β Cell Neogenesis from Ducts and Phenotypic Conversion of Residual Islet Cells in the Adult Pancreas of Glucose Intolerant Mice Induced by Selective Alloxan Perfusion

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Abstract. The aim of this study was to clarify the pattern of β cell neogenesis in the alloxan-perfused, β cells-depleted segment of glucose intolerant mice induced by selective alloxan perfusion. First, duct cells proliferated in the perfused segment, then cells co-expressing multiple islet hormones and transcription factors such as PDX-1, Nkx2.2, Isl1, and Pax6 were observed in duct cells, and newly formed islet-like cell clusters (ICCs) containing β cells were recognized. In residual β cell-depleted islets, glucagon or somatostatin and PDX-1 double-positive immature endocrine cells were recognized. Glucagon or somatostatin, insulin and PDX-1 triple-positive cells then appeared and these cells appeared to undergo terminal differentiation into β cells. In conclusion, we demonstrated at least two different processes of β cell neogenesis, i.e., formation of new ICCs from ductal epithelium and redifferentiation of residual non- β islet cells in this model. In addition, transcription factors that appear in the processes of endocrine cell development may also play essential roles during β cell neogenesis from duct cells.

Key words: Pax6, Isl1, Nkx2.2, Duct cell, Differentiation

(Endocrine Journal 49: 561–572, 2002)

NEOGENESIS of islets from duct-like epithelial cells occurs during normal embryonic development as well as in the neonatal period. Endocrine precursor cells differentiate into four major types of endocrine cells: those secrete glucagon, insulin, somatostatin, or pancreatic polypeptide (PP). Various transcription factors are induced to promote islet cell growth and differentiation. Among them, pancreatic and duodenal homeobox gene-1 (PDX-1) is known to play a key transcriptional role [1, 2]. Pax6 is expressed in

the developing eye, nose, pancreas and central nervous system [3], and plays a crucial role in pancreas development and differentiation of endocrine cells. Isl1 is a LIM homeodomain protein, and it is required for differentiation of islet and expressed in all type of islet cells in adult pancreas [4]. Nkx2.2 is a member of mammalian NK2 homeobox transcription factor family, and is also required for the final differentiation of pancreatic insulin cells. In the absence of Nkx2.2, insulin cells are trapped in an incompletely differentiated state [5].

In adults, endocrine cells are rarely localized in the duct cell lining, and the mitotic index of both duct and islet cells is very low [6]. However, the differentiation of β cells from precursor cells in the pancreas, i.e., β cell neogenesis, has been reported to occur in several animal models, such as neonatal rats

Received: February 14, 2002

Accepted: August 19, 2002

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with β cell destruction by streptozotocin (STZ) [7], adult hamsters with cellophane wrapping of the pancreas [8] and young rats with partial pancreatectomy [9]. In most of these studies, attempts to demonstrate how new β cells are generated have been hampered by the inability to unambiguously identify the precursor or immature endocrine cells. We have developed a new diabetic mice model induced by selective perfusion of alloxan. In this mouse model, glucose intolerance occurred and two different processes of β cell regeneration were demonstrated. Increase of body weight was retarded at early stage after alloxan treatment, and improved gradually without any treatment and glucose tolerance was completely normalized spontaneously in 48 weeks. One is that the regeneration of islet endocrine cells occurs mostly through the proliferation of preexisting intra-islet β cells in the nonperfused segment, the other one is that islet cells neogenesis from duct cells in β cell-depleted segment [10].

In the present study, we further analyzed the process of β cell neogenesis from precursor cells among pancreatic duct cells in detail with special reference to the expression of transcription factors that appear in proendocrine or endocrine cells during pancreatic endocrine cell development. In addition, we also demonstrated the phenotypic changes in residual non- β islet cells in the β cell-depleted pancreas, which might be associated with redifferentiation from non- β to β cells.

