The distribution and frequency of endocrine cells in the splenic lobe of grass lizard *(Takydromus wolteri)*: An immunohistochemical study

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The regional distribution and frequency of the pancreatic endocrine cells in the splenic lobe of grass lizard, *Takydromus wolteri*, were studied by immunohistochemical (PAP) method using six types of specific mammalian antisera against bovine Sp-1/chromogranin (bCG), serotonin, insulin, glucagon, somatostatin and human pancreatic polypeptide (hPP). The pancreas was subdivided into two regions – islet kike and exocrine regions. The frequency of each immunoreactive (IR) endocrine cells was calculated as mean number/total 100 islet cells and as mean number/total 1000 cells (including exocrine and endocrine cells) using automated image analysis process. In addition, the percentage of each IR cell was also calculated. All of six endocrine cells were demonstrated. They were dispersed in the whole pancreatic parenchyma between exocrine acinar cells, or they were also observed as islet like clusters. In islet-like regions, bCG-, insulin- and glucagon-IR cells were detected as one or two cell layer cords and they were located between this cell-cords with 14.30±5.62, 61.50±9.76 and 26.50±9.31/100 cells frequencies, respectively. However, somatostatin-IR cells were mainly located in the peripheral parts not in cell-cords with 12.40±4.86/100 cells, and no serotonin- and hPP-IR cells were demonstrated. In exocrine regions, all of bCG-, serotonin-, insulin-, glucagon-, somatostatin- and hPP-IR cells were detected and they occurred mainly among the exocrine parenchyma as solitary cells with 10.30±2.54, 0.80±0.63, 15.50±5.30, 5.80±2.66, 3.10±1.29 and 11.00±3.33/1000 cells frequencies, respectively. In addition, serotonin-IR cells were mainly located between epithelia and connective tissue of pancreatic duct. Overall, there were 0.58±0.49% serotonin–, 56.44±9.35% insulin–, 23.73±8.22% glucagon–, 11.28±3.03% somatostatin– and 7.97±2.02% hPP-IR cells

Key words: grass lizard, *Takydromus wolteri*, pancreas, endocrine cell, immunohistochemistry.

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The grass lizard, *Takydromus wolteri* Fischer, belonging to the Laceridae in order Lacertinae is habited in east part of Russia and China with Korea and it is generally considered as being primitive palearctic lacertids, most closely related to *Lacerta vivipara*. They have distinct white ventrolateral band runs from tip of snot to groin. In Korea, the number and habitation of this grass lizard have been dramatically decreased because of pollution and immigration of other foreign species of amphibian and reptiles having similar feeding habits.

It is generally known that the pancreas of lacertids is could be subdivided into two parts as splenic and duodenal lobes (El-Sahly and Grimelius, 1981) and pancreas of vertebrates is subdivided into two portions, one is exocrine portions where digestive enzymes are released and the other is endocrine portions where regulatory hormones such as insulin, glucagon, somatostatin and pancreatic polypeptide (PP) are released into blood circulation. The appearance, regional distribution and relative frequency of these regulatory hormones secreted by endocrine cells in the pancreas were well recognized by Histochemistry (Kobayashi and Syed Ali, 1981), immunofluorescence method (Orci, 1982) and immunohistochemistry (Sternberger *et al.*, 1970). Except above regulatory hormones, peptide YY-, neuropeptide YY- (Ali-Rachedi *et al.*, 1984), motilin- (Yamada *et al.*, 1986), chromogranin family- (Rindi *et al.*, 1986; Ito *et al.*, 1987) and secretin- (Lee *et al.*, 2003) immunoreactive (IR) cells were also demonstrated in the vertebrate pancreas. The pancreas has been treated as a valuable organ for endocrine studies, therefore endocrine pancreas has been extensively studied, associated with diabetes (Jansson and Sandler, 1988). In addition, the investigations of GEP endocrine cells have been considered as an important part of a phylogenetic studies (D'Este *et al.*, 1994).

