Differentiation and proliferation of endocrine cells in the regenerating rat pancreas after 90% pancreatectomy*

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Summary. The transplantation of pancreatic tissue has been anticipated to serve as a radical treatment for diabetes mellitus. However, the identification of the stem cells, and elucidation of their differential lineage and controlling mechanisms are prerequisites to ensure effective transplantation. We conducted an immunohistochemical study to determine the proliferation and differentiation dynamics of pancreatic endocrine cells in the rat pancreas 1 to 28 days after a 90% pancreatectomy.

Regeneration of endocrine cells started immediately after pancreatectomy. The process of regeneration included the proliferation of preexisting islet cells and neogenesis of endocrine cells from epithelial cells of the most peripheral duct. Intercalated ductal cells and centroacinar cells were speculated to be the major sources of neogenesis, from which islet tissue was formed. Glucagon cells were the first endocrine cells differentiated, some of which transformed to

insulin cells by a mechanism of non-replication. These results indicate that endocrine stem cells exist among the intercalated ductal and/ or centroacinar cells, and these special regions should be utilized in transplantation for the successful treatment of diabetes.

Introduction

The pancreas is known to consist of both exocrine and endocrine tissues. Four types of cells participating in endocrine functions are organized in the islet of Langerhans (Orsi and Unger, 1975). All parenchymal cells of the pancreas have been indicated to originate from common progenitor cells situated in the foregut endoderm (Pictet and Rutter, 1972; Le Douarin, 1988).

The transplantation of ductal cells has held great promise as a radical treatment for diabetes mellitus (Ramiya *et al.*, 2000), and much interest has been given to the cultivation of purified tissue stem cells (Bonner-Weir *et al.*, 2000) for transplantation purposes. Identification of tissue stem cells and determination of their differentiation lineage as well as controlling mechanism are fundamentally important issues for the reconstruction of islet tissue.

The developmental lineage of endocrine cells has been investigated in experimental animals as well as human materials. Recent advances have indicated the presence of various biologically active substances that control cellular differentiation (Furukawa *et al.*, 1995; Mashima *et al.*, 1996; Miralles *et al.*, 1998), and a large number of extensive molecular biological studies have been conducted to determine the expression mechanisms of various transcription factors (for review, see St-Onge *et al.*, 1999; Edlund, 1999, 2001).

The process of regeneration is one of the preferred models for analyzing the proliferation and differentiation of cells in organs and tissues. Not only pancreatic exocrine but

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also endocrine tissues have been shown to regenerate after massive tissue lesions produced by drug administration (Fitzgerald *et al.*, 1966), ductal ligation (Wang *et al.*, 1995), or partial resection (Brockenbrough *et al.*, 1988; Bonner-Weir *et al.*, 1993). The process of regeneration may differ according to the method of lesion production, but stem cells existing in the ductal epithelium are thought to differentiate and regenerate into mature tissue (Bonner-Weir *et al.*, 1993). However, the specific location of stem cells in the ductal system has not yet been identified. Information on cellular lineage during the process of pancreatic development has been integrated; in contrast, little knowledge is available concerning the course of regeneration, and, furthermore, only a few definitive studies have described the controlling mechanism (Bouwens, 1998).

We have previously developed an improved method of tissue preparation after a 90% partial pancreatectomy, which permits the comparison of a specific portion of the parabiliary segment of pancreas among rats (Hayashi *et al.*, 1999). In applying this method and using immuno-histochemistry under both light and electron microscopy, the present study aimed to determine the location of stem cells in the ductal system and the mode of differentiation into specialized endocrine cells during the regenerating stage after pancreatectomy.

Materials and Methods

Experimental animals

Male Wistar rats at 6 weeks of age weighing 150–160 g were used. They were kept in an experimental animal facility controlled at 23°C room temperature with a 12-h light and dark cycle, and with free access to standard chow food and water. The experimental protocol was performed in strict accordance with the ethical requirements issued by the Animal Experiment Committee, Kitasato University School of Medicine.