Material and Methods

A total of 44 male ICR/JCL mice (8 weeks old) (experiment group: 24, control group: 20) were used in this study. Glucose intolerance was created by selective alloxan perfusion according to our previous method [10]. All mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU; Boehringer Mannheim Biochemica, Mannheim, Germany) at 4 weeks and 12 weeks after alloxan treatment. From paraffin block of pancreatic tissues, 5 sets of 22 sections (about 5 μ m thick), including 10 pairs of mirror sections and two sections adjacent to the first and last mirror sections, were prepared from each pancreas. Each set of sections was cut at an interval of 500 μ m. All procedures were done at room temperature, unless otherwise stated.

Immunohistochemistry and morphometric analysis

The first pair of mirror sections in each set was double immunostained with a mixture of anti-glucagon (1:1000) (Linco Research Inc., St. Charles, MO), anti-somatostatin (1:1000) (Dako, Glostrup, Denmark) and anti-pancreatic polypeptide (PP) (1:1000) (Dako) antibodies, rabbit anti-duct cell specific cytokeratin (DCK) antibody (Nichirei, Tokyo, Japan) and guinea pig anti-insulin IgG antibody (1:5000) (Dako) according to our previous method [10]. Islet cell neogenesis by the formation of ICCs was confirmed only when ICCs were directly associated with the duct epithelium, because we often observed a close association of islet structure with ducts even in normal islets.

The next three pairs of mirror sections from each set were double-immunostained for two hormones, PDX-1 and DCK. One section from each of the three pairs of mirror sections was first immunostained for either glucagon, somatostatin, or PP and then was immunostained for insulin. The other sections from the three pairs of mirror sections were first immunostained for PDX-1 with rabbit anti-PDX-1 antibody (1:200) and then were immunostained for DCK.

We performed double immunostaining for BrdU using BrdU Labeling and Detection Kit II (Boehringer Mannheim Biochemica) and insulin in one section by the method described previously [10], and stained BrdU and DCK or pancreatic amylase (1:500) (Sigma) in the other section of the fifth pair sections. The sixth pair of mirror sections in each set and the adjacent section to this pair were prepared for double immunostaining for four islet hormones so that the number of hormone-positive cells (/mm²) could be calculated in each pancreatic region. One of the mirror sections was double immunostained for somatostatin and insulin. The other one was double immunostained for PP and glucagon. The adjacent section to the mirror sections was double immunostained for glucagon, somatostatin, PP and insulin. Hormone-producing islet cells were detected by positive staining for insulin, glucagon, somatostatin, and/or PP.

The seventh, eighth and ninth pair of mirror sections were double immunostained for Pax6, Isl1, or Nkx2.2 and DCK. One of each pair sections was firstly incubated with mouse anti-chick Pax6 a.a.

1-223 or mouse anti-C-terminal portion of Isl1(1:5) or mouse anti chick Nkx2.2 antibody (1:5) (Developmental Studies Hybridoma Bank) using histomouse-SP plus kit for broad spectrum (Zymed Laboratories) and secondly stained with DCK. The other one of three mirror sections was stained for pancreatic hormone with the same method described above [10].

For morphometric analyses, the relative number of hormone-producing cells and islets and the area of each pancreatic section were determined on light microscopic images (PROVIS AX 80 equipped with an HDTV system and a color-charged 3CCD camera, Olympus, Tokyo, Japan) and were analyzed using an image analysis system (Macscope version 2.55, Mitani Corp., Fukui, Japan).

Statistical analysis

Results are expressed as the mean $\pm$ SE. The significance of differences between the alloxan-treated and the control groups was evaluated by Dunnett's multiple comparison test, and $p < 0.05$ was accepted as statistically significant.

Results

In the mice with glucose intolerance induced by selective perfusion of alloxan, the number of islets in the perfused segment was markedly decreased at an early stage. However, at 12 weeks after alloxan treatment, the relative number of islets in the alloxan-perfused segment was increased. Also, no significant difference was observed between the nonperfused segment of diabetic mice and either segment in the control group, and this was also the case at 4 weeks after alloxan perfusion (Table 1). However, at 12 weeks after treatment, insulin-positive cells could be detected even in the alloxan-perfused segment, and nonhormone cells (islet cells producing neither insulin, glucagon, somatostatin, nor PP hormone) also appeared in the ICCs. In the perfused segment, the percentage of nonhormone cells at 4 and 12 weeks was significantly higher in the glucose intolerant diabetic group than in the control group. In contrast, nonhormone cells were rarely detected in the nonperfused segment of the glucose intolerant diabetic group and either segment of the control group, at the same time (Table 1). There were no significant differences between the glucose intolerant and control groups with respect to the percentages of glucagon-, somatostatin-, PP-positive cells in the perfused or nonperfused segments, as shown in Table 1. Thus, the recovery of β cells at this stage may