Until now, the regional distribution and relative

frequency of major four types of endocrine cells, insulin, glucagon, somatostatin and PP, were reported in the lizard pancreas such as lacertid lizards (Della Rossa and Putti, 1995), desert lizard *(Chalcides ocellatus and Uromasyx aegyptia)* (El-Salhy and Grimelius, 1981; El-Salhy *et al.*, 1983), green anole *(Anolis carolinensis)* (Rhoten and Hall, 1982) and grass lizard *(Mabuya quinquetarniata)* (El-Salhy and Grimelius, 1981) . In addition, peptide tyrosine tyrosine- and neuropeptide tyrosine- (Della Rossa and Putti, 1995) and chromogranin (CG)- (Trandaburu *et al.*, 1999) IR cells were found in the lacertid lizard pancreas and new types of endocrine cells have been reported in the pancreas of the various vertebrates.

With the increasing demands of diabetic animal models and/or usefulness of irradiation in many fields, the regional distribution and relative frequency of pancreatic endocrine cells, especially insulin- and glucagon-producing cells in the laboratory animals have been concerned in recent years (Warbritton *et al.*, 1994; Gomez-Dumm *et al.*, 1995; Fu *et al.*, 1996). Many researchers suggested that species-dependent characteristic distribution of cells producing different hormones in the pancreas of each species of animals might be due to feeding habits and now it is generally accepted (Wieczorek *et al.*, 1998).

It was also reported that different regional distribution and relative frequency of endocrine cells in the pancreas were demonstrated in different portions even if they were (the) same pancreas of same lizard (Putti *et al.*, 1992) and they showed species-dependent characteristic distributional patterns in lizard species (El-Salhy and Grimelius 1981). In addition, more numerous and well-organized pancreatic endocrine systems are detected in the splenic lobes compared to that of duodenal lobes (El-Sahly and Grimelius, 1981). Despite of biological, physiological and anatomical differences between the grass lizard, *Takydromus wolteri*, and other lacertid species, reports have rarely dealt with the endocrine cells in the pancreas of this species. However, the distribution and relative frequency of some gastrointestinal endocrine cells in this species are reported (Lee and Ku, 2004). And, with the exception of the desert lizard (El-Salhy *et al.*, 1983), quantitative studies of the lacertid pancreas are scarce.

The object of this study was to determine the regional distribution and quantitative frequency of the endocrine cells in the splenic lobe of pancreas of grass lizard by immunohistochemistry using antisera specific for bovie Sp-1/chromogranin (bCG), serotonin-, insulin, glucagon, somatostatin and human PP (hPP).

Materials and Methods

Ten adult (45 ~ 50mm in length) grass lizards of the Laceridae, *Takydromus wolteri* Fischer, were captured around Kyungpook, Korea and both males and females were used in this study. After phlebotomy from head, the splenic lobes of pancreas were sampled according to El-Sahly and Grimelius (1981) and fixed in Bouin's solution. After paraffin embedding, 3-4 μm serial sections were prepared with routine methods. Each section was deparaffinized, rehydrated and stained with hematoxylin and eosin for light microscopic examination of the normal alimentary architecture.

The deparaffinised and rehydrated sections were treated with methanol containing 0.3% H_2O_2 for 30 min to block any endogenous peroxidase. Subsequently, the sections were incubated for 1 hr at room temperature, in normal goat serum (1:100), then stained immunohistochemically to identify specific endocrine cells using peroxidase anti-peroxidase (PAP) method (Sternberger, 1979). In the first layer, the sections were incubated with antisera specific for individual pancreatic hormones for 12 hrs at 4°C. Details of specific antisera used as the first layer are listed in Table 1. After rinsed in phosphate buffered saline (PBS; 0.01M, pH 7.4 containing 0.05% tween), Anti-rabbit or –guinea pig IgG serum raised in goat (Sigma, St. Louis MO, USA) was used as the second layer at 1:200 for 1hr at room temperature. They were then washed with PBS buffer and the PAP complex (Sigma, St. Louis MO, USA) was used as the third layer at 1 : 400 for 1 hr at room temperature. The peroxidase reaction was carried out in a solution 3,3'-diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2 in Tris-HCl buffer (0.05M, pH 7.6). After immunostained, the sections were lightly counterstained with Mayer's hematoxylin and the IR cells were observed under light microscope.