Partial pancreatectomy

Under anesthesia by an intraperitoneal injection of 1 ml/100 g body weight of a mixture containing 35 mg Napentobarbital (Dainihon Pharmaceutical Co., Ltd., Osaka) and 15 mg ketamin hydrochloride (Sankyo Pharmaceutical Co., Ltd., Tokyo) in 1 ml physiological saline, the abdomen was opened by a median incision. Among the four segments of the pancreas, the gastric, splenic and duodenal segments were resected with a battery-operated cauterizer (Fine Science Tools, Heidelberg, Germany) leaving only the parabiliary segment—which was the smallest segment—attached to the duodenum together with the

common pancreatic duct (Scow, 1957; Richards *et al.*, 1964; Lehv and Fitzgerald, 1968). This method allows a resection of about 90% of the whole pancreas (Lehv and Fitzgerald, 1968). All operational maneuvers were completed within approximately 20 min. A group of rats was sham-operated by the same procedures as the pancreatectomized animals, but the pancreatic segments were separated from the mesentery and rubbed several times gently with fingertip instead of being resected. Non-operated normal rats also were examined as controls.

After the operation, all animals were kept for a maximum of 4 weeks in the same conditions as stated above. During this period, less than 5% of the rats died from obstructive jaundice or hemorrhagic diathesis; these animals were excluded from the examination.

Light and electron microscopic immunohistochemical studies

At the indicated days after surgery, animals were laparotomized under ether anesthesia, and the pancreatic remnants were removed *en bloc* together with the surrounding gastric pylorus, duodenum, common bile duct (CBD), and hepatic portal region (Hayashi *et al.*, 1999). The tissue block was fixed in 10% formalin with microwave irradiation (Yamashina *et al.*, 1990), followed by paraffin embedment by routine histological techniques. Sections of 3 µm in thickness were cut and examined after staining with hematoxylin and eosin. At least 5 rats were examined for one indicated time point.

Light microscopic immunohistochemistry

For immunohistochemical identification of each type of endocrine cell, sections were incubated overnight with the respective first antibody at 4°C, followed by incubation with a biotinylated second antibody. After incubation in peroxidase-conjugated streptavidin (# SA-5004, Vector Labo. Inc., Burlingame, CA, USA) at 200× dilution for 1 h at room temperature, the sections were colored by diaminobenzidine (DAB) and counter-stained with hematoxylin. The following first and second antibodies were used, rabbit anti-C-peptide antibody (# 1700-0054, Biogenesis Ltd., Poole, UK) at 800× dilution, guinea pig anti-insulin antibody (# A564, Dako, Carpinteria, CA, USA) at 1000× dilution, rabbit anti-glucagon antibody (# 08-0064, Zymed Labo., Co., San Francisco, CA, USA) at 4× dilution, rabbit anti-somatostatin (# 18-0078, Zymed Labo., Co.) at 15× dilution, rabbit anti-human pancreatic polypeptide antibody (# AB939, Chemicon International, Inc., Temecula, CA, USA) at 1000× dilution, and rabbit anti-human α-amylase antibody (# A-8273, Sigma-Aldrich, Inc., St. Louis, MO, USA) at 1000× dilution. Biotinylated anti-rabbit IgG (H +

Fig. 1. Photomicrographs of the pancreas in a sham-operated control rat. **A:** Double labeling of BrdU and insulin. Insulin-positive cells are organized in the islets of Langerhans, with only a minimal number of solitary insulin cells. A few BrdU-positive cells (black in nuclei), are distributed in the acinar and ductal regions. In the islets, a small number of BrdU-positive nuclei can be recognized in insulin-positive (light brown) and -negative cells. $\times 250$. **B:** Confocal laser scanning micrograph of double labeling by glucagon and insulin antibodies showing glucagon cells (labeled by FITC; green) surrounding insulin cells (labeled by rhodamin; red) in the islets. Note the presence of only a small number of solitary endocrine cells. $\times 350$

L) and biotinylated anti-guinea pig IgG (H+L) antibodies were obtained from Vector Laboratories (#BA-1000 and BA-7000, respectively).