Table 1. Relative number of hormone cells and nonhormone cells in islets including newly formed ICCs and number of islets in the pancreas of control and experimental group

	4 weeks		12 weeks	
	control group	experimental group	control group	experimental group
Alloxan-nonperfused segment				
Insulin cells (/mm ²)	49.95 $\pm$ 1.35	55.35 $\pm$ 0.85*	59.9 $\pm$ 1.10	75.4 $\pm$ 4.60
Glucagon cells (/mm ²)	5.23 $\pm$ 0.41	4.92 $\pm$ 1.62	6.91 $\pm$ 0.64	6.55 $\pm$ 0.74
Somatostatin cells (/mm ²)	2.90 $\pm$ 0.10	3.15 $\pm$ 0.15	3.30 $\pm$ 0.41	3.81 $\pm$ 0.22
PP cells (mm ²)	1.53 $\pm$ 0.49	1.26 $\pm$ 0.19	1.50 $\pm$ 0.17	1.43 $\pm$ 0.08
Nonhormone cells (mm ²)	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Islets (/mm ²)	1.16 $\pm$ 0.16	1.06 $\pm$ 0.04	1.04 $\pm$ 0.04	1.20 $\pm$ 0.05
Alloxan-perfused segment				
Insulin cells (/mm ²)	49.25 $\pm$ 3.55	1.47 $\pm$ 0.54*	59.0 $\pm$ 2.14	3.25 $\pm$ 0.38*
Glucagon cells (/mm ²)	4.91 $\pm$ 0.68	4.60 $\pm$ 0.37	7.54 $\pm$ 0.40	7.81 $\pm$ 0.44
Somatostatin cells (/mm ²)	2.08 $\pm$ 0.55	2.10 $\pm$ 0.56	3.99 $\pm$ 0.29	3.75 $\pm$ 0.25
PP cells (mm ²)	0.65 $\pm$ 0.17	0.74 $\pm$ 0.60	0.75 $\pm$ 0.34	1.07 $\pm$ 0.23
Nonhormone cells (/mm ²)	0.00 $\pm$ 0.00	2.34 $\pm$ 0.45*	0.00 $\pm$ 0.00	3.58 $\pm$ 0.94*
Islets (/mm ²)	1.05 $\pm$ 0.42	1.04 $\pm$ 0.02	1.12 $\pm$ 0.04	1.28 $\pm$ 0.05

Data are means $\pm$ SE. N = 5 for each group. * $p < 0.05$ vs. control. In the control group, 154 mmol/l NaCl was perfused during sham operation.

have been the result of neogenesis of islet cells. As expected, small ICCs were recognized in direct association with the ducts, and insulin-positive cells could be detected within such ICCs from the alloxan-perfused segment of the pancreas (Fig. 1A and 1B). PDX-1 positive cells were also observed in the ICCs, although not all of these cells were insulin-positive (Fig. 1B and 1C). The relative number of newly formed ICCs showing a direct association with the ducts was significantly higher in the alloxan-perfused segment of the diabetic group, and newly formed ICCs were hardly recognized in any of the pancreatic segments without β cell depletion (Fig. 1D).

We next analyzed the characteristics of cells in the newly formed ICCs. Using double immunostaining of mirror image sections for islet hormones, we could observe double hormone-positive cells in ICCs from the alloxan-perfused, β cell-depleted segment. In

