

## Insulin and Glucagon Secretions, and Morphological Change of Pancreatic Islets in OLETF Rats, a Model of Type 2 Diabetes Mellitus

This study was performed to observe the changes of glucose-related hormones and the morphological change including ultrastructure of the pancreatic islets in the male Otsuka Long-Evans Tokushima Fatty rat. Area under the curve (AUC) of glucose at the 30th ( $709 \pm 73$  mg.h/dL) and at the 40th week ( $746 \pm 87$  mg.h/dL) of age were significantly higher than that at the 10th week ( $360 \pm 25$  mg.h/dL). AUC of insulin of the 10th week was  $2.4 \pm 0.9$  ng.h/mL, increased gradually to  $10.8 \pm 8.3$  ng.h/mL at the 30th week, and decreased to  $1.8 \pm 1.2$  ng.h/mL at the 40th week. The size of islet was increased at 20th week of age and the distribution of peripheral alpha cells and central beta cells at the 10th and 20th weeks was changed to a mixed pattern at the 40th week. On electron microscopic examination, beta cells at the 20th week showed many immature secretory granules, increased mitochondria, and hypertrophied Golgi complex and endoplasmic reticulum. At the 40th week, beta cell contained scanty intracellular organelles and secretory granules and apoptosis of acinar cell was observed. In conclusion, as diabetes progressed, increased secretion of insulin was accompanied by increases in size of islets and number of  $\beta$ -cells in male OLETF rats showing obese type 2 diabetes. However, these compensatory changes could not overcome the requirement of insulin according to the continuous hyperglycemia after development of diabetes.

**Key Words :** Rats, Inbred OLETF; Diabetes Mellitus, Type 2; Insulin; Glucagon; Islets of Langerhans; Ultrastructure

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## INTRODUCTION

Experimental animal of this study, Otsuka Long-Evans Tokushima Fatty (OLETF) rats were discovered in an outbred colony of Long-Evans rats and had a congenital defect of the cholecystokinin-A receptor gene. OLETF rats characteristically begin to gain weight rapidly from 5 weeks of age, show insulin resistance at 8 to 9 weeks, and finally develop obese type 2 diabetes. The cumulative incidence of diabetes in male OLETF rats over age 23 weeks are 86% (1-5).

Several studies have demonstrated that chronic hyperglycemia could impair both insulin secretion and insulin action. Ultimately, worsening of insulin resistance and impairment of beta cell function cause diabetes (6-14).

Induction of beta cell proliferation in male OLETF rats by partial pancreatectomy was poor compared to diabetes-resistant counterparts, Long-Evans Tokushima Otsuka (LETO) rats (15). This result in OLETF rats suggests that genetic background is of considerable importance. However, further study is necessary to observe metabolic and morphological

changes during the natural course of diabetes.

The pancreas derives from the upper, duodenal part of the foregut via evagination of endoderm and is formed by differentiation of acinar and islet cells. Some pancreatic endocrine cells, secreting insulin, somatostatin, and glucagon, develop via a budding process from embryonic duct-like cells, forming primitive islet cells within the hepatic lobule in the periphery of pancreatic duct. The formation of pancreatic islet and acinar cell is related with the proliferation of mature islet cells together with the differentiation and neogenesis of embryonic ductal cells. Pancreatic beta cell mass is dynamically changed after the birth, and this phenomenon is thought as an adaptation for maintenance of euglycemia (16).

Many studies were performed to investigate changes during the progress of diabetes, and some studies were done on the morphological changes of islets by immunohistochemical staining method (17-21). However, studies on ultrastructure of islets during the progress of diabetes are rare (22-24).

In type 2 diabetes, hepatic glucose production at fasting and postprandial states is increased by the decrease of hepat-

ic insulin sensitivity (12, 25, 26). However, it is not true in the early stage of non-obese type 2 diabetes (27-29). Inappropriate suppression of glucagon secretion is accompanied by the decrease of beta cell function in the progress of diabetes (6-8).

In this study, we aimed to observe the change of glucose-related hormones in male OLETF rats during the progress of diabetes, and the morphological change using light microscopy (LM) and electron microscopy (EM). The another purpose of the present study was to determine whether the changes of islet cells were related with the progress of diabetes by measuring the alpha cell, the most abundant non-beta-cell in pancreatic islet.

