutions of 1:500 to 1:2,000 of the antisera. Alternatively, it is noteworthy that positive immunostaining in normal pancreas was seen only with anti-GHRH-44 sera, but never with anti-GHRH-29 or anti-GHRH-40 sera. In addition, both anti-GHRH-sera (RG107 and #4676) recognize GHRH(1-44)NH₂ but not GHRH(1-40)OH. These findings indicate that the antigenic determinant for these antibodies may be closely related with the C-terminal amino acid sequence of GHRH(1-44)NH₂.

Fig. 8 shows the amino acid sequences of human GHRH(1-44)NH₂ and human PP(1-36)NH₂. Although there is no common sequence between the two peptides, a structure of -Arg-x-Arg-x-NH₂ is seen in the C-terminus of both peptides. Taking the secondary structure into account, epitope is not always necessary to be a continuous sequence (9, 10,

Fig. 8 Amino acid sequence of human GHRH(1-44)NH₂ and human PP(1-36)NH₂. Common sequence in the C-terminus (-Arg-x-Arg-x-NH₂) is underlined.

17); the antibody that recognizes both GHRH and PP in #4676 may be originated from this discontinuous amino acid sequence and amide, common to the two peptides. Similar observation was described by Halmi and Krieger (7) on the immunostaining of human syncytiotrophoblast with anti- β -lipotropin serum. As for the antibody that recognizes PP but not GHRH in RG107, the reason why it does not cross-react with GHRH is unknown. A possible explanation is that the epitope in the C-terminus of GHRH(1-44)NH₂ might have undergone a degradative change in the immunized rabbit and become no longer identical with the proper antigen but still similar to PP(1-36)NH₂. Thus, anti-GHRH-44 sera could be classified into: 1) antiserum containing GHRH-specific antibody alone (such as anti-GHRH-44 serum reported by Dayal *et al.* (4), 2) antiserum containing not only GHRH-specific antibody but also an antibody that recognizes both GHRH and PP (#4676), and 3) antiserum containing not only GHRH-specific antibody but also an antibody that recognizes PP but not GHRH (RG107).

Recent studies on immunoreactive-GHRH in pancreatic endocrine tumors have shown that GHRH(1-40)OH is sometimes detected but the full structure of GHRH(1-44)NH₂ is only rarely observed (6, 14, 15). In immunohistochemical investigation, only one tumor has so far been shown to contain cells that react with anti-GHRH-44 serum (14). In contrast, about 25-44% of tumors were positively stained with anti-GHRH-40 sera (1, 3, 4). In

the present study on 16 tumors, however, no tumors reacted with anti-GHRH-29 or anti-GHRH-40 sera, except for one GHRH-producing tumor. The reason is not known for the lower incidence of immunoreactive GHRH detected by the C-terminal-specific anti-GHRH-44 sera. Dayal *et al.* (4) have mentioned that anti-GHRH-40 sera could recognize not only GHRH(1-44)NH₂ but also GHRH(1-40)OH and (1-37)OH, but the C-terminal-specific antisera could recognize only GHRH(1-44)NH₂. They, however, did not refer to the cause of rare occurrence of GHRH(1-44)NH₂ molecule in the tumor. The processing mechanism of GHRH from prepro-GHRH-108 (5) to GHRH(1-44)NH₂ and (1-40)OH or (1-37)OH has not been established, and there has been no evidence to show that GHRH(1-40)OH or (1-37)OH is produced from the precursor through GHRH(1-44)NH₂. It is likely that in most pancreatic endocrine tumors prepro-GHRH-108 is cleaved directly to GHRH(1-40)OH or (1-37)OH, but not via GHRH(1-44)NH₂. Further studies are required to characterize the peptides produced by pancreatic endocrine tumors.

The present study gives evidence that some antiserum directed against a peptide with high region specificity may contain an antibody that cross-reacts with a different peptide, even if there is no common continuous amino acid sequence between the two peptides. Overestimation of the absorption test should be avoided.

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