ICCs directly associated with the duct cell lining, glucagon/insulin double-positive cells and somatostatin/insulin double-positive cells were detected in the vicinity of single hormone-positive cells (Fig. 2A-D and 3A-D). These insulin positive cells co-expressing glucagon or somatostatin were largely restricted to the alloxan-perfused, β cell-depleted segment, and such double-positive cells were not recognized in the alloxan-nonperfused segment or in either segment of the control group. The proportion of cells co-expressing glucagon and insulin was significantly higher in the alloxan-perfused segment than in the nonperfused segment as well as in both segments of the control group ($0.09 \pm 0.01 / \text{mm}^2$, $0.00 \pm 0.00 / \text{mm}^2$, $0.00 \pm 0.00 / \text{mm}^2$, respectively; $p < 0.05$) at 12 weeks after perfusion, and this was also the case at 4 weeks after perfusion ($0.04 \pm 0.01 / \text{mm}^2$, $0.00 \pm 0.00 / \text{mm}^2$, $0.00 \pm 0.00 / \text{mm}^2$, respectively; $p < 0.05$). The

Fig. 1. Immunofluorescent detection of duct cell specific cytokeratin (DCK), insulin and PDX-1 in newly formed ICCs using a set of mirror sections from the pancreas of diabetic mice at 4 and 12 weeks after selective perfusion with alloxan. The intralobular duct with small branches showed positive DCK fluorescence (green color), and two small ICCs (arrows) were directly or closely associated with the duct epithelial lining (A). In the mirror section (B), these ICCs are seen to contain insulin-positive cells (brown). In section A, PDX-1 was also immunohistostained, and several PDX-1-positive cells were detected in each ICC (C). The number of PDX-1-positive cells appeared to be larger than that of insulin-positive cells (B and C). Morphometric analysis showed that the proportion of ICCs directly associated with ducts was significantly higher in the alloxan-perfused segment than in the nonperfused segment of the diabetic group or both segments of the control group (D). (A, B and C: $\times 580$, scale bar = 25 μm)

Fig. 2. Detection of glucagon and insulin cells in the duct cell lining from the alloxan-perfused segment of the pancreas.

Double immunostaining for two pancreatic hormones showed small clusters of glucagon cells (green) and insulin cells (red) in the duct epithelium. At least two cells (arrowheads in A and B) were glucagon/insulin double-positive, showing a yellow color in the double-exposed photograph (C). These cells were localized in the duct cell lining, as revealed by DCK immunostaining of the mirror section (D). The asterisk (*) in Figure 2D indicates the duct lumen. (A, B, C and D: $\times 450$, scale bar = 20 μm)

proportion of cells co-expressing somatostatin and insulin was also significantly higher in the alloxan-perfused segment than in the nonperfused segment as well as in both segments of the control group at 12 weeks ($0.04 \pm 0.01 / \text{mm}^2$, $0.00 \pm 0.00 / \text{mm}^2$, $0.00 \pm 0.00 / \text{mm}^2$, respectively; $p < 0.05$) (Fig. 4A and 4B), but somatostatin and insulin double-positive cells were not detected at 4 weeks after perfusion. We could not detect PP- or amylase-positive cells co-expressing insulin or glucagon in either the alloxan-perfused or nonperfused segments of the diabetic group (data not shown).

Next, we evaluated the proliferative activity of the β (insulin-positive) cells, duct (DCK-positive) cells, and acinar cells of each segment. The BrdU labeling index (L.I.) of DCK-positive duct cells, insulin-positive cells, and acinar cells was determined by double immunostaining in both groups (Fig. 5A-C). In the perfused segment of the diabetic group, the BrdU L.I. of DCK-positive cells was significantly higher than in the control group. However, the nonper-

Fig. 3. Double immunostaining for somatostatin (A) and insulin (B) and staining for DCK in a newly formed ICC from the alloxan-perfused segment of a diabetic mouse.

An aggregate of somatostatin-positive (green) cells is observed (A), and three of the four somatostatin-positive cells are also insulin-positive (B). These somatostatin/insulin double-positive cells show a yellow-orange color in the double-exposed photograph (C). In the mirror section, immunostaining for DCK shows that this ICC (arrow) is localized in close contact with the duct epithelium (green) (D). The asterisk in each figure indicates the duct lumen. (A, B, C and D: $\times 600$, scale bar = 10 μm)

fused segment showed no significant difference from the control group at 4 or 12 weeks. Also, the BrdU L.I. of DCK-positive cells was significantly higher in the alloxan-perfused segment of the diabetic group than in the nonperfused segment, while there was no significant difference between the two segments of the control group (Fig. 5A). As shown in Fig. 6A and 6B, cytoplasmic DCK-positive duct cells with positive nuclear BrdU labeling could be observed in the alloxan-perfused segment, but such cells were hardly detected in the nonperfused segment. At 4 or 12 weeks after alloxan treatment, the BrdU L.I. of insulin-positive cells in both the nonperfused and perfused segments showed no significant difference between the two groups (Fig. 5B). BrdU/insulin double-positive cells were undetectable in the alloxan-perfused