The specificity of each immunohistochemical reaction was determined as recommended by Sternberger (1979), including the replacement of specific antiserum by the same antiserum, which had been preincubated with its corresponding anti-

Table 1. Antisera used in this study

*Antisera raised**	*Code*	*Source*	*Diluton*
bCG	805398	Dia Sorin, Stillwater, USA	1:1000
Serotonin	B068082C	BioGenex Lab., San Ramon, USA	1:20
Insulin	842613	Diasorin, Stillwater, USA	1:2000
Glucagon	8240-0004	Biogenesis, Kingston, USA	1:800
Somatostatin	PU0421295	BioGenex Lab., San Ramon, USA	1:20
hPP[1)]	A619	DAKO corp., Carpenteria, USA	1:600

**All antisera were raised in rabbits except for insulin, which were raised in a guinea pig.[1)] bCG: bovine Sp-1/chromogranin; hPP: humane pancreatic polypeptide.*

gen. The frequencies of IR cells were calculated as mean ± standard deviation (S.D.) of 10 parts (n=10) of pancreatic parenchyma. In islet-like regions, among 100 endocrine cells, cells showing immunoreactivities against each antiserum were counted using automated image analysis process (Soft Image System, Germany) attached to light microscopy, and, in exocrine regions, among 1000 parenchymal cells, cells showing immunoreactivities against each antiserum were counted. In addition, the percentage of IR cells to each antiserum was also determined using counted IR cell numbers.

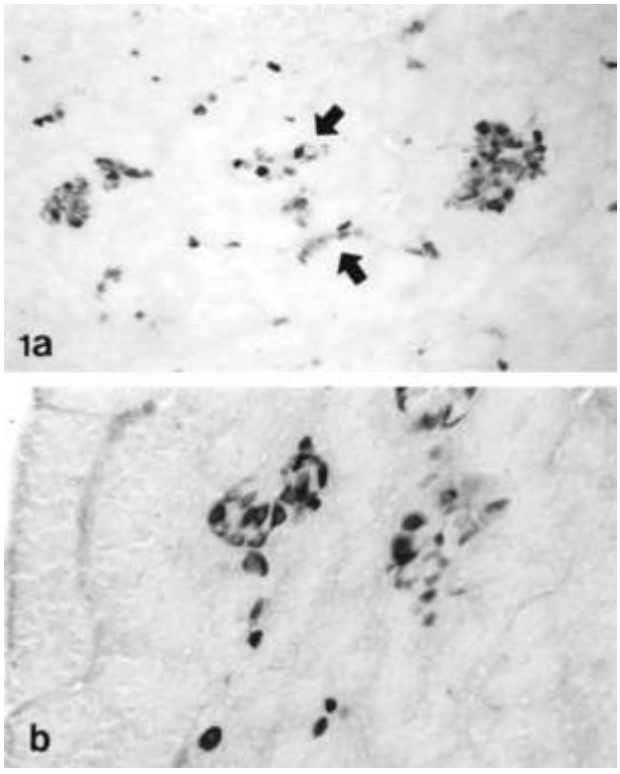


Figure 1. bCG-IR cells were distributed as solitary cells in the exocrine regions (a) and/or as clusters in the islet-like regions (b). In addition, they were also dispersed in some islet-like regions (a, arrows) of grass lizard. a. ×150; b. ×300; PAP method.

Results

In this study, six types of IR endocrine cells were detected with the antisera to bCG, serotonin, insulin, glucagon, somatostatin and hPP in the splenic lobes of pancreas of grass lizard. The pancreas was divided into exocrine and islets-like regions. Most of islet-like regions showed cord shaped consisted of one or two layers of cell-cords. According to the types of IR cells, different regional distribution and quantitative frequency were observed and these differences are shown in Table 2. Spherical to spindle or occasionally oval to round-shaped immunoreactive cells were located in the pancreas. They were dispersed in the whole pancreatic parenchyma between exocrine acinar cells, or they were also observed in islet-like regions.

