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Regeneration of Pancreatic β Cells from Intra-Islet Precursor Cells in an Experimental Model of Diabetes

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We previously reported that new β cells differentiated in pancreatic islets of mice in which diabetes was produced by injection of a high dose of the β cell toxin streptozotocin (SZ), which produces hyperglycemia due to rapid and massive β cell death. After SZ-mediated elimination of existing β cells, a population of insulin containing cells reappeared in islets. However, the number of new β cells was small, and the animals remained severely hyperglycemic. In the present study, we tested whether restoration of normoglycemia by exogenous administered insulin would enhance β cell differentiation and maturation. We found that β cell regeneration improved in SZ-treated mice animals that rapidly attained normoglycemia following insulin administration because the number of β cells per islet reached near 40% of control values during the first week after restoration of normoglycemia. Two presumptive precursor cell types appeared in regenerating islets. One expressed the glucose transporter-2 (Glut-2), and the other cell type coexpressed insulin and somatostatin. These cells probably generated the monospecific cells containing insulin that repopulated the islets. We conclude that β cell neogenesis occurred in adult islets and that the outcome of this process was regulated by the insulin-mediated normalization of circulating blood glucose levels. (*Endocrinology* 142: 4956–4968, 2001)

THE PANCREAS IS composed of exocrine tissue, which produces digestive enzymes, and endocrine tissue, which is comprised by cells clustered into the islets of Langerhans. The are four different islet cell types, which produce glucagon (α cells), insulin (β cells), somatostatin (∂ cells), and pancreatic polypeptide (PP cells), respectively. Insulin (IN) containing β cells form the core of the mature islets, which is surrounded by a rim of α, ∂, and PP cells.

Because the absence of β cells leads to hyperglycemia and overt diabetes, a crucial question is whether the adult pancreas contains β stem cells and, if so, the location of these cells in the tissue. One approach to answer this question is to characterize the phenotype of the presumptive precursor cells in embryos and then ascertain whether similar cells are present in adults. In the last few years, a molecular fingerprint of embryonic islet precursor cells has begun to emerge (Refs. 1–6 and references herein). In embryos and young postnatal mice, precursors present in the pancreatic duct (7) migrate into the tissue parenchyma where they differentiate into mature islets. Previous studies indicated that the ductal precursors can be identified by the expression of the low affinity glucose transporter-2 (Glut-2) (8), which is also present in mature β cells. This observation supported the view that β cells are generated by Glut-2^+ precursor cells (8). It is generally assumed that pancreatic duct cells retain the ability to generate endocrine cells and form new islets even late in life (reviewed in Ref. 9). Recently, cells expressing nestin, an intermediate filament protein expressed by neuronal stem cells, were located in pancreatic ducts of adult rats and were found to differentiate into IN-expressing cells *in vitro* (10). Whether the nestin$^+$ cells display the ability to differentiated into mature β cells *in vivo* remains to be determined.

There is also evidence suggesting the presence of presumptive β precursor cells in mouse pancreatic islets that differentiate into insulin cells following injury. These cells differentiated in islets following depletion of the resident β cell population by streptozotocin (SZ), a β cell toxin. The first immature cell type to appear expressed somatostatin (SOM) and pancreatic and duodenal homeobox gene 1 (Pdx-1), a transcription factor expressed by islet progenitor cells (reviewed in Ref. 3) which it was followed in time by the appearance of cells coexpressing SOM and IN (10). It was proposed that the SOM/Pdx-1^+ cells initiated IN expression, generating the SOM/IN$^+$ cells that reappeared in islets following SZ treatment (11). However, the number of newly differentiated SOM/IN$^+$ cells was low, they failed to differentiate into monospecific IN$^+$ cells and the SZ-treated animals remained severely hyperglycemic at all stages examined.

High glucose levels have a negative effect on β cell function (12) by decreasing the expression of Pdx-1 (13) and of β cell genes, which are transactivated by Pdx-1, such as insulin, Glut2, glucokinase, a key enzyme in glucose metabolism, and islet amyeloid polypeptide (14–19). These observations raised the possibility that hyperglycemia could also impair the differentiation of islet precursor cells into cells containing insulin. We reasoned that the reestablishment of normoglycemia would enhance β cell neogenesis and maturation and tested this hypothesis in the present study. Our results indicate that prompt reestablishment of normoglycemia in SZ-treated mice by exogenously administered insulin allowed the differentiation of two sets of precursor cells in islets, the

Abbreviations: AADC, Aromatic L-amino acid decarboxylase; bg, blood glucose; BrdU, 5-bromo-2′deoxyuridine; DAB, 3,3′-diaminobenzidine; GLU, glucagon; Glut-2, glucose transporter-2; IN, insulin; Pdx-1, pancreatic and duodenal homeobox gene 1; PP, pancreatic polypeptide; SOM, somatostatin; SZ, streptozotocin.