## MATERIALS AND METHODS

### Animal care

Twenty one 4-week-old male OLETF rats were used in this study. All animals were maintained in our animal facilities under specific pathogen-free condition and supplied with standard rat chow (Purina-Korea, Pyungtaek, Korea) and tap water ad libitum. We weighed each animal at 2-4 weeks intervals.

### Metabolic changes

We analyzed the only available data of oral glucose tolerance test (OGTT) at the 10th (n=5/25), the 20th (n=5/20), the 30th (n=5/10) and the 40th (n=6/10) week of age. After a 12- to 15-hr overnight fast, the animals were prepared under light ether anesthesia. And 50% glucose solution, 2 g of glucose/kg of body weight was administered per os and blood was sampled from the tail vein for the measurement of glucose, insulin, and glucagon at 0, 60, and 120 min. All samples were placed on ice and centrifuged at 4°C, and serum was separated and stored at -70°C until assay. Glucose concentration was measured by the automated glucose oxidase method (Beckmann, Brea, CA, U.S.A.). Rats were diagnosed as diabetic when their peak blood glucose level was  $\geq 300$  mg/dL (16.7 mM) and 120 min blood glucose level was  $\geq 200$  mg/dL (11.1 mM) and as having impaired glucose tolerance (IGT) when either one of the two criteria were met (1). All animals used in this study after the 20th week, became diabetic at the 20th week of age. Plasma insulin and glucagon were measured by radioimmunoassay (Linco research Inc., St. Louis, MO, U.S.A.) (29).

Area under the curve (AUC) of glucose, insulin, and glucagon at the 10th, 20th, 30th, and 40th week was calculated by the values measured each time during the OGTT as follows.

$$\text{AUC} = \{1\text{-hr value} + (\text{fasting value} + 2\text{-hr value})/2\} \times 1 \text{ hr}$$

### Tissue preparation for light microscopy

Two rats were sacrificed at the 10th (pre-diabetic stage), the 20th (diabetic stage), and the 40th (post-diabetic stage) week of age, and their pancreatic islets were observed under LM or EM.

The material was obtained from pancreatic tail of OLETF rats (n=2) at the 10th, 20th, and 40th week, fixed for 10 hr in Bouin's solution, and embedded in paraffin. Each pancreatic block was serially sectioned (4  $\mu\text{m}$  thickness) throughout its length to avoid any bias from the variations of islet distribution or cell composition and then mounted on slides. For each pancreas, four sets of serial sections were randomly chosen at a fixed interval through the block (every 20th section, about 320  $\mu\text{m}$  apart). Adjacent sections were stained for hematoxylin-eosin and immunohistochemical identification of insulin and glucagon (1, 2, 30, 31). Area and mean diameter of pancreatic islets were measured by computer image analyzer, Image-pro plus (Media cybernetics®, Silver Spring, MD, U.S.A.). Mean diameter was calculated by mean value of the longest and shortest diameters of each islet. All islets were divided into 3 groups (large,  $>150$   $\mu\text{m}$ ; medium, 100-150  $\mu\text{m}$ ; small,  $<100$   $\mu\text{m}$ ) by mean diameter and compared in each age group.

### Immunohistochemistry

Sections were deparaffinized with xylene and ethanol, and rehydrated with phosphate-buffered saline (PBS) for 5 min. Sections were treated with 3%  $\text{H}_2\text{O}_2$  (auto-blocker) solution for 15 min and then incubated with protein blocker from Novostatin Super ABC kit (Novocastra Lab., Bagsvaerd, Denmark) for 20 min. The sections were incubated for 1 hr with guinea pig anti-insulin serum, predilution DAKO LSAB® kit (DAKO Corp., Carpinteria, CA, U.S.A.) or rabbit anti-glucagon serum (final dilution 1:30, Novocastra Lab., Bagsvaerd, Denmark). Thereafter, sections were incubated for 30 min with peroxidase-conjugated secondary antibody from ABC kit (Novocastra Lab., Bagsvaerd, Denmark) and incubated with A+B solution from ABC kit for another 30 min. The activity of the antibody-peroxidase complex was revealed with 3,3'-diaminobenzidine-tetrahydrochloride using a peroxidase substrate kit (DAB; final dilution 1:20, ScyTek, Logan, Utah, U.S.A.). A standard concentration of hematoxylin was added as a counterstain. After staining, sections were mounted in glycerol. Numbers of beta cell and alpha cell were counted under LM and the changes in distribution of both islet cells in randomly selected 10 islets from pancreatic section were examined ( $\times 400$ ).