Table 2. Quantitative frequencies of the endocrine cells in the pancreas of grass lizard, Takydromus wolteri

IR cells	*Number of IR cells in islet-like regions**	*Number of IR cells in exocrine regions***	*Percentage of each IR cells (%)*
bCG 1)	14.30±5.62	10.30±2.54	Not calculated
Serotonin	0.00±0.00	0.80±0.63	0.58±0.49
Insulin	61.50±9.76	15.50±5.30	56.44±9.35
Glucagon	26.50±9.31	5.80±2.66	23.73±8.22
Somatostatin	12.40±4.86	3.10±1.29	11.28±3.03
hPP 2)	0.00±0.00	11.00±3.33	7.97±2.02

*Quantitative frequencies were calculated using automated image analysis process (Soft Image System, Germany) attached to light microscopy; *Cell numbers/100 islet cells; **Cell numbers/1000 parenchymal cells; 1) bCG: bovine Sp-1/chromogranin; 2) hPP: human pancreatic polypeptide.*

bCG-IR cells

These cells were located in islet-like and exocrine regions. In case of exocrine, they were dispersed throughout the whole pancreas parenchyma, between exocrine acinar cells as solitary cells (Figure 1a). In case of islet-like regions, relatively small numbers cells were dispersed in the islets mainly in the central regions (Figure 1a, b) or they were situated in the cell-cords that consisted of one or two cell layers (Figure 1a, arrows). bCG-IR cells showed 14.30±5.62/100 cells frequencies in islet-like regions and 10.30±2.54/1000 parenchymal cells frequency in exocrine.

Serotonin-IR cells

These cells were restricted to exocrine pancreas especially to the region of the pancreatic duct

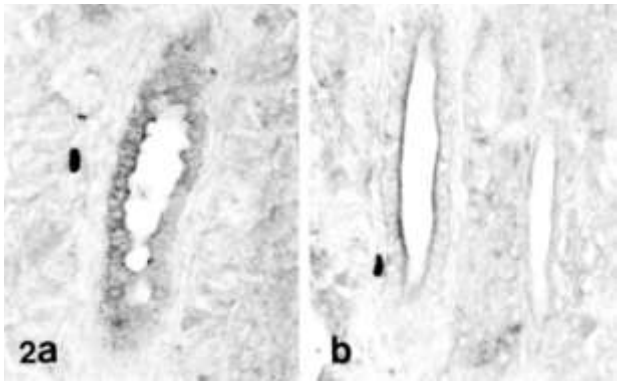


Figure 2. Serotonin-IR cells were distributed as solitary cells in the pancreatic duct between connective tissue and epithelia lining of grass lizard (a, b). a, b. ×300; PAP method.

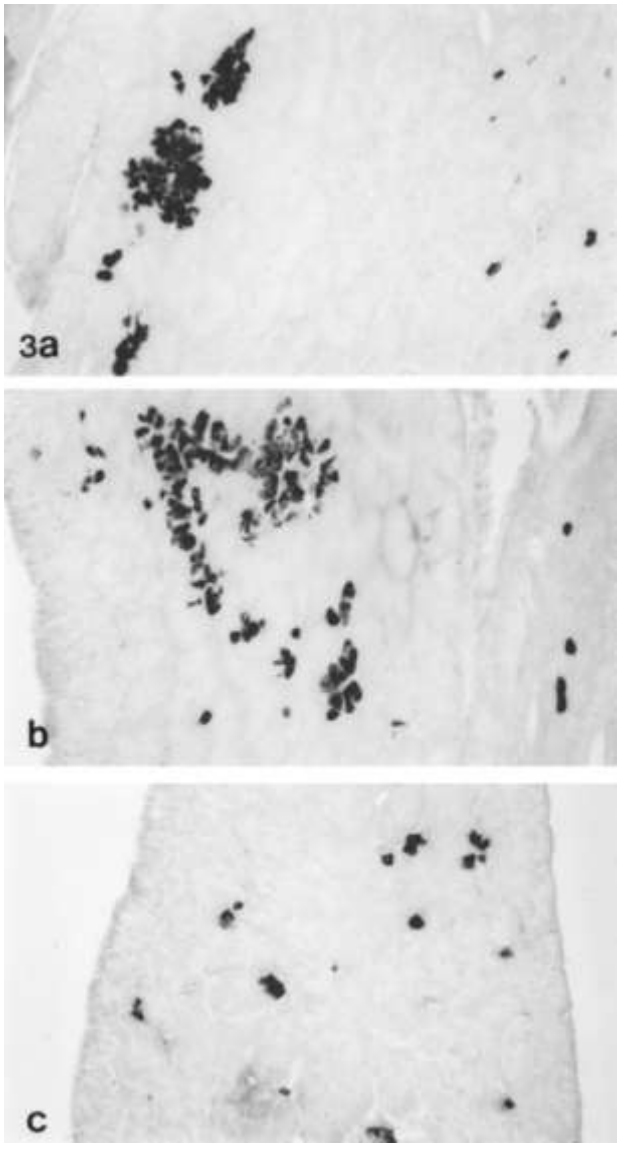
between connective tissue and epithelia (Figures 2a, b). Serotonin-IR cells showed a frequency of 0.80±0.63/1000 parenchymal cells frequency in exocrine and they occupied approximately 0.58± 0.49% of total IR cell population (Table 2). This is the least abundant cell type in the pancreas of this grass lizard.