Glut-2 and Pdx-1/SOM cells, respectively, into IN-containing cells and the reappearance of morphologically normal islets.

Materials and Methods

Animals and tissue processing

Six-week-old male CD-1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Obese (C57Bl/6J-*Lep*^{ob}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). CD-1 mice were injected ip with 200 mg/kg streptozotocin (200 SZ mice, Upjohn Co., Kalamazoo, MI; USB/Amersham Pharmacia Biotech, Arlington Heights, IL) in 0.1 M citrate buffer, pH 4.5, after a 12-h overnight fast. The drug was kept at 4 C to prevent its degradation and was administered immediately after preparation of the solution. Approximately 10 mice were injected using the same drug solution. Only those animals with plasma glucose levels of 350–400 mg/dl or higher 24 h after injection of SZ (=1 d post SZ) were used. Following this protocol, approximately 80% of the injected mice were severely hyperglycemic at 1 d post SZ. Control mice were injected with the equivalent volume of citrate buffer. Blood for glucose determination was collected by snipping the tail in the fed state. Blood glucose was measured with Tracer II Blood Glucose monitor (Roche Molecular Biochemicals). Following the injection of SZ or diluent, animals were provided with Pedialyte (Walgreen's Corp., Deerfield, IL), a pediatric electrolyte solution. We examined the following groups of mice:

1) Hyperglycemic mice. These mice did not received IN treatment and were termed hyperglycemic x days post-SZ mice. They were examined at 1, 2, and 6 d post SZ (at least 6 animals per group).

2) Normoglycemic mice that received IN treatment from 1 d post SZ and were normoglycemic thereafter. These mice were injected with 2–3 U of NPH-ILETIN IN (Eli Lilly, Indianapolis, IN) ip in the afternoon of 1 d post SZ. The dosage of insulin was not adjusted to compensate for possible differences in body weight. Animals with blood glucose (bg) levels between 40 to 130 mg/dl at 2 d post SZ were considered normoglycemic. One group of normoglycemic mice was killed at 2 d post SZ (n = 40). Other normoglycemic 2 d post-SZ mice were anesthetized with Metofane (Schering-Plough Corp., Union, NJ), received two to three insulin implants (Linβit, Linshit Canada Inc., Ontario, Canada) following manufacturers' instructions and were killed at 4 (n = 10) and 6 (n = 16) d post SZ.

Blood glucose levels were determined daily. From the morning of d 1 post SZ until the end of the experiment, all SZ mice were fed with Pregestimil, (Mead Johnson & Co., Evansville, IN), a hypoallergenic infant formula used to feed diabetic mice (20). Mice were perfused through the heart with 4% paraformaldehyde buffered to pH 7.4 with 0.1 M PBS. The fixed tissues were infiltrated overnight in 30% sucrose, mounted in embedding matrix (Lipshaw Co., Pittsburgh, PA) and 15–20 μm cryostat sections were collected onto gelatin-coated slides.

Source of antibodies and purified peptides

Primary antiserum. Guinea pig antibodies to bovine IN and rat C-peptide were purchased from Linco Research, Inc. (Eureka, MO). Rabbit antiserum to human glucagon was purchased from Calbiochem (San Diego, CA). Rabbit antisera to human PP and to somatostatin were supplied by Peninsula Laboratories, Inc. (Belmont, CA). Rabbit antisera to Ki 67p was purchased from Novocastra. Mab antibody to rat PP was generously provided by CURE/Gastroenteric Biology Center, Antibody/RIA Core (NIH Grant DK-41301). Mab antibody to human glucagon was purchased from Sigma (St. Louis, MO). Rabbit antisera to rat L-amino acid decarboxylase (AADC) and to rat SOM were purchased from Protos (New York, NY). Rabbit antisera to Glut2 was purchased from Chemicon (Temecula, CA). Affinity purified antiserum to the N-terminal domain of Pdx-1 was a generous gift from C. V. E. Wright (21, 22). Antisera to peptide hormones were tested by immunoadsorption and the dot blot technique according to criteria previously described (23). Antibodies were used at the following dilutions: guinea pig antibovine insulin and antirat C-peptide, 1:400; rabbit antisera to Glut2, 1:1000; rabbit antihuman glucagon, 1:12,000; rabbit antihuman somatostatin-1:8,000 for control sections; and 1:20,000 for sections of SZ-treated pancreas; rabbit antihuman pancreatic polypeptide, 1:100,000; rabbit antimouse PDX-1, 1:8000; rabbit antibovine AADC, 1:250; rat antisomatostatin, 1:2000; Mab to glucagon, 1:6000; Mab to PP, 1:2000; rabbit anti Ki 67p, 1:1:1000.

