Immunohistochemical Demonstration of Glucagon- and GLI-Containing Cells in the Canine Gut and Pancreas

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Summary. Cells possessing glucagon- and glucagon-like immunoreactivity (GLI) were studied by an indirect immunofluorescence technique in the normal canine gastrointestinal mucosa and pancreas.

Glucagon-immunoreactivity was demonstrated in the basal-granulated cells in the deeper portion of the fundic gland of the stomach as well as in the A cells of the pancreatic islet. GLI-positive basal-granulated cells were found in the fundus of the stomach, jejunum, ileum and colon. None of them were found in the pyloric antrum and duodenum. Careful examination of the immunofluorescence specimens gave us the impression that essentially every GLI-positive cell in the gastric fundus and pancreas contained glucagon.

It was observed that the GLI-positive cells in the jejunum, ileum and colon were open in type reaching the lumen with their tapered luminal process, whereas the glucagon/GLI-positive cells in the stomach were always separated from the lumen by a layer of other epithelial cells so that they were closed in type.

The possible difference in the way of stimulus-reception between the GLI-positive cells in the intestine and glucagon/GLI-positive cells in the stomach was discussed with particular reference to the idea that basal-granulated cells could receive information for their secretory activity not only from the gut lumen but also from the blood side.

In 1949, Sutherland and De Duve demonstrated that the extracts of the dog gastrointestinal tissues had the glucagon-like hyper-glycemic-glycogenolytic activity. Unger and his co-workers (1961) confirmed the original observations of Sutherland and De Duve by the technique of radioimmunoassay and named the material cross-reactive to the anti-glucagon serum glucagon-like immunoreactivity (GLI, Unger, et al., 1968). Since then, it has been a matter of dispute whether the material with GLI contains glucagon. Recent radio-immunoassay studies using a specific antiserum to glucagon by Vranic, Pek and Kawamori (1974) and Matsuyama and Foá (1974) demonstrated hyperglucagonemia occurring in depancreatized dogs untreated with insulin. Mashiter and his co-workers (1975) revealed that plasma glucagon level in depancreatized dogs rose in response to intravenous infusion of L-arginine and that plasma glucagon level in the portal vein was higher than that in the femoral artery. Furthermore, Sasaki and his co-workers (1975) found in the porcine gastrointestinal extracts a component that was indistinguishable from pancreatic glucagon by immunological and biological methods. All these findings suggest that glucagon exists in the extra-pancreatic tissues, particularly in the gastro-intestinal tract.

As to the cellular origin of GLI, Polak and her co-workers (1971) reported on the basis of the results of their immunofluorescence studies that GLI-positive cells existed in the gastric fundus, jejunum, ileum and colon in the dog. However, since they did not distinguish GLI-cross-reactive antisera from glucagon-specific antisera, it remained obscure whether their GLI-positive cells possessed glucagon. In an attempt to
solve this problem, Larsson and his co-workers (1975) examined the gut and pancreas of the pig, cat, dog and rat using two different kinds of anti-glucagon antisera, i.e., glucagon-specific antisera and GLI-cross-reactive antisera. They found cells reacting to both of the two kinds of anti-glucagon antisera (glucagon/GLI-positive cells) in the fundus of the stomach. On the other hand, cells reacting only to GLI-cross-reactive antisera (GLI-positive cells) were found in the duodenum, jejunum, ileum and colon.

The first purpose of the present study was to ascertain the observations of Larsson and his co-workers (1975) on the occurrence of the glucagon/GLI- and GLI-positive cells in the canine gut and pancreas, by the use of the glucagon-specific and GLI-cross-reactive antisera prepared in our laboratory (Ito, Suzuki and Sasaki, 1975). The second purpose of the study was to examine the morphological differences between the glucagon/GLI-positive cells in the stomach and GLI-positive cells in the intestine, and to discuss the functional significance of these differences.