Insulin-IR cells

These IR cells were located in islet-like and exocrine regions. In exocrine regions, they were dispersed throughout the whole pancreas parenchyma, between exocrine acinar cells as solitary or two to three cell clusters (Figures 3a-c). In case of islet-like regions, insulin-IR cells were situated in the cell-cords that consisted of one or two cell layers (Figure 3b). Insulin-IR cells showed 61.50± 9.76/100 cells frequencies in islet-like regions and 15.50±5.30/1000 parenchymal cells frequency in exocrine regions. Insulin-IR cells occupied approximately 56.44±9.35% of total IR cell population (Table 2). This is the most predominant cell type in the pancreas of this grass lizard.

Glucagon-IR cells

These cells were located in islet-like and exocrine regions similar to other types of IR cells. In case of exocrine regions, they were dispersed in pancreas parenchyma, between exocrine acinar cells as solitary or two to three cell clusters (Figure 4a). In case of islet-like regions, glucagon-IR cells were situated in the cell-cords that consisted of one or two cell layers (Figures 4b, c) similar to that of insulin-IR cells. Glucagon-IR cells showed 26.50± 9.31/100 cells frequencies and 5.80±2.66/1000



Figures 3. Insulin-IR cells were distributed as cell cords in the islet-like regions (a, b) and some cells were also dispersed in the exocrine regions as solitary and/or two to three cell clusters (a-c). a – c. ×150; PAP method.

parenchymal cells frequency in islet-like and exocrine regions, respectively. They occupied approximately 23.73±8.22% of total IR cell population (Table 2). This is the second predominant cell type in the pancreas of this grass lizard.

Somatostatin-IR cells

These IR cells were also located in islet-like and exocrine regions. Somatostatin-IR cells were dispersed in restricted pancreas parenchyma of exocrine regions between exocrine acinar cells as solitary or two cell clusters (Figure 5b). In case of