Detection of proliferating cells

Proliferating cells were detected using a cell proliferation kit (#RPN 20, Amersham Pharmacia Biotech Inc., Piscataway, PH, USA) with an antigen retrieval technique (Shi *et al.*, 1991). Bromodeoxyuridin (BrdU) at a dose of 60 mg/kg body weight was injected intraperitoneally 4 h before sacrifice.

Double labeling for hormones and BrdU

Sections showing proliferating cells by the BrdU method were doubly labeled with one of the anti-hormone antibodies to determine the proliferating activity of specific endocrine cells. For this purpose, sections were treated for the BrdU method by incubating in the DAB medium containing nickel followed by the peroxidase-anti-peroxidase (PAP) method to localize respective hormone immunoreactivity. For the PAP method, swine anti-rabbit immunoglobulin antibody (#Z0196, Dako) and rabbit PAP (#Z0113, Dako) at $100\times$ dilution were applied one after another for 1 h at room temperature followed by DAB staining. Double labeling of dual hormones or hormones and amylase was performed using an FITC-conjugated affinity-purified goat anti-rabbit IgG antibody and a rhodamine-conjugated affinity-purified goat anti-guinea pig IgG antibody (#AP132F and AQ108R, respectively, Chemicon International, Inc.) at

$150\times$ dilution. The sections were examined under a MRC1024 confocal laser scanning microscope (Bio-Rad Microscience Ltd, Hertfordshire, UK).

Immunoelectron microscopy

Tissue blocks were fixed in a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde with microwave irradiation (Yamashina *et al.*, 1990), and then placed on ice for 1 h in the same fixative. The specimens were embedded in LR white resin (Tamaki and Yamashina, 1994), and ultrathin sections were mounted on 200 mesh nickel grids supported by formvar-carbon film. Non-specific staining was blocked by treatment in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min. Goat anti-rabbit IgG labeled with 15-nm colloidal gold and goat anti-guinea pig IgG labeled with 5-nm colloidal gold were purchased from EY Laboratories, Inc. (San Mateo, CA, USA). A medium grade LR white resin was obtained from London Resin Co. (Basingstoke, Hampshire, UK). Double immunogold reactions were performed by an indirect method. The sections were treated for 3 h by flotation on drops of the antiserum diluted in 1% BSA-PBS. Anti-insulin and anti-glucagon antibodies were the same as those used in immunohistochemistry for light microscopy. After several washes with PBS, the grids were incubated for 1 h in a mixture of colloidal gold-conjugated secondary antibodies (diluted $50\times$ in 1% BSA-PBS) and washed with four changes of PBS and three changes of distilled water. Sections were counterstained with 5% aqueous uranyl acetate

Fig. 2. Two days after pancreatectomy. **A:** Confocal laser scanning micrograph of double labeling with glucagon and insulin antibodies indicates the appearance of both solitary and clusters of endocrine cells. Note that solitary endocrine cells always show a positive reaction for glucagon (thin arrows), and insulin cells occur in endocrine cell clusters (thick arrows). $\times 280$. **B:** Immunostaining with a glucagon antibody demonstrates solitary positive cells in close association with acinar or intercalated ductal cells (arrows). $\times 500$. **C:** Double labeling by BrdU and anti-C peptide antibody. Note the increase in the number of BrdU-positive cells (black in nuclei) in the region of the acini and duct. Insulin cells are shown in pale brown. $\times 520$

for 5 min and Reynolds' lead citrate for 5 min, and were then examined by a JEM-1200EX electron microscope (JEOL, Tokyo) operated at 80 kV.

Immunohistochemical control

For the immunohistochemical control, sections were treated with normal serum in place of the first antibody. For the BrdU method, sections showing an even positive reaction in the cryptal region of the duodenal gland next to the pancreas were used as the internal control.