**Materials and Methods**

**Anti-glucagon antisera**

*Preparation of glucagon-specific antisera.* Assan and Sluscher (1972) showed that glucagon has two antigenic sites inside the molecule. One is located at the C-terminal and the other at the N-terminal and central portions of the molecule. Since it was demonstrated that glutaraldehyde bridges free amino groups of different protein molecules (Richards and Knowles, 1968), it might be assumed that glutaraldehyde modifies lysine moieties in the N-terminal and central portions of glucagon and destroys the antigenicity located at these portions of the molecule which are the sites most often bound by GLI-cross-reactive antisera (Assan and Sluscher, 1972).

In the present study, we prepared antisera specific to the C-terminal portion of glucagon molecule which lacks free amino groups by following procedures: (1) Crystalline glucagon (12 mg, Sigma) dissolved in 0.3 ml of 0.02 N hydrochloric acid was added to a mixture of 1 ml of NN-dimethyl-formamide and 0.7 ml of 0.1 M phosphate buffer of pH 7.4 containing 4 mg of bovine serum albumin (BSA, Sigma). (2) Glutaraldehyde (0.5% aqueous solution, 0.05 ml) was added to this mixture and allowed to stand for 2 hrs at room temperature. (3) Then, this glucagon-glutaraldehyde-BSA mixture was dialized for 24 hrs against 1 l of 0.1 M potassium phosphate buffer of pH 7.4. (5) The dialized material was emulsified with an equal amount of complete Freund Adjuvant (Difco). (6) A subcutaneous injection of glucagon-glutaraldehyde-BSA conjugate was performed once a month for 5 months in 4 healthy adult rabbits (G-1, G-5, G-7 and G-8). Two weeks after the final booster, about 1 dl of blood was collected from each rabbit and the blood serum containing anti-glucagon antibodies was prepared according to a routine method.

*Preparation of GLI-cross-reactive antisera.* As mentioned above the anti-glucagon antisera prepared by the glutaraldehyde method most probably react to the C-terminal portion of glucagon and mask the antigenicity of this portion of the molecule.

In order to prepare antisera specific for both the N-terminal and central portions of glucagon, a glucagon-anti-glucagon antibody complex prepared by incubating 2 mg of glucagon, 5000 units of Trasylol (Bayer) and 2 ml of the anti-glucagon antisera at 4°C for one day, was injected subcutaneously into three rabbits (AE-1, AE-2,
Glucagon and GLI Cells in the Canine Gut and Pancreas

AE-3). Forty days after the first injection, glucagon emulsified with 2 ml of Freund Adjuvant was injected into the animals, in place of the glucagon-anti-glucagon antibody complex which was used in the first injection. Two weeks after the fifth injection of the glucagon-Freund Adjuvant complex, sera containing GLI-cross-reactive antibodies were prepared.

GLI-extracts. The GLI-extracts used in the present study were prepared by the method described by Kenny (1955).

Specificity of the anti-glucagon antisera. Firstly, the sensitivity of anti-glucagon antisera produced by the two methods described above was examined by radioimmunoassay. Glucagon-specific antisera and GLI-cross-reactive antisera obtained from the rabbits, G-8 and AE-1 respectively, were the most sensitive among the samples examined. These two antisera then were used throughout this study after the examination of the cross-reactivity to the GLI-extracts using a dilution slope of radioimmunoassay (Table 1).

Table 1. The cross-reactivity of two anti-glucagon antisera used in the present study to GLI extracts (Ito, Suzuki and Sasaki, 1975)

<table>
<thead>
<tr>
<th>GLI extracts (dry weight)</th>
<th>Specific antisera (G-8)</th>
<th>Cross-reactive antisera (AE-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µg/ml</td>
<td>100 pg/ml</td>
<td>more than 2 ng/ml</td>
</tr>
<tr>
<td>50</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>not detectable</td>
<td>500 pg/ml</td>
</tr>
<tr>
<td>5</td>
<td>not detectable</td>
<td>250</td>
</tr>
<tr>
<td>2.5</td>
<td>not detectable</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>not detectable</td>
<td>35</td>
</tr>
</tbody>
</table>

The values for G-8 and AE-1 antisera are calculated on the basis of dilution curve of radioimmunoassay for glucagon.