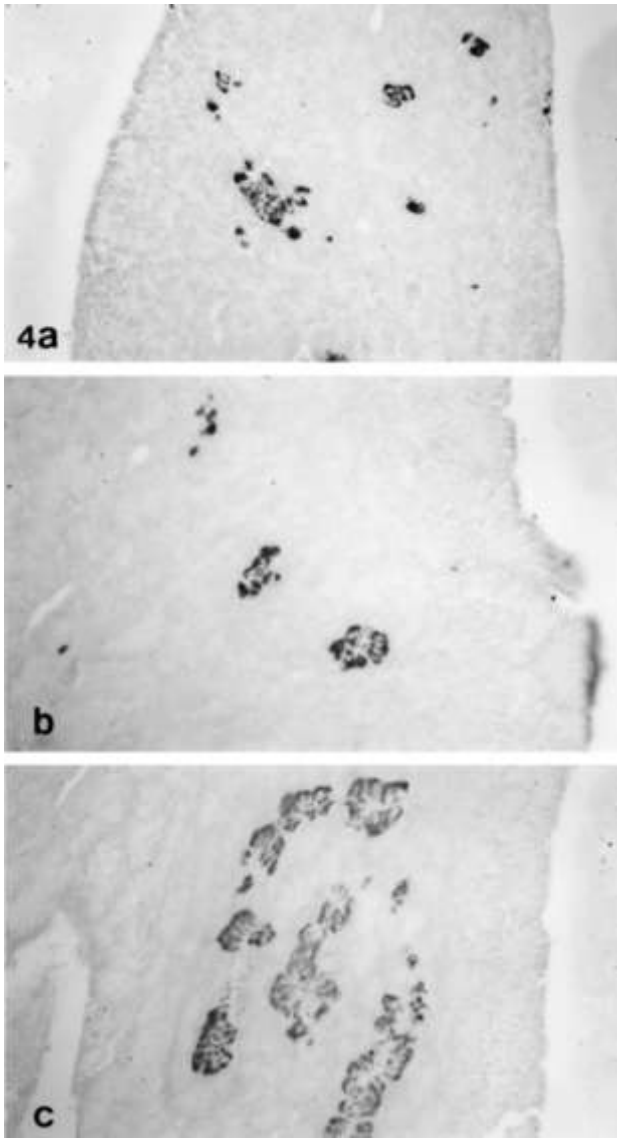


Figure 4. Glucagon-IR cells were distributed as single, two to two cell clusters in the exocrine regions of grass lizard (a). In addition, they were distributed as cell cords in the islet-like regions (b, c) of grass lizard. a – c. ×150; PAP method.

islet-like regions, most of somatostatin-IR cells were situated in the peripheral parts of cell-cords and relatively low frequencied cells were also observed in the cell-cords that consisted of one or two cell layers (Figure 5a). Somatostatin-IR cells showed 12.40±4.86/100 cells frequencies in islet-like regions and 3.10±1.29/1000 parenchymal cells frequency in exocrine. They occupied approximately 11.28±3.03% of total IR cell population (Table 2). This is the third most abundant cell type in the pancreas of this grass lizard.

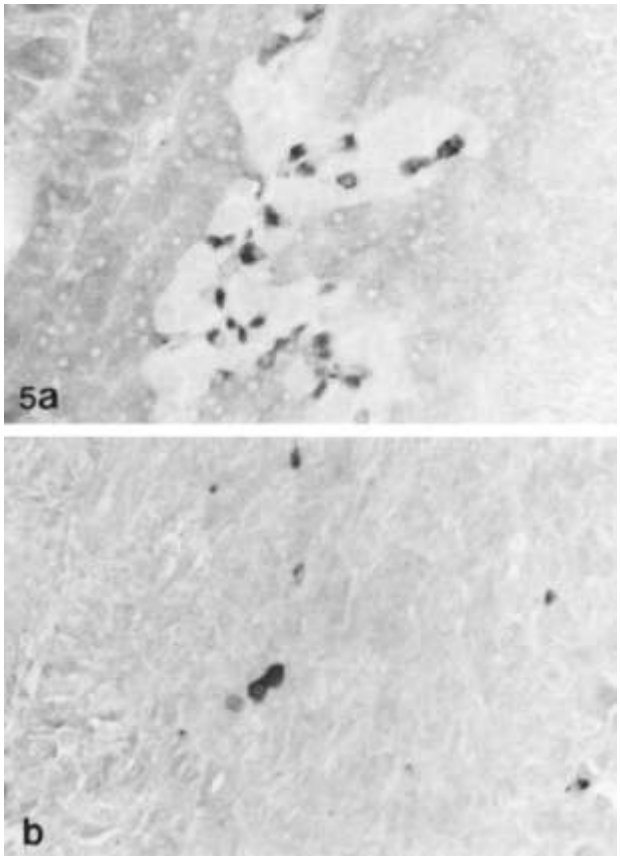


Figure 5. Somatostatin-IR cells were distributed in the outside, peripheral parts of cell cords in islet-like regions (a) and as solitary or two cell clusters in the exocrine regions (b) of grass lizard. a, b. ×300; PAP method.

hPP-IR cells

hPP-IR cells were restricted to the exocrine regions. They were dispersed throughout the whole exocrine pancreas parenchyma, between exocrine acinar cells as solitary cells (Figures 6a, b). hPP-IR cells showed 11.00±3.33/1000 parenchymal cells frequency in exocrine regions and they occupied approximately 7.97±2.02% of total IR cell population (Table 2). This is the fourth most abundant cell type in the pancreas of this grass lizard.