Results

The incorporation of BrdU in the exocrine acinar cells of non-operated and operated rats has been described in our previous paper (Hayashi *et al.*, 1999). This report will describe only the proliferation activities in the ductal epithelium and endocrine cells.

Distribution and proliferation activity of endocrine cells in non-operated control rat pancreas

The following results in the normal rat pancreas were used

as a control for comparison with those of pancreatectomized rats.

Distribution of endocrine cells in mature Langerhans' islet

In a cross section covering the total area of the parabiliary segment laterally from the duodenal attachment region medially, to the stump 4 to 5 mature Langerhans' islets were observed in which α , pancreatic polypeptide (PP), and somatostatin (SO) cells surrounded a mass of centrally located β cells (Fig. 1A, B). The α cells were located in one to two layers at the most peripheral portion of the islet, and SO cells were located between groups of α and β cells, confirming previous findings (Orci and Unger, 1975). In a given islet of Langerhans, α cells outnumbered PP and SO cells. Beside mature islets, a very few clusters of endocrine cells were observed throughout the parenchyma. Occasional α or PP cells were discernible in close proximity to the intercalated duct or acini. In contrast, no endocrine cells were distributed in ductal epithelium, except in the discrete region of the papillary opening of the CBD, in which SO cells were frequently distributed. Solitary β cells were found outside islets on very rare occasions, and by means of double labeling, neither cells co-expressing insulin/

Fig. 3. Three days after pancreatectomy. **A:** Confocal laser scanning micrograph of double labeling with anti-glucagon and anti-insulin antibodies. Small endocrine cell clusters have increased in size and number. In the peripheral islets, some cells showing double positive reactions for glucagon and insulin are observed (arrows). $\times 280$. **B:** Double labeling with BrdU and anti-C-peptide antibody. Proliferating cells are observed in acinar and intercalated ductal cells at this stage. Insulin cells form small clusters consisting of several endocrine cells. $\times 1,000$

Fig. 4. Five days after pancreatectomy. **A:** Confocal laser scanning micrograph of double labeling with glucagon and insulin antibodies shows that endocrine cell clusters (thick arrow) have further increased in size. Solitary glucagon cells are still recognized (thin arrows). $\times 310$. **B:** Double immunostaining with BrdU and glucagon antibodies. BrdU-positive cells continue to be abundant both in acinar regions and in islets. In a given islet, BrdU-positive nuclei are found more frequently in insulin cells than in non-insulin cells. $\times 600$

glucagon nor insulin/amylase were observed in the pancreas of sham-operated and non-operated control rats.

Incorporation of BrdU in endocrine cells

In the mature islet, only a few α and β cells incorporated BrdU, indicating a very low turnover rate in these cells (Fig. 1A), but neither PP nor SO cells showed any positive BrdU reaction. Endocrine cells in small clusters showed a very low activity of proliferation.

Regeneration of endocrine cells after 90% pancreatectomy

1 day after pancreatectomy

No remarkable change in the number of mature islets was discernible immediately after surgery. The number of endocrine cell clusters also did not differ from that of non-operated control rats. However, in the close vicinity of the acini, a small number of cells appeared that showed positive reactions for glucagon and PP but not for insulin. In the

Fig. 5. Photomicrographs at 7 (A), 14 (B) and 21 (C) days after pancreatectomy, doubly immunostained with BrdU and anti-C peptide antibody (A and C), and with BrdU and anti-glucagon antibody (B). **A:** Frequencies of BrdU-positive cells in the acinar region and insulin cells in islets have peaked, indicating that these cells proliferate at the highest rate. $\times 710$. **B and C:** Double labeling immunohistochemistry indicates that BrdU incorporation gradually returns to the normal control level at 2 to 3 weeks, but more mature islets are observed. B: $\times 100$, C: $\times 250$

islets, the number of BrdU-positive α and β cells started to increase.