Fig. 4. The proportion of cells coexpressing glucagon/insulin (A) and somatostatin/insulin (B) in islets (including newly formed ICCs) in the pancreas at 4 and 12 weeks after selective perfusion of alloxan.

(A) The number of glucagon/insulin double-positive cells (/mm²) in the alloxan-perfused segment of the diabetic group was significantly higher than in the control group, while such cells were negligible in the nonperfused segments of both groups. (B) The number of glucagon/insulin double-positive cells (/mm²) in the alloxan-perfused segment of the diabetic group was also significantly higher than in the control group. These cells were extremely rare in the nonperfused segments, and there was no significant difference.

A BrdU L.I. in cytokeratin positive cells

B BrdU L.I. in insulin positive cells

C BrdU L.I. in acinar cells

□ Control group, ■ Experimental group, *p<0.05

Fig. 5. BrdU labeling index of insulin, duct, and acinar cells.

(A) The BrdU labeling index of duct cells from the alloxan-perfused segment of the diabetic group was significantly higher than in the control group, but there was no significant difference between the nonperfused segments of the two groups.

(B) There was no significant difference between the BrdU labeling index of insulin-positive cells from the alloxan-perfused segment of the diabetic group and the index in the control group, and the same was true for the nonperfused segments of the two groups.

(C) There was no significant difference between the BrdU labeling index of acinar cells from the alloxan-perfused segment of the diabetic group and the index in the control group, and the same was true for the nonperfused segments of the two groups.

Fig. 6. Detection of BrdU-positive cells among duct cells in the alloxan-perfused and nonperfused segments of a pancreas from a diabetic mouse at 12 weeks. Double immunostaining for BrdU (green nucleus) and DCK (blue) showed that BrdU-positive cells were observed among DCK-positive duct cells in the alloxan-perfused segment (arrows in A), while BrdU/DCK double-positive cells were hardly detected in nonperfused segment (B). (A and B: $\times 370$, scale bar = 20 μm)

segment of the diabetic group despite the presence of insulin-positive cells. Positive staining of acinar cells by BrdU could be observed, and the BrdU L.I. was higher than that of DCK-positive duct cells, but there was no significant difference between the two groups in either the nonperfused and perfused segments (Fig. 5C). Thus, mitotic activity at 4 weeks after alloxan perfusion was only significantly elevated in the duct cells of the alloxan-perfused, β cell-depleted pancreatic segment of the diabetic group.

Pax6-positive but hormone negative cells were observed in duct cells, and Pax6 and hormone double positive cells were also observed in duct cell lining (Fig. 7A-C). Isl1-positive cells were also observed in duct cells and newly formed ICC was associated with the duct. Isl1-positive (Fig. 7D) but pancreatic hormone-negative cells (Fig. 7F) were observed in duct cells (Fig. 7E), and Nkx2.2 and pancreatic hormone double positive cells were also observed in duct cells (Fig. 7G-I).

During the analysis of multiple hormone-positive cells in newly formed ICCs, we noticed that double hormone-positive cells were also detectable in residual islets of the alloxan-perfused segment. These islets were not directly associated with the ducts, and mainly consisted of glucagon cells, while insulin-positive cells were not detected at an early stage after alloxan treatment. In this model, we have previously confirmed by electron microscopy that β cells are totally eliminated within 5 days of alloxan perfusion [11]. However, careful observation revealed that