### Preparation for EM

Pieces of pancreatic tail (2  $\times$  2 mm) were fixed by immersion in a solution of 1% paraformaldehyde (Merck, KGaA,

Germany), 2% glutaraldehyde (Merck, KGaA, Germany), and 20 mg/mL calcium chloride (Yakuri Pure Chemical Co., Osaka, Japan) in 0.1 M cacodylate (Ted Pella Inc., Redding, CA, U.S.A.) buffer (Karnovsky solution). After wash with PBS, the tissues were postfixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA, U.S.A.) for 2 hr and embedded in Epon-araldite mixture (epon 812 resin 1.3 g; dodecyl succinic anhydride 27.02 g; methyl nadic anhydride 21.95 g; 2, 4, 6-tri dimethylaminoethyl phenol-30 1.5-2 mL; Electron Microscopy Science Inc., Fort Washington, PA, U.S.A.). The semithin sections (1  $\mu$ m) were cut on an ultramicrotome (Ultracut; Leica, Nussloch, Germany) and observed after staining with toluidine blue. And the ultra-thin sections (80-90 nm) were contrast-stained with saturated solutions of uranyl acetate and lead citrate (Polysciences Inc. Warrington, PA, U.S.A.) and cell organelles such as nucleus, cytoplasm, Golgi complex, mitochondria, endoplasmic reticulum, and secretory granules were examined under EM (EM902A, Zeiss, Germany).