In the CBD from the hepatic portal to papillary opening, there was an increase in the number of SO-positive cells, some of which showed a positive BrdU reaction. The ductal epithelium from the interlobular duct to centroacinar cells revealed an increased number of BrdU-positive cells, but there was no evidence that these proliferating cells expressed any immunoreactivity for any type of islet hormone.

2 days after pancreatectomy

One characteristic finding at this stage was the appearance of solitary and small clusters of endocrine cells in the close vicinity of the acini. The solitary endocrine cells were always positive for glucagon (Fig. 2A, thin arrows) and localized between acinar cells (Fig. 2B, arrows) and the surrounding intercalated duct. In contrast, insulin-positive cells first appeared when endocrine cells began to form a small cluster (Fig. 2A, thick arrows), since the initial endocrine cell cluster contained three types of cells that expressed glucagon, insulin, or both. Cells co-expressing glucagon and insulin were also demonstrated in the periph-

eral region of preexisting mature islets at this stage. There was no increase in the number of mature islets.

BrdU incorporation further increased in the ductal epithelium from the interlobular duct to centroacinar cells (Fig. 2C), but the BrdU-positive cells in the ductal region did not co-express any hormone immunoreactivity by the double labeling method. BrdU incorporation was detected in a few α and β cells in the clusters as well as in mature islets.

3–4 days after pancreatectomy

Compared with the previous stage, cell proliferation in the endocrine cluster had evidently advanced. Clusters had enlarged in size and some had developed into small islets, in which cells co-expressing glucagon and insulin could be detected. Solitary glucagon-expressing cells were still found in the close vicinity of the acini and intercalated ducts. Increased BrdU incorporation was observed in centroacinar cells (Fig. 3B), and in some β cells in the endocrine cluster as well as mature islets. On rare occasions, glucagon cells in the cluster also showed BrdU incorporation.

Fig. 6. Immunoelectron micrographs at 2 days after pancreatectomy, doubly labeled with anti-glucagon (marked by 15-nm colloidal gold particles) and anti-insulin (marked by 5-nm colloidal gold particles) antibodies. **A:** A solitary endocrine cell found between acinar cells (A) can be identified as a glucagon cell from the morphology of its secretory granules and distribution of 15-nm colloidal gold particles on the secretion granules. $\times 10,600$. **B:** A portion of an endocrine cell cluster in the acinar region depicting the presence of glucagon cells and insulin cells. $\times 7,400$. **C:** A portion of an intermediate cell containing glucagon (thick arrows) and insulin positive granules (thin arrows). $\times 33,000$

5 days after pancreatectomy

The endocrine clusters continued to grow in size (Fig. 4A). Mature islets also increased in size, indicating the gradual proliferation of endocrine cells, and the thickness of the glucagon cell layer also increased. Solitary glucagon cells could be seen adjacent to the acini similar to day 2 after surgery (thin arrows in Fig. 4A). In the thick ductal epithelium, glucagon and insulin cells were found sporadically, but not PP or SO cells. Double labeling for insulin and glucagon indicated the distribution of co-expressing cells in the endocrine cell cluster (thick arrow in Fig. 4A). BrdU incorporation was more active in the insulin cells than in the glucagon cells in mature islets (Fig. 4B).

7 days after pancreatectomy (Fig. 5A)

The growth of endocrine clusters further advanced, resulting in an increase in the number of mature islets. In the region adjacent to the acini, there was a decrease in the number of solitary cells showing glucagon immunoreactivity. The number of glucagon cells in mature islets also recovered to the control level. SO-positive cells and cells co-expressing glucagon and insulin were no longer observable at this stage. Endocrine clusters also decreased in number. The prime characteristic of this stage was that the number of BrdU-positive β cells in the islets reached a peak, whereas the number of proliferating α cells had decreased, compared with day 5.

14 days after pancreatectomy (Fig. 5B)

At this stage, neither solitary nor clustered endocrine cells could be seen in the region close to the acini. The number of maturing islets of the Langerhans tended to increase until 2 weeks after surgery and maintained a steady state thereafter. The size of the islet increased due to the proliferation of endocrine cells. Only a small number of β cells showed a positive BrdU reaction, indicating that a low level of proliferation activity still persisted, but the proliferation rate in general had recovered to the control level at this stage.