Immunofluorescence histochemistry

Pieces of the gastro-intestinal tract and pancreas obtained from adult, male mongrel dogs were fixed in Bouin’s fluid for 2 hrs at room temperature and dehydrated through a graded ethanol series and embedded in paraffin (melting point, 42°C). Sections of 6 µm thick were cut on a cryo-microtome (Leitz, Cryomat) and mounted on glass slides coated with a mixture of 0.5% gelatin and 0.05% chromium potassium sulfate.

Sections deparaffinized in xylene were treated with either the glucagon-specific antisera obtained from the G-8 rabbit or GLI-cross-reactive antisera obtained from the AE-1 rabbit in the final dilution of 1/15 in 0.01 M phosphate buffered physiological saline at pH 7.4 for 90 min at 37°C. These sections were incubated with the antirabbit IgG-FITC for 40 min at 37°C and examined in the fluorescence microscope.

Control study

In order to examine the specificity of the reaction between the glucagon-specific and GLI-cross-reactive antisera used in the present study and the tissue glucagon and
Results

Cells reacting to the glucagon-specific antisera

In the pancreas, cells reacting to the glucagon-specific antisera were found mainly in the periphery of the islet (Fig. 1). They also occurred in the extra-islet tissues. Their distribution seemed to coincide with that of A-cells examined by conventional histological methods.

In the stomach, specific immunofluorescence was found in the basal-granulated cells in the deeper portion of the fundic gland. These cells were oval in shape and did not reach the lumen of the gland (Fig. 3).

Glucagon-positive basal-granulated cells were not demonstrated in other portions of the gut examined, i.e., the duodenum, jejunum, ileum and colon.

Cells reacting to the GLI-cross-reactive antisera

In the pancreas, immunofluorescence for the GLI-cross-reactive antisera was frequently seen in the cells located in the marginal portion of the islet. A few GLI-
Fig. 2. Cells reacting to a GLI-cross-reactive antiserum, AE-1, in the pancreatic islet of the dog. Their distribution is identical with that of the glucagon-cells shown in Figure 1. ×660

Fig. 3. A glucagon-positive cell in the gastric fundus of the dog (white arrows). It is localized in the bottom of the fundic gland. ×660

Fig. 4. GLI-positive cells in the gastric fundus of the dog. They are seen mainly in the bottom of the fundic gland. Weakly autofluorescent cells in this micrograph are chief cells of the fundic gland. ×660
Fig. 5. GLI-positive cells in the ileum of the dog. They reach the cryptal lumen with their apical cytoplasmic process. Most of the small, strongly fluorescent structures in the connective tissue represent lipofuscin granules in the histiocyte and other connective tissue cells such as fibroblast and migrating leukocyte. ×660

Fig. 6. A GLI-positive cell in the ileum of the dog shown at a high magnification. The immunofluorescent reaction is seen only in the cytoplasm. This cell reaches the cryptal lumen with its apical process (open type). ×1,320

Fig. 7. GLI-positive cells in the proximal portion of the colon of the dog. Lumen of the intestinal crypt is shown by S. The inner circular and outer longitudinal muscle layers are indicated by cm and lm respectively. ×660
positive cells were also seen in the extra-islet tissues. Distribution and number of these cells corresponded to that of glucagon-positive cells in the pancreas mentioned above (compare Fig. 1 and Fig. 2).

In the stomach, GLI-positive cells were found only in the fundus. The majority of them were located in the base of the fundic gland. A small number of them were also seen among other epithelial cells in the neck of the gland. They appeared to be oval in shape and did not reach the lumen of the gland (Fig. 4). By a careful examination of the specimen, the GLI-positive cells in the stomach coincided in their distribution and morphology to the glucagon-positive cells mentioned above. Thus, it seemed probable that a single type basal-granulated cells reacted to both glucagon-specific and GLI-cross-reactive antisera.