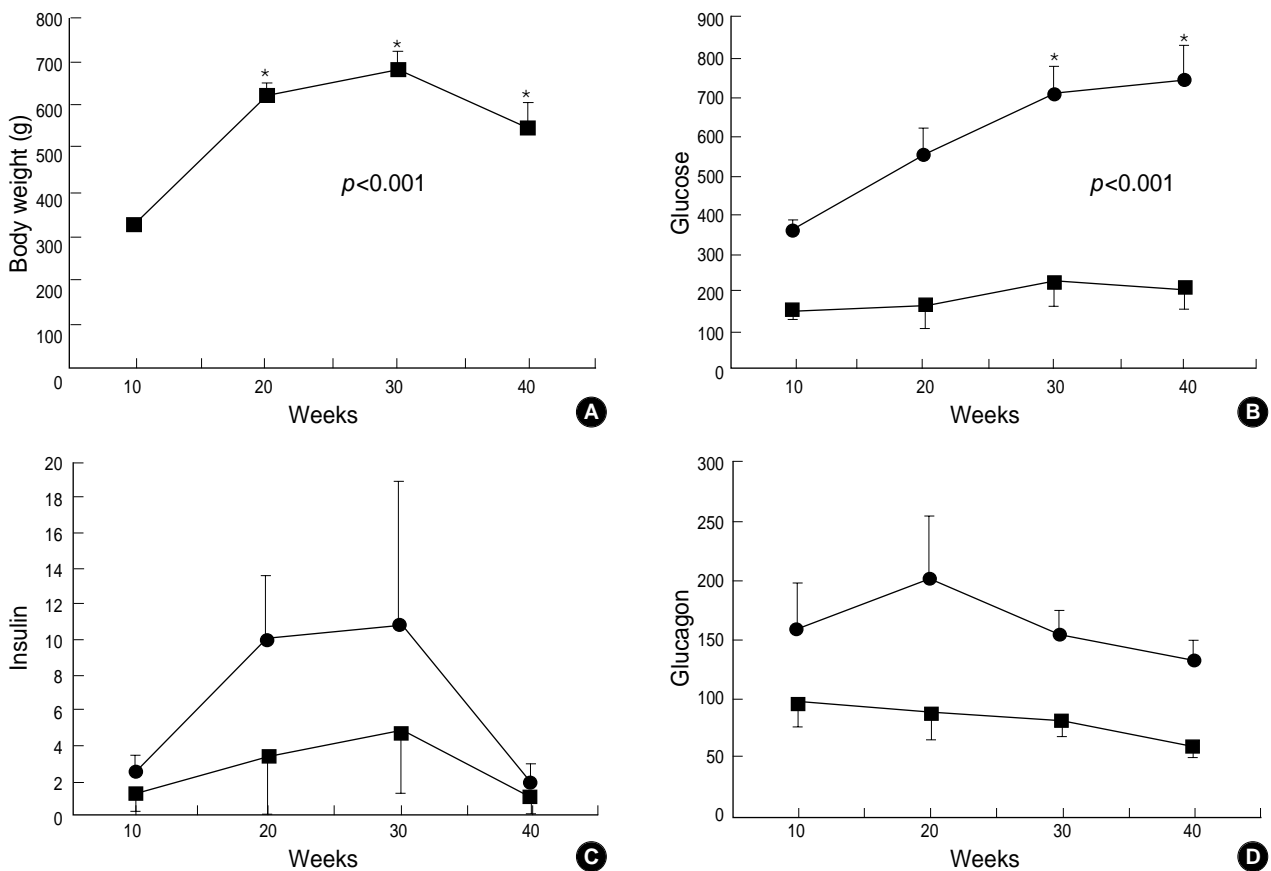
### Statistical analysis

All results were expressed as means  $\pm$  SD. The statistical significance of differences was evaluated using the Student t-test and one-way ANOVA by SPSS package program. Differences were considered significant when *p* value was below 0.05.

## RESULTS

### Body weight and metabolic changes

Mean body weight of 10-week-old male OLETF rats was  $325 \pm 9$  g. The weight increased significantly with age to  $621 \pm 25$  g at the 20th week and  $680 \pm 40$  g at the 30th week ( $p < 0.001$ ), and decreased gradually to  $543 \pm 60$  g at the 40th week (Fig. 1A). As a result of OGTT, AUC of glucose at the 30th and 40th week increased significantly to  $709 \pm 73$  mg.h/



**Fig. 1.** Body weight and metabolic changes of male OLETF rats. (A) changes of body weight; (B) basal (■, mg/dL) and AUC (●, mg.h/dL) glucose levels. AUC glucose at the 30th and 40th week are significantly higher than that at the 10th week; (C) basal (■, ng/mL) and AUC (●, ng.h/mL) insulin levels. AUC insulin increases until the 30th week and then decreases. However, the change was not statistically significant; (D) basal (■, pg/mL) and AUC (●, pg.h/mL) glucagon levels. AUC glucagon slightly increases until the 20th week and then decreases gradually after the onset of diabetes. However, the change was not significant.

dL,  $746 \pm 87$  mg.h/dL, respectively, than that of the 10th week ( $360 \pm 25$  mg.h/dL) (Fig. 1B,  $p < 0.001$ ). AUC of insulin at the 10th week was  $2.4 \pm 0.9$  ng.h/mL and increased gradually to  $10.8 \pm 8.3$  ng.h/mL at the 30th week and decreased rapidly to  $1.8 \pm 1.2$  ng.h/mL at the 40th week (Fig. 1C,  $p = 0.055$ ). AUC of glucagon at the 10th week was  $159.4 \pm 38.7$  pg.h/mL, increased to  $200.7 \pm 54.4$  pg.h/mL at the 20th week. It decreased gradually after the onset of diabetes (Fig. 1D,  $p > 0.05$ ).