21–28 days after pancreatectomy (Fig. 5C)

The number of large islets of Langerhans increased as seen at weeks 2. The rate of BrdU incorporation was similar to the control level, indicating that regeneration had been completed by 3–4 weeks after operation. Throughout the examination, no co-expressing cells for amylase nor either one of the hormones could be detected.

Immunoelectron microscopic findings

Examination by electron microscopy essentially confirmed the findings obtained with light microscopy. As early as 2 days after the surgery, solitary endocrine cells started to appear in close association with acinar and intercalated ductal cells (Fig. 6A). For these newly formed endocrine cells, the luminal surface was always covered by a thin cytoplasmic process of the acinar and intercalated ductal cells with no direct communication to the luminal space, and the basal surface was lined by the common basement membrane of neighboring epithelial cells.

Solitary endocrine cells contained secretory granules with high electron density in the central core surrounded by characteristic halo of α cells; furthermore, they were confirmed to express glucagon immunoreactivity by immunoelectron microscopy. The endocrine cells located adjacent to the acinar cells began to form clusters composed of a few cells at 2–3 days after the surgery, in which insulin-positive cells started to appear as seen in Figure 6B. A few cells contained two types of secretory granules showing positive reactions for glucagon and for insulin. These dual-positive cells seemed to correspond to cells showing double immuno-reactivities at the light microscopic level. Dual-positive cells for insulin and glucagon and only insulin-positive cells were also recognized intermingling with the glucagon-positive cells. Apart from the acinar region, a few solitary endocrine cells were also found in the epithelial lining of the ductal system; however, they were sporadic, and whether these single cells developed into clusters could not be ascertained.

Discussion*Proliferation activity of pancreatic cells*

The present study indicated that about 2–3% of the cells in the pancreas of normal 6-week-old rats incorporated BrdU, and these proliferating cells were not only exocrine cells but also cells of the Langerhans's islets, indicating the organ to be undergoing steady renewal. A rapid increase in the labeling index in epithelial cells of the ductal system was started as early as 1 day after partial pancreatectomy, and the first peak of proliferation was observed at 2 days after surgery followed by a second wave of increment during days 4–7 (Hayashi *et al.*, 1999). The rate of BrdU incorporation gradually decreased after 14 days and recovered to the control level at 28 days.

Origin of endocrine cell regeneration

Immediately after the partial pancreatectomy, hormone-containing cells appeared in the ductal lining from the CBD to centroacinar cells, among which the intercalated ductal and centroacinar cells appeared to be the major sites of regeneration. In this particular region, BrdU-positive cells rapidly increased in number, and solitary endocrine cells that emerged in close proximity to the acinar cells gradually grew to form clusters. Despite attempts to demonstrate the changes in the labeling index quantitatively, the considerable difficulty in identifying intercalated ductal and centroacinar cells one by one necessitated our describing them qualitatively. The BrdU-positive centroacinar cells showed no expression of hormone immunoreactivity in the earliest stage, indicating that cellular differentiation proceeded after replication. These results strongly suggested that neogenesis might have initiated from progenitors existing among the intercalated duct and centroacinar cells. Examination at the EM level disclosed that the newly formed endocrine cells were always covered at the luminal pole by a thin layer of cytoplasmic processes of the neighboring cells and that they had no direct communication with the luminal space. The newly formed cells at first aligned with the acinar cells on the common basement membrane, but eventually extended cytoplasmic processes toward the interstitium through the basement membrane as they increased in number to form endocrine clusters. Thereafter, the cluster seemed to detach from the original acinar location, and its further proliferation and differentiation might produce a mature islet of Langerhans. A study using aggregation chimeras proposed that β cells with different origins assembled to form a mature islet (Deltour *et al.*, 1991). Detailed studies will be required to determine if a similar phenomenon might take place in the regenerating islet tissue of the

pancreatectomized rat. In normal human pancreas, solitary insulin cells have been observed to bud off from ductular cells (Bouwens and Pipeleers, 1998). Hormone-producing cells are known to increase in number in the centroacinar regions during development and regeneration (Hughes, 1956; Pour, 1994), and centroacinar cells have a high proliferating activity (Pour, 1994) as well as a higher risk of canceration (Pour, 1978). These observations strongly suggest that the centroacinar cells and/or cells of the intercalated duct have potential stem cell functions.