Discussion

In the splenic lobe of pancreas of the grass lizard, *Takydromus wolteri*, all of six endocrine cells were dispersed in the whole pancreatic parenchyma, between exocrine acinar cells or they were also observed in islet-like regions. The cord or duct-like pancreatic islets were specific observation in some

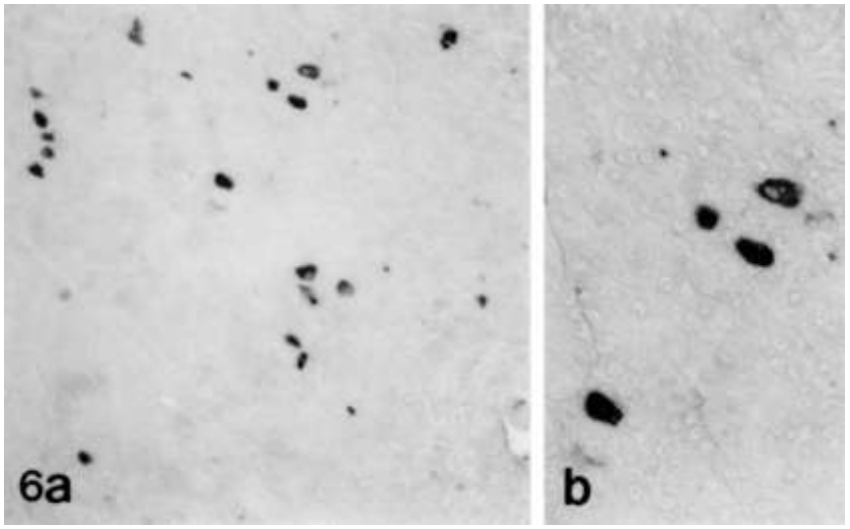


Figure 6. hPP-IR cells were distributed as single cells (a, b) in the exocrine pancreas of grass lizard. a. ×150; b. ×300; PAP method.

species of snake (Lee and Lee, 1992) but they were not generally demonstrated in the pancreas of lacertid lizards. However, in this study similar to that of some species of snake, most of islet-like regions showed cord shape and it is considered to be a species-dependent characteristic appearance.

bCG belongs to a family of large anionic proteins (CG A, B and secretogranin II), the members of which are known to be present in the secretory granules of a broad spectrum of amine and peptide-producing cells of adrenal medulla and gastroenteropancreatic (GEP) endocrine system, as well as in some neurons of the peptidergic and catecholaminergic nervous system of several mammals (Rindi *et al.*, 1986; Reinecke *et al.*, 1991). CGs have been found to occur in large variety of endocrine organs and cells outside the adrenal medulla, and they have been claimed as common "markers" of all neuroendocrine cells (Cohn *et al.*, 1984). In addition, Trandaburu *et al.* (1999) reported that CG A-IR cells were found only in the turtle pancreas among the four reptile species belonging to the turtles, lizards and snakes whereas secretogranin II-IR cells appeared both in the turtle and snake. In addition, no CG A-IR cells were demonstrated in the pancreas of red-eared slider (Ku *et al.*, 2000). In the present study, somewhat differed from previous studies (Trandaburu *et al.*, 1999; Ku *et al.*, 2000), bCG-IR cells were detected in the pancreatic islets and exocrine of this grass lizard. However, it is considered that single use of bCG is not suitable as a marker of endocrine cells in the pancreas of this grass lizard because a density of bCG-IR cells was lower than that of insulin- and glucagon-IR cells. Until now, observations of reptilian species were restricted, and the possibility of the usefulness of the CGs as a common marker for neuroendocrine cells in the pancreas of other reptilian species should be investigated.

Serotonin consisted of monoamines and was widely distributed in nervous system and GEP endocrine cells (El-Salhy *et al.*, 1985). Appearance of serotonin-IR cells showed species-dependent differences in the pancreas of the vertebrates (Ding *et al.*, 1991). In the reptilian pancreas including lacertid lizards, they were demonstrated in the exocrine regions especially to the peripheral region of duct epithelia lining with very lowered frequency (Perez-Tomas *et al.*, 1989; Ding *et al.*, 1991; Ku *et al.*, 2000). Similar to these previous studies (Perez-Tomas *et al.*, 1989; Ding *et al.*, 1991; Ku *et al.*, 2000), serotonin-IR cells were restricted to the pancreatic duct of this grass lizard and they showed the least abundant frequency.