**Histological changes**

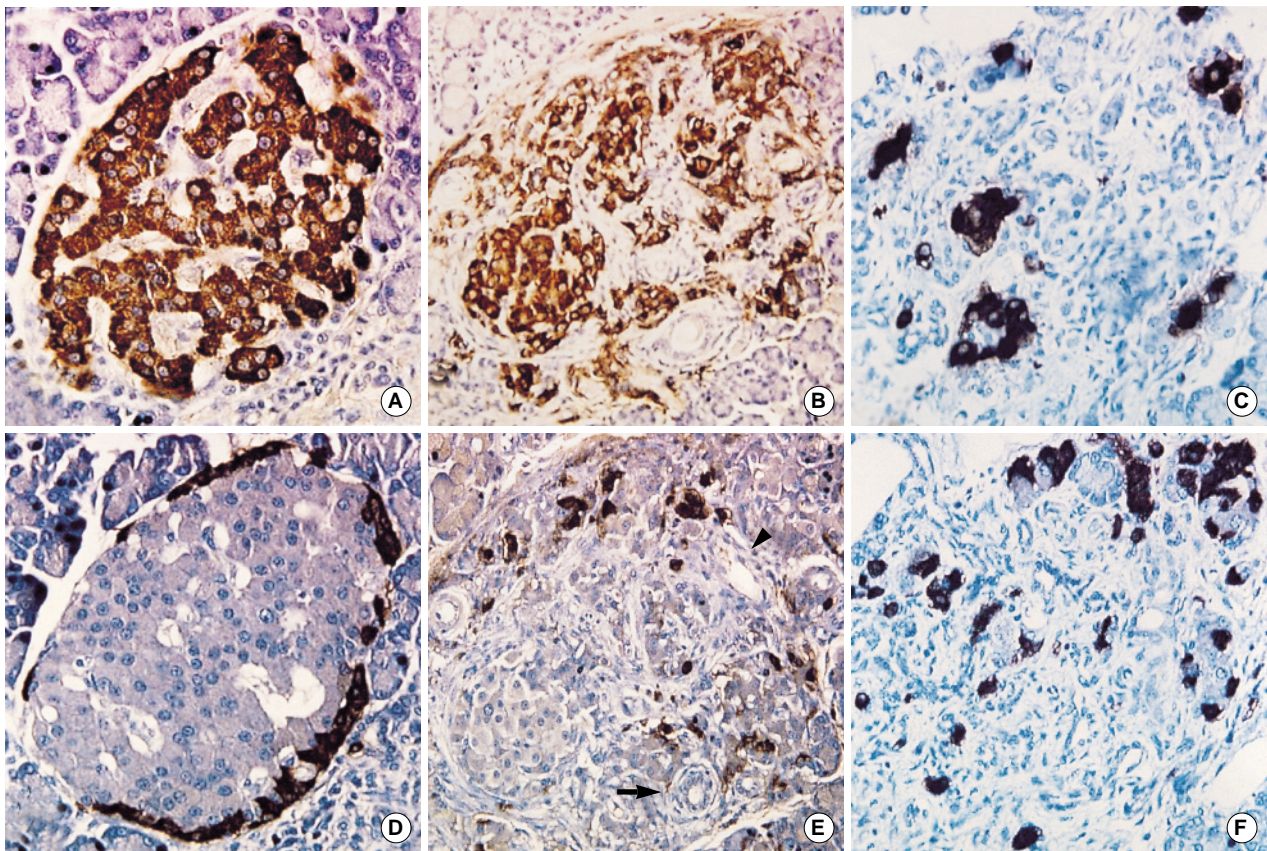
Mean diameter and area of pancreas at the 20th week ( $183.0 \mu\text{m}$ ,  $47,800 \mu\text{m}^2$ ) increased significantly than those

of the 10th week ( $115.4 \mu\text{m}$ ,  $17,500 \mu\text{m}^2$ ). The number of large islet ( $>150 \mu\text{m}$  of diameter) increased from 22% (6/27) at the 10th week to 47% (16/34) at the 20th week (Table 1). At the 20th week, some islets were enlarged and contained newly developed blood vessels and ducts in their periphery (Fig. 2). At the 40th week, some islets were enlarged and the others were poorly margined. In the enlarged islets, there were various extents of connective tissue proliferation which widely separated endocrine cells, giving an appearance of multilobules or clusters of endocrine cells. Hemosiderin inclusions were detected in the periphery. Occasionally, budding of islet cells from small intralobular ducts was observed.