Differences in enzyme activity have been demonstrated in the epithelial lining of the ductal system from the CBD to centroacinar portion (Githens III *et al.*, 1980). Therefore, ductal epithelial cells should be considered to differ in nature according to their position. On the other hand, increased proliferation activity was demonstrated in the epithelial lining immediately after pancreatectomy (Sharma *et al.*, 1999); furthermore, the occurrence of SO cells in the CBD epithelium and sporadic expression of hormones in the inter- to intra-lobular duct were characteristic features. In the present study, however, detailed observation of the solitary endocrine cells found in the region from the CBD to the interlobular ductal system failed to demonstrate any evidence that these cells grew to form clusters or islets. These results indicate that, although a partial pancreatectomy induces the differentiation of certain ductal epithelial cells into endocrine cells, the induction effect may be transient, and endocrine cells originated from the ductal epithelium are unlikely to maintain permanent endocrine properties to develop into islet tissue. It is possible that proliferation of the cells in that portion of ductal elements may transform into exocrine as well as ductal cells.

Sharma *et al.*, (1999) indicated that CBD epithelial cells reached peak labeling index as early as 24 h after a 90% pancreatectomy, and the proliferating cells co-expressed PDX-1, a pancreatic transcription factor, followed by the gradual disappearance of PDX-1 from the ductal epithelial cells at 3 days. Although there was a slight difference in time course, the appearance of SO cells in the CBD in our study seems to correlate well with the expression of PDX-1. Co-expressing cells of SO and PDX-1 have been proposed to be a progenitor of endocrine cells (Fernandes *et al.*, 1997). However, our study in partially pancreatectomized rats failed to show any evidence of development of mature Langerhans' islets at the region of CBD, and it is very unlikely that the SO cells found in the CBD were the progenitor of endocrine cells. The exact nature of SO cells requires further analysis.

Regeneration models by streptozotocin administration (Gu *et al.*, 1997) and ductal ligation (Bertelli and Bhandari, 1997) have indicated a possibility that exocrine cells differentiate into endocrine cells, since cells expressing

both amylase and insulin were demonstrated in these models. Furthermore, the differentiation of progenitor cells into intermediate exocrine and endocrine cells has already been proposed in the process of development of the transgenic mouse (Gu *et al.*, 1994). However, we found no cells co-expressing amylase and hormones in the acinar regions. Therefore, it seems unlikely that mature exocrine cells transform into endocrine cells during regeneration after pancreatectomy. In order to rule out the possible transformation of exocrine cells, a detailed study conducted during the stage immediately after the pancreatectomy is advised. All cells in the ductal system may have the potential to become progenitors of both endocrine and exocrine cells (Gu *et al.*, 1994); however, differences in function as well as differentiative capacity have been suggested according to position of the cells (Pour, 1994). One of the conclusions of the present study is that intercalated ductal and centroacinar cells have a high differentiation potential, and progenitor cells may exist in this special regions of the ductal system.

Differentiation lineage of endocrine cell regeneration

Neogenesis of endocrine cells

Partial evidence of the differentiation lineage for the regenerating islet cells has been reported (Gu *et al.*, 1997), but comprehensive knowledge is still lacking. According to Bonner-Weir's group (Brockenbrough *et al.*, 1988; Bonner-Weir *et al.*, 1993), regeneration of endocrine cells after a 90% pancreatectomy took place by the proliferation of preexisting islet cells and ductal epithelial cells from the CBD to centroacinar region; regeneration started immediately after surgery, and the cell number rapidly doubled by 7 days, followed by steady proliferation until 21 days. The process of regeneration recapitulated that of normal development. Still, the detailed lineage of endocrine cell differentiation remains to be elucidated.