glucagon/insulin double-positive cells were localized among the clusters of glucagon cells at 4 and 12 weeks (Fig. 8), while somatostatin/insulin double-positive cells were localized among the clusters of somatostatin cells at 12 weeks (Fig. 9). Double immunostaining for insulin and glucagon in one of the mirror sections from a pair demonstrated islet cells with varying degrees of insulin immunoreactivity scattered among glucagon-positive cells (Fig. 8A and 8B), and some of the glucagon-positive cells co-expressed insulin as shown by the double-exposure photograph in Fig. 8C. In such islets, PDX-1-positive cells were more frequent than insulin-positive cells, and PDX-1 was not only detected in insulin-positive cells but also in glucagon/insulin double-positive cells and a few glucagon-positive cells (Fig. 8C and 8D). Double immunostaining for somatostatin and insulin in one of the mirror sections from a pair also showed the existence of somatostatin/insulin double-positive cells in the residual islets (Fig. 9A and 9B). PDX-1 immunostaining of the other section from the pair revealed that the somatostatin/insulin double-positive cells were PDX-1 positive, although some single somatostatin-positive cells also expressed PDX-1 (Fig. 9C and 9D).

Thus, in the completely β cell-depleted adult pancreatic tissue, we demonstrated at least two different processes of β cell neogenesis; one was the formation of new ICCs from ductal epithelium and the other was redifferentiation of β cells from residual non- β cells (phenotypic conversion).

Discussion

Recent advances in our knowledge of pancreatic development and β cell differentiation in the fetal and neonatal periods have provided important clues towards understanding the mechanism of β cell regeneration in diabetic pancreas [12–15]. However, the precise mechanism of β cell regeneration has not been clarified. To understand the process of β cell neogenesis, we developed a mouse model with glucose intolerance induced by selective alloxan perfusion, in which two types of regeneration occurred separately in the pancreas of the same animal [10, 11, 16]. In this model, preexisting β cells proliferate at an early stage in the alloxan-nonperfused segment, while β cells gradually differentiate from extra-islet

Fig. 7. Double immunostaining for Pax6, Isl1, Nkx2.2 and DCK, and pancreatic hormones using mirror sections from an alloxan-perfused segment at 12 weeks after alloxan treatment.

One of the mirror sections was double-immunostained for Pax6 (A), Isl1 (D), Nkx2.2 (G) and DCK (B, E and H). The other mirror sections were immunostained for non-insulin pancreatic hormones and insulin, and Figures C, F and I show the double exposed photographs. The Pax6 positive cell (arrow in A) was detected in duct cell lining (green fluorescence) (arrow in B), and it was nonhormone cell (arrow in C). The Pax6 and pancreatic hormone double positive cell was detected in the duct cells lining (arrowheads in A, B and C). The Isl1 positive cell (arrow in D) was detected in a duct cell (green fluorescence) (arrow in E), and it was non-insulin pancreatic hormone positive cell (arrow in F). A newly formed ICC which consists of pancreatic endocrine cells was detected adjacent to the duct (E and F), and the Isl1 and non-insulin pancreatic hormone double positive cell was detected in the ICC (arrowheads in D and F). The Nkx2.2 positive cell (arrow in G) was detected in a duct cell (green fluorescence) (arrow in H), and it was pancreatic hormone positive (arrow in I). (A to I: $\times 1080$, scale bar = 10 μm)

precursor cells (mainly duct cells) in the alloxan-perfused segment. Thus, duct cells are important for islet neogenesis in the diabetic pancreas when the β cells have been almost totally destroyed. In the alloxan-perfused segment, the relative number of islets or ICCs including single endocrine cells, was decreased on day 5, but was significantly increased at 20 weeks. Also, insulin-positive cells were significantly increased at 20 weeks compared with day 5. In our model, difference in body weight between the control and diabetic groups was no longer significant at 12 weeks after the alloxan treatment. Blood glucose

concentrations at 0, 60 and 120 min in the IPGTT were clearly decreased in the glucose intolerant group at 12 weeks after alloxan treatment [10]. We therefore compared the mice at 4 weeks and 12 weeks after alloxan perfusion to investigate how β cells differentiate from precursor cells in the ducts and whether non- β cells in residual islets show any change in their morphological characteristics in the alloxan-perfused, β cell-depleted segment.

It now seems clear that the β cell precursor is located in the duct wall, and that these cells or dormant duct cells are the major source of endocrine cells in

Fig. 8. Double immunostaining for glucagon and insulin and staining for PDX-1 in a residual islet using mirror sections from an alloxan-perfused pancreatic segment at 12 weeks after alloxan treatment.