On immunohistochemical staining, the distribution of endocrine cells in each islet were normal pattern at 10 and

**Table 1.** Morphometric characteristics of endocrine pancreas in OLETF rats

Age (weeks)	Number of islets	Islets (diameter in $\mu\text{m}$ , %)			Diameter (mean, $\mu\text{m}$ )	Area (mean, $\mu\text{m}^2$ )
		$>150$	100-150	$<100$		
10	27	6 (22)	8 (30)	13 (48)	115.4	17,500
20	34	16 (47)	10 (29)	8 (24)	183.0	47,800



**Fig. 2.** Immunohistochemical stainings of OLETF rat pancreas. Each pancreas was stained with antibodies against insulin (A-C) and glucagon (D-F). A and D, sections of pancreas at the 10th week show islets with a typical distribution of peripheral glucagon-secreting cells and central insulin-secreting cells ( $\times 400$ ); B and E, sections of pancreas at the 20th week show islets with a relatively well preserved typical distribution. Some islets are enlarged and show newly developed blood vessels and ducts in the periphery of islet ( $\times 200$ ); C and F, sections of pancreas at the 40th week show destructed islets with fibrosis and poor margination ( $\times 400$ ).

20 weeks old; beta cells located centrally and alpha cells located at the periphery. However, the distribution of both endocrine cells were changed to a mixed pattern at the 40th week of age. The ratio of beta cell to alpha cell increased from 2.1:1 at the 10th week to 4.7:1 at the 20th week. However, it decreased markedly to 0.5:1 at the 40th week (Table 2).

#### Electron microscopic changes

Beta cells at the 10th week possessed rounded or oval nuclei with a moderately distinct nucleolus. The chromatin was finely dispersed and the nuclear membranes and pores were easily discerned. The electron density of the cytoplasm varied; most often it was moderately dense. The endoplasmic reticulum was mostly of rough type and either vesicular or lamellar. The mitochondria are medium-sized, rounded, oval,

**Table 2.** Changes of cell distribution in randomly selected 10 islets from a pancreatic section

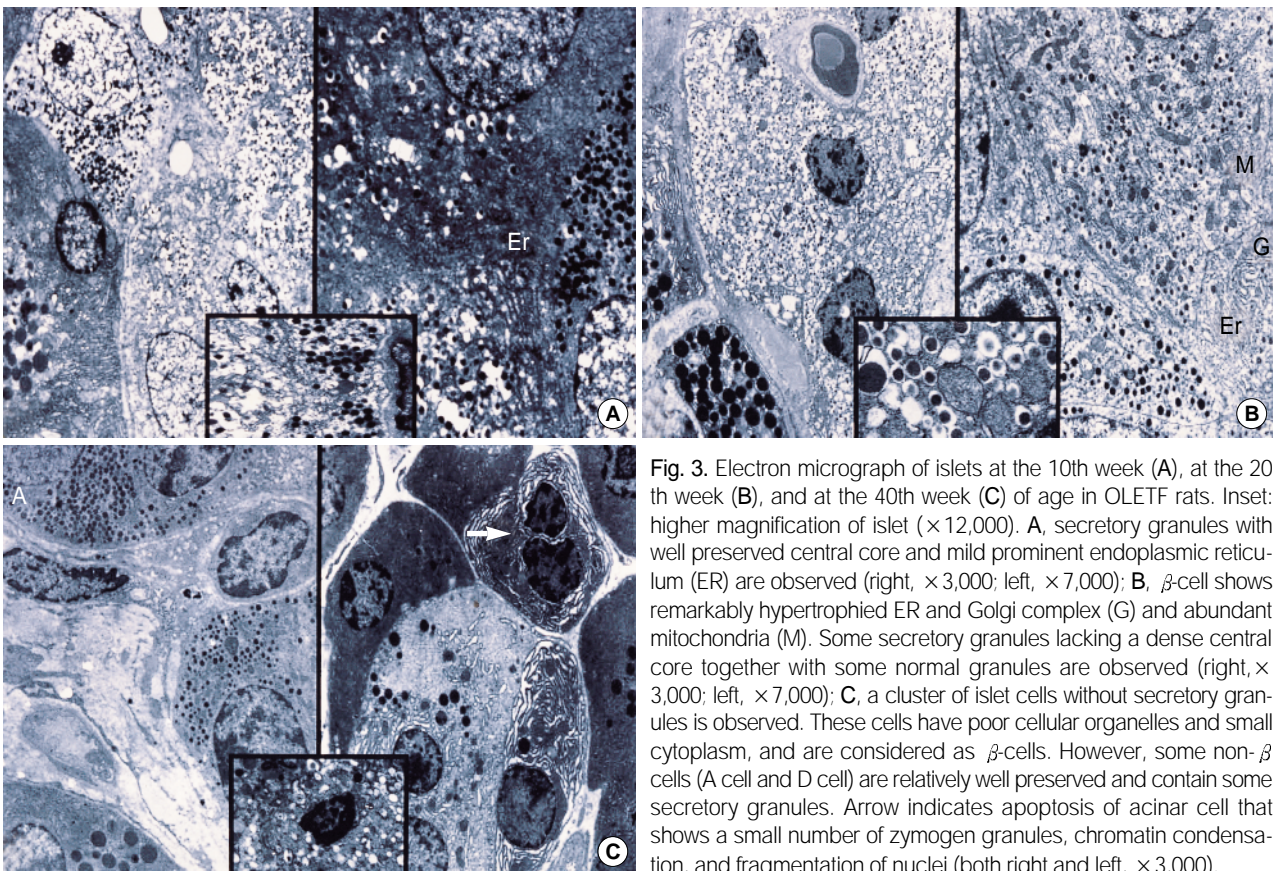
Age (weeks)	Mean $\beta$ -cell number/islet	Mean $\alpha$ -cell number/islet	$\beta$ -cell $\alpha$ -cell
10	142	68	2.1:1
20	264	56	4.7:1
40	40	89	0.5:1