In the present study, solitary endocrine cells found in the close vicinity of the acini up to 2 days after surgery always expressed glucagon or PP but not insulin immunoreactivity. While the solitary cell proliferated and began to form a cluster of a few cells, insulin-positive cells started to appear closely associated with glucagon-expressing cells in the cluster. Some of the insulin-positive cells co-expressed glucagon immunoreactivity, indicating a possibility that some newly formed glucagon cells had transformed to insulin cells. Enhanced BrdU uptake by both glucagon and insulin cells suggests that the proliferation of both cell types might contribute to the formation of a mature islet. In the process, both glucagon and insulin cells seemed to

proliferate at a similar rate up to 5 days, followed by the predominant proliferation of insulin cells during the first two weeks after the operation.

The occasional PP-positive cells found at the earliest stage after a pancreatectomy are not likely to contribute to the formation of new islets, since the PP immunoreactivity gradually declined at 2 days after surgery. In the process of normal development, α cells are known to appear in the immature ductal epithelium followed by the emergence of β cells (Teitelman, 1991). In contrast, Herrera *et al.* (1994) reported that the targeted ablation of cells expressing the PP but not glucagon gene resulted in a marked defect in the β and SO cellular development. Also, an RT-PCR study indicated the appearance of PP and SO cells at the earliest stage of pancreatic development (Gittes and Rutter, 1992). The reported roles played by PP cells in development remain controversial (Teitelman *et al.*, 1993; Herrera *et al.*, 1994; Mashima *et al.*, 1996), although this might partly be due to the antibodies used in immunohistochemical studies (Teitelman *et al.*, 1993). Some unknown functions of PP cells may be uncovered in the future. In the regeneration model by duct ligation, a large number of α cells appeared, and they were proposed to play a significant role as progenitor cells for other types of endocrine cells (Wang *et al.*, 1995). It is very likely that the mode of endocrine cell differentiation may differ according to the regeneration model.

Regeneration in preexisting islets

Preexisting endocrine cells have been shown to proliferate after a partial pancreatectomy (Brockenbrough *et al.*, 1988; Bonner-Weir *et al.*, 1993). In the present study, α and β cells incorporated BrdU at enhanced levels 2 days after surgery. The peripheral layer of the islet containing mostly α cells clearly increased in thickness. The observation of cells expressing both glucagon and insulin immunoreactivity suggests the possible transformation of α to β cells, and this is likely to represent the same phenomenon as the neogenesis of endocrine cells in the centroacinar region. The proliferation activity of α cells predominated over β cells until 5 days, but the tendency was reversed from 7 days until 2 weeks. This mode of proliferation is quite similar to the embryonic development of endocrine cells (Teitelman, 1991). The prolonged proliferation of β cells after the pancreatectomy might be a reaction to the continued high glucose level in the blood. However, in spite of a persistent high blood glucose level, β cells ceased to proliferate after 3 weeks. It would be valuable to examine the long-term fate of β cells after the pancreatectomy.

A recent study has indicated the presence of tissue stem cells in islets that expressed nestin immunoreactivity (Zulewski *et al.*, 2001). In the present study, BrdU-positive cells in the islets always expressed hormonal immunoreac-

tivity, and no hormone-negative proliferating cells could be detected. The presence of stem cells in the islet has attracted a great deal of attention, since the transplantation of isolated human islets into the liver produced islet tissue (Shapiro *et al.*, 2000). It will be of great interest to in future studies identify the stem cells in the islet.

In conclusion, the present study indicated that endocrine stem cells exist among the intercalated ductal and centroacinar cells, and are capable of developing into new islet tissue. These special regions should be considered in transplantation for the treatment of diabetes.

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