In the jejunum, ileum and colon, GLI-positive basal-granulated cells occurred mainly in the deeper portion of the crypt. Population density of them was decreased in the order of the ileum, jejunum and colon. They clearly reached the cryptal lumen with their tapered cytoplasmic process (Fig. 5–7).

Control study

As the immunofluorescence was not found in the sections used in the control studies, the specificity of the reaction between the glucagon-specific and GLI-cross-reactive antisera used in the present study and tissue glucagon and GLI was confirmed.

Discussion

In the present study, we demonstrated basal-granulated cells reacting to both glucagon-specific and GLI-cross-reactive antisera in the fundus of the canine stomach. These cells are identical with the pancreatic A cells at least from the immunohistochemical point of view. On the other hand, in the intestine we found only GLI-positive cells. Thus, our results confirmed the description of Larsson and his co-workers (1975) who claimed that glucagon/GLI-positive cells existed in the canine fundic gland and that GLI-positive cells in the intestine were devoid of glucagon.

According to Fujita and Kobayashi (1973), the open type basal-granulated cells are able to receive adequate chemical stimuli in the gut lumen with their apical process, while closed type of them cannot (see, Fig. 8). Thus, the GLI-positive cells in the intestine would be able to recognize the luminal chemical stimuli, whereas the glucagon/GLI-positive cells in the stomach would not, the latter being separated from the lumen by a layer of other epithelial cells.

Unger and his co-workers (1968) reported that the intraduodenal administration of glucose in the dog stimulated the secretion of GLI from the gut, however, the intravenous injection did not. It was also reported that an oral glucose load in the normal persons elevated the plasma GLI level, although no change or even a drop was found in the level of glucagon (Heding, 1968, 1971). In gastrectomized patients as compared with healthy subjects, the increase of plasma GLI after oral administration of glucose is conspicuous (Shima et al., 1972). All these data reported by the previous authors might well be accounted for by the present observations that GLI-positive basal-granulated cells in the intestine contain no glucagon and are open in type.

Fujita and Kobayashi (1973) have postulated that the secretion of hormones of
the closed type basal-granulated cells could be caused by the physical stimuli in the gut lumen such as the distension of the cells accompanying the uptake of foodstuff. It might be assumed that glucagon released from the glucagon/GLI-positive cells in the fundic mucosa by a mechanical effect of meal could, passing through the liver, increase glycogenolysis in the liver cells and further, after entering the general circulation, enhance insulin release from the pancreatic islets.

According to Fujita and Kobayashi (1973) (Fig. 8), both open and closed types of basal-granulated cells can receive information for their secretory activity from the blood front in addition to the luminal surface, although the kinds of blood born adequate stimuli should be different from cell type to cell type as in the case of the luminal stimuli.

Raised plasma glucose levels remarkably inhibit the release of glucagon (Ohneda et al. 1969), while their influence on the plasma GLI-levels is indistinct (Unger et al., 1968). This is consistent with the idea that the GLI-positive cells in the intestine,
which are open in type, have blood born adequate stimuli different from those to pancreatic A cells and/or gastric glucagon/GLI-positive cells, both of which are closed in type.

MASHITER and his co-workers (1975) demonstrated a remarkable increase in the plasma level of the reactivity to the glucagon-specific antisera in the depancreatized dog after an intravenous injection of L-arginine. It seems probable that the release of glucagon took place from the glucagon/GLI-positive cells in the stomach of these animals, because they had no pancreatic islets. The result of the experiment of MASHITER and his co-workers (1975) in the depancreatized dog favors the view that the closed type basal-granulated cells can release hormones in response to the blood born stimuli.

In our previous study (ITO, SUZUKI and SASAKI, 1975), we failed to demonstrate glucagon-positive cells in the canine gut fixed in one of the following three kinds of fixatives: 2% carbodiimide solution, a mixture of 1% glutaraldehyde and 2% paraformaldehyde and 95% ethanol. The present positive result and previous negative one in the demonstration of glucagon-positive cells in the canine gut seems to be due to the different effect of the fixatives used on the preservation of the antigenicity of tissue glucagon.

References