or elongated and possess a moderate number of mainly transverse cristae. The inner mitochondrial compartment exhibits a moderate electron density. Free ribosomes, hypertrophied Golgi complex, lipid bodies, lysosomal bodies of varying appearance and microtubules were also observed the cytoplasm. Beta cells contained increased number of mature secretory granules. The granules were composed of a central core, usually of moderate homogeneous, or slightly heterogeneous electron density, and an external single-layered membrane with a rather large space between the core and the membrane (Fig. 3A). The granules were diffusely distributed in the cytoplasm.

At the 20th week, beta cell contained remarkably increased number of immature secretory granules, mitochondria and of hypertrophied cytoplasmic organelles such as Golgi complex and endoplasmic reticulum (Fig. 3B). At the 40th week, beta cell contained scanty organelles and secretory granules, and apoptosis of acinar cell was observed (Fig. 3C).

## DISCUSSION

OLETF rats are a useful animal model of human type 2 diabetes mellitus, especially for the etiologic analysis of insulin resistance and diabetes susceptibility. They show obesity,



**Fig. 3.** Electron micrograph of islets at the 10th week (A), at the 20th week (B), and at the 40th week (C) of age in OLETF rats. Inset: higher magnification of islet ( $\times 12,000$ ). A, secretory granules with well preserved central core and mild prominent endoplasmic reticulum (ER) are observed (right,  $\times 3,000$ ; left,  $\times 7,000$ ); B,  $\beta$ -cell shows remarkably hypertrophied ER and Golgi complex (G) and abundant mitochondria (M). Some secretory granules lacking a dense central core together with some normal granules are observed (right,  $\times 3,000$ ; left,  $\times 7,000$ ); C, a cluster of islet cells without secretory granules is observed. These cells have poor cellular organelles and small cytoplasm, and are considered as  $\beta$ -cells. However, some non- $\beta$  cells (A cell and D cell) are relatively well preserved and contain some secretory granules. Arrow indicates apoptosis of acinar cell that shows a small number of zymogen granules, chromatin condensation, and fragmentation of nuclei (both right and left,  $\times 3,000$ ).

hyperglycemia and hyperinsulinemia. The results of this study confirm the previous findings that the body weights of OLETF rats and basal insulin increased gradually with age until 30 weeks old and then decreased.