One of the mirror sections was double-immunostained for glucagon (A) and insulin (B), and a double-exposed photograph is shown in (C). The other mirror section was immunostained for PDX-1 (D). The residual islet mainly consisted of glucagon cells (green fluorescence) (A), but a few insulin-positive cells could be detected (red fluorescence) (B). Some of the glucagon-positive cells (arrowheads and double arrowhead in A) were also insulin-positive (arrowheads and double arrowhead in B), and these double-positive cells were recognized as cells with yellow fluorescence in double-exposed photographs (arrowheads and double arrowhead in C). In a mirror section (D), many PDX-1 positive cells were detected, and a cluster of non-insulin/glucagon cells (indicated by * in C) was positive for PDX-1 (indicated by * in D). An insulin-positive cell (arrows in B and C) was also PDX-1 positive (arrow in D), and a glucagon/insulin double-positive cell was also positive for this transcription factor as indicated by the double arrowheads in A, B, C and D. (A, B, C and D: $\times 720$, scale bar = 20 μm)

the process of β cell neogenesis [17]. Such cells are considered to be evidence of epithelial cell origin and may be a part of normal islet neogenesis. In the present study, the BrdU L.I. of duct cells was significantly increased in the alloxan-perfused segment from 4 weeks after perfusion, but there was no significant difference in the BrdU L.I. of insulin-positive cells and acinar cells between the alloxan-perfused segment and the nonperfused segments of the two groups. In the alloxan-perfused segment, BrdU/insulin double-positive cells were undetectable despite the increase of insulin-positive cells. In addition, the proportion of ICCs associated with the ducts was

increased significantly. These findings suggest that the proliferation of duct cells was induced following elimination of β cells and this led to mobilization of duct cells to create ICCs. The appearance of insulin-positive cells is considered to be a post-mitotic phenomenon. Since PDX-1-positive cells were detected among nonhormone cells and immature endocrine cells co-expressing insulin and/or glucagon in the ICCs, induction of PDX-1 seems to be a prerequisite for redifferentiation of duct cells into endocrine cells. However, it was unclear whether or not the proliferation of duct cells precedes the induction of PDX-1. During the process of pancreatic re-

Fig. 9. Double immunostaining for somatostatin and insulin and staining for PDX-1 in a residual islet using a set of mirror sections from an alloxan-perfused pancreatic segment at 12 weeks after alloxan treatment. One of the mirror sections was double-immunostained for somatostatin (green fluorescence) (A) and insulin (red fluorescence) (B), and a double-exposed photograph is shown in (C). The other mirror section was immunostained for PDX-1 (D). Many somatostatin-positive cells were observed in the residual islet (A), and a few insulin-positive cells were also detected (B). The insulin-positive cell (double arrowhead in B) was also positive for somatostatin (double arrowhead in A), and it (yellow) (double arrowhead in C) was also recognized to be PDX-1 positive (brown) (double arrowhead in D). The somatostatin-positive cell also expressed PDX-1 (arrowheads in A, C and D). The PDX-1 positive cell (arrow in D) was hormone negative (arrows in A and C) (A, B, C and D: $\times 640$, scale bar = 20 μm)

generation in rats after 90% pancreatectomy, proliferation of duct cells occurred at 24–36 hours after pancreatectomy, while the PDX-1 protein level increased transiently after 2–3 days [18]. In the present study, the stimulation of duct cell proliferation and formation of ICCs were induced by the selective destruction of β cells, and not by the indiscriminate elimination of both exocrine and endocrine cells, suggesting that a different mechanism of β cell neogenesis might be involved. If the regeneration pattern demonstrated in the rat pancreatectomy model is applicable to the process of islet neogenesis in the present model, the following process could be suggested: an increase of mitotic activity occurred in duct cells before the induction of PDX-1 caused duct cells or transitional cells to differentiate into mature endocrine cells. We also observed glucagon/insulin and somatostatin/insulin double-positive cells with or without PDX-1 expression in the duct cell lining or in newly formed ICCs from the alloxan-perfused seg-