Insulin is synthesized in the B cells of the pancreatic islets and regulates the serum glucose levels (Hsu and Crump, 1989). In the pancreas of the reptilian species, insulin-IR cells were present as solitary cells or grouped in the pancreatic islets and they were located in the central core of the pancreatic islets (Perez-Tomas *et al.*, 1989; Morescalchi *et al.*, 1997). And these IR cells were located in the islet center and comprised 3% of dorsal and 0.2% of ventral lobe volume in the squamate reptile, the desert lizard (El-Sahly *et al.*, 1983). In addition, Putti *et al.* (1992) reported that insulin-IR B cells appeared in 11 species, 3 genera of lacertids and different distributional patterns were seen in the

pancreatic islets but these IR cells were mainly distributed in the central core of the pancreatic islets. From these previous reports (El-Sahly *et al.*, 1983; Perez-Tomas *et al.*, 1989; Putti *et al.*, 1992; Morescalchi *et al.*, 1997), insulin-IR cells that were the most predominant cell types in pancreas were located in the central core of the pancreatic islets, some cells were scattered in the inter acinar regions of the exocrine pancreas. Similar to those of other reptilian species, insulin-IR cells were the most predominant cell types and they were observed in the exocrine pancreas in this grass lizard. But the appearance in islet-like regions and distributional patterns in this islet-like region considered as species-dependent distributional patterns.

Glucagon is synthesized in the A cells of the pancreas and regulates glucose levels in blood (Hsu and Crump, 1989). Glucagon-IR cells were distributed in the peripheral mantle zone of the pancreas of 11 species of lacertids (Putti *et al.*, 1992) and some of these IR cells were located in the central portions of the pancreatic islets. In the turtles, no glucagon-IR cells were detected in the central portion of the pancreatic islets (Perez-Tomas *et al.*, 1989; Ku *et al.*, 2000). In addition, these IR cells were distributed in the peripheral regions of the pancreatic islets, exocrine pancreas and pancreatic duct of the *Testudo graeca* (Garcia-Ayala *et al.*, 1987). Similar to those of other reptilian species, glucagon-IR cells were the second most predominant cell types and most of these IR cells were observed in the exocrine pancreas but the appearance in the islet-like regions and distributional patterns were considered as species-dependent distribution patterns.

Somatostatin, which consisted of 14 amino acids, was isolated from hypothalamus of sheep for the first time. It could be divided into straight form and cyclic form (Brazeau *et al.*, 1973). In the present study, most of somatostatin-IR cells were dispersed in the exocrine pancreas and located in the outside regions cell-cords in the islet-like regions. Different from the present study, somatostatin-IR cells in the pancreatic islets of the reptilian species were located in the peripheral region of the 11 species of lacertids (Putti *et al.*, 1992), desert lizard (El-Sahly *et al.*, 1983) and anolian lizard (Rhoten and Smith, 1978). And also they were dispersed in the exocrine pancreas (Rhoten and Smith, 1978; El-Sahly *et al.*, 1983; Putti *et al.*, 1992). Somatostatin-IR cells showed the fourth highest frequency in the present study. These results were quite similar to those of desert lizard (El-Sahly *et al.*, 1983). However, the distributional patterns in the islet-like regions were considered as a species-dependent characteristic of this grass lizard.

PP is a peptide hormone containing 36 amino acids, which is synthesized by F cells in the pancreatic islets (Hsu and Crump, 1989). Since PP-IR cells have been described for the first time in the lizard pancreas (Rhoten and Smith, 1978), the occurrence of these cells have been demonstrated in the pancreas of the reptiles (Rhoten and Smith, 1978; El-Sahly *et al.*, 1983; Agulleiro *et al.*, 1985; Garca-Ayala *et al.*, 1987; Putti *et al.*, 1992; Ku *et al.*, 2000). From these previous results, PP-IR cells were dispersed in the exocrine pancreas in a case of the reptilian species but they showed different distribution according to sampling portions (El-Sahly *et al.*, 1983). Similar to those of the previous reports, hPP-IR cells were restricted to the exocrine pancreas of this grass lizard. They showed the fourth highest frequency and these results were corresponded to those of desert lizard (El-Sahly *et al.*, 1983).

In conclusion, distributional patterns of IR cells in the splenic lobe of pancreas of this species of grass lizard were quite similar to those of other reptilian species but the cord-shaped islet-like regions showing different morphology and distributional patterns of endocrine cells of this grass lizard was considered as species-dependent characteristics.

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