Until the 30th week of age, insulin response to glucose was increased. There was no significant difference in insulin response to glucose between the age of 20 and 30 weeks. This is assumed to correspond to the period of manifestation of early diabetes, when hyperglycemic effect is not severe. The fact of decreased insulin secretion in the presence of a high concentration of glucose at 40th week of age was supported by the result from the content of insulin in the islets. These rats showed decreased insulin response to glucose in addition to decreased basal insulin at the 40th week of age, which means the period of late diabetes. Our results were similar to those of Kawano et al. (2) but slightly different from those of Shima et al. (32) or Kanazawa et al. (6), which reported that sedentary OLETF rats showed normal or increased basal insulin and decreased insulin response to glucose.

This study also show that basal glucagon decreased continuously during the observation period. Kanazawa et al. (6) already reported that basal glucagon was significantly lower at the developing and post-developing stages of diabetes in male OLETF rats, whereas glucagon secretion by oral glucose loading showed significant decrease at the pre-developing and the developing stages of diabetes. Our study also showed the similar results except transiently increased AUC of glucagon at 20 weeks old, when the rats became diabetes. The discrepancy between other studies and ours might be due to several possibilities, that is, different degree of weight gain, different onset age of diabetes, and environmental factors.

As a result of histological study, islets of OLETF rats was multilobulated at the 40th week of age. Each islet contains fewer beta cells and centrally located alpha cell, and are separated by connective tissue. The lymphocytic infiltration reported by Kawano et al. (2) was not observed in this study. It was assumed that the migration of alpha cells into the center of the islet occurred with the appearance of connective tissue in the corresponding area. The decreased content of insulin and glucagon was consistent with the morphological changes in islets and immunohistochemical findings.

As a result of EM, the number of mature secretory granules and hypertrophied Golgi complexes were increased at the 10th week. Although hyperglycemia was not developed at this stage, this changes suggested that compensatory change of beta cell according to increased secretion of insulin against glucose. Hamaguchi et al. (33) suggested that the ultrastructure of the islet can be affected by various glucose and oxygen concentration. Firstly, hyperglycemia with high oxygen induced hypertrophy of endoplasmic reticulum and Golgi complex, an abundance of free ribosomes, and degranulation and the margination of secretory granules in the beta cells. It was speculated that these changes represented the effort to increase the insulin secretion. Secondly, hyperglycemia

with hypoxia induced dilatation of endoplasmic reticulum cisternae and dominance of Golgi vesicles in addition to the above-mentioned changes. Because the transfer of proinsulin from the granular endoplasmic reticulum to the Golgi complexes and the intra-Golgi transfer of the prohormone are energy-dependent processes, the above findings were resulted from the hypoxic state. In the present study, abundant immature secretory granules lacking a dense central core, hypertrophied and partially dilated endoplasmic reticulum and Golgi complexes, and increased mitochondria were observed at the 20th week. With the above findings, it can be speculated that the compensatory changes in pancreatic beta-cells of male OLETF rats may induce increase of insulin production. At the 40th week, beta cell contained scanty organelles and secretory granules, and apoptosis of acinar cell increased. Although eSS (e Stilman Salgado) rats suffered from mild diabetic syndrome, there were striking lesions in pancreatic islets (23). With the advance of diabetes, pancreatic beta cells from eSS rats showed increases in the volume densities of endoplasmic reticulum, immature secretory granule, and lysosomes. Conversely, the volume density of total secretory granules and microtubules appeared diminished. These observations were reported in many studies with different animal models and cell lines (23), and might be associated with an effort to increase insulin secretion by hyperglycemia. All these changes suggested that the atrophy of pancreas would develop with further advance of diabetes. Since the number of rats for morphological study were so small, our observation should be interpreted cautiously.

In conclusion, as diabetes progressed, increased secretion of insulin was accompanied by increases in size of islets and number of  $\beta$ -cells in male OLETF rats showing obese type 2 diabetes. However, these compensatory changes could not overcome the requirement of insulin according to the continuous hyperglycemia after development of diabetes. As a result, there is a marked decrease of insulin response to glucose occurred from the results at 40th week of age which is characterized by changes in pancreatic tissue such as severe fibrosis and decreased beta cells containing fewer secretory granules.

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