ment. These results suggest that multi-hormone expressing, immature endocrine cells could be formed in the process of β cell neogenesis, although direct differentiation from nonhormone cells to insulin-producing cells might also occur. It is clear that our present co-localization study did not necessarily clarify the sequence of events in the process of β cell neogenesis. However, during the process of pancreatic development and fetal islet cell differentiation, the appearance of multi-hormonal cells has been well documented [19, 20], and there is evidence that the manifestation of multi-hormonal cells during islet neogenesis represents a recapitulation of the events that occur during embryogenesis [2, 21, 22]. Thus, the following hypothesis can be proposed for the process of β cell neogenesis from duct cells in the alloxan-perfused, β cell-depleted segment. Precursor cells in the ducts might differentiate into PDX-1-positive cells, which then differentiate into PDX-1/somatostatin or PDX-1/glucagon double-positive cells, and

then terminally differentiate into β cells via somatostatin/insulin/PDX-1 or glucagon/insulin/PDX-1 triple positive cells, respectively. Transcription factors such as Pax6, Isl1 and Nkx2.2 were observed in duct cells of alloxan perfused segment, suggesting that these factors were also essential during β cell neogenesis from duct cells in adult mice.

The fate of non- β cells such as glucagon, somatostatin and PP cells in the β cell-depleted residual islets has not been studied in detail so far. It was reported that β cell precursors among the non- β islet cells might act as a β cell reservoir, and one possible candidate is the somatostatin cell [23]. We also detected somatostatin/insulin double-positive cells with or without PDX-1 expression in the residual islets of the β cell-depleted pancreatic segment. In addition, our co-localization study clearly demonstrated that some of the residual glucagon cells came to express PDX-1 or both PDX-1 and insulin. This would be more or less expected, since PDX-1-transfected, glucagon-secreting α TC1 cells are reported to induce insulin and glucokinase gene expression in the presence of betacellulin [24]. It is interesting that PDX-1 was newly expressed in non- β cells, such as glucagon and somatostatin cells, of the residual islets in the alloxan-perfused segment. Since the BrdU L.I. of islet cells did not increase significantly, induction of PDX-1 may have occurred in a preexisting population of glucagon and somatostatin cells. We also detected the cells co-expressing somatostatin and PDX-1, although this was not surprising because some somatostatin cells from normal islets express this transcription factor in mice [25]. From these observations, it could be speculated that somatostatin or glucagon cells co-expressing PDX-1 (especially somatostatin/PDX-1 double-positive cells) act as a β cell reservoir in normal islets, and that these cells can differentiate into somatostatin/insulin/PDX-1 triple-positive cells and then into β cells. Recently, Zulewski *et al.* reported the possible existence of intra-islet, nestin-

positive nonhormone cells as endocrine stem cells [26]. In this study, we could not identify such cells in residual islets of β cell-depleted pancreas, although this interesting issue would be worth evaluating in detail. The increase of β cell mass achieved by this mechanism may be small, since there was no significant decrease in the proportion of non- β endocrine cells when the alloxan-perfused and nonperfused segments were compared.

In conclusion, there may be two different pathways of β cell neogenesis in the β cell-depleted diabetic pancreas. One pathway involves the differentiation of duct cells or islet precursor cells residing in the ducts, probably via several steps such as immature nonhormone cells and cells co-expressing multiple hormones. The other is the redifferentiation or phenotypic conversion of non- β endocrine cells into β cells in the residual islets. Our results suggest that these two mechanisms of β cell neogenesis respond spontaneously to the circumstances of β cell depletion in the adult pancreas of mice with glucose intolerance, although the actual increase in β cells does not seem to be enough to restore the initial functional β cell mass. This model may help to clarify the factors that regulate these two pathways of β cell neogenesis. From a clinical point of view, strategies to stimulate β cell neogenesis from precursor or non- β cells might help to develop a new therapy for diabetic patients with very low insulin secretion.

Acknowledgments

This study was supported by Scientific Research Funds from the Ministry of Education, Science, and Culture of Japan (Grants No. 09671056, 11671087, and 11770641). Part of the data was reported at the 57th American Diabetes Association Meeting, Boston, 1997.

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