Secondary antibodies. Biotinylated goat antirabbit IgG and avidin-labeled peroxidase were purchased from Vector Laboratories, Inc. (Burlingame, CA). Alexa Fluor 488 antimouse, antirat, and antirabbit IgG, Alexa Fluor 594 antiguinea pig, antirabbit and antimouse IgG and the nuclei acid dye Topro-3 were purchased from Molecular Probes, Inc. (Eugene, OR). Cy-5 donkey antirabbit and antimouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). For double-labeling using rabbit and guinea pig antisera, the secondary antibody used to visualize the guinea pig antibodies (purchased from Jackson ImmunoResearch Laboratories) did not cross-react with rabbit antibodies.

Immunolabeling of cryostat sections using peroxidase techniques

These techniques have been previously described (11). In brief, the sections were incubated sequentially in an empirically derived optimal dilution of control serum or primary antibody raised in species "X" containing 1% goat serum in Tris-saline solution (TS; 0.9% NaCl in 0.1 M Tris, pH 7.4) for 18 h; a 1:50 dilution of anti- (species x) biotinylated IgG solution in 1% goat serum in TS for 30 min; and a 1:100 dilution of peroxidase-avidin complex for 30 min (avidin-biotin complex: ABC technique). Following these incubations, the bound peroxidase was visualized by 3,3′-diaminobenzidine (DAB). After the DAB step, sections were dehydrated and mounted with Permount (Fisher Scientific, Fairlawn, NJ).

Double label immunohistochemistry using two peroxidase substrates

This technique allowed the simultaneous visualization of nuclear and cytoplasmic antigens. Sections were incubated first with antisera to PDX-1 and the bound antibody was visualized by DAB (brown precipitate), followed by incubation with antisera to a hormone, which was visualized with the blue reaction product of the Vector SG substrate (Vector Laboratories, Inc.). Slides processed for double immunohistochemical staining or for combined immunohistochemistry and autoradiography (see below) were examined with a Nikon Microphot SA microscope equipped with Nomarski optics and using a 10× ocular and an oil immersion 100× objective.

Determination of cell proliferation

For 5-bromo-2′deoxyuridine (BrdU) administration, mice were injected ip with 200 mg/kg body weight of BrdU (Sigma, St Louis, MO). Two hours after the injection, animals were perfused and the pancreas collected and sectioned. Sections were first stained for a hormone, rinsed and incubated with the corresponding IgG linked to an Alexa fluorophore. Sections were rinsed overnight with 0.1 M PBS, pH 7.2, fixed with 4% paraformaldehyde for 30 min, rinsed and treated with 2 N HCl at 37 C for 20 min and with 0.05 mg/ml pepsin in 0.1 N HCl at 37 C for 20 min. Sections were then rinsed, blocked with 1:30 goat serum in PBS for 30 min, and incubated overnight at 4 C with monoclonal antibody to BrdU (DAKO Corp., Carpinteria, CA; 1:100 diluted with a 1% solution of goat serum in PBS). Sections were rinsed and incubated with goat antimouse IgG labeled with Alexa fluorofore for 2 h. Sections were mounted with coverslips using Prolong. In addition, in some experiments, the presence of proliferating cells was examined in sections processed for simultaneous visualization of a hormone and Ki 67p, a nucleolar protein expressed by cycling cells.

Confocal microscopy

For double and triple label immunofluorescence, SOM was visualized with a rat antibody and Alexa Fluor goat 488 antirat IgG, IN with a guinea pig antibody and Alexa Fluor 594 goat antiguinea pig IgG, PDX-1 with a rabbit antibody and Cy5 donkey antirabbit IgG. PP, glucagon (GLU), and SOM were visualized with a cocktail of specific rat and mouse antisera and a mixture of antirat and antimouse Alexa Fluor 488. All secondary IgGs were used at 1:200 dilution and To-Pro 3 at 2 μM dilution for 30 min. After completion of the staining procedure, sections were covered with two to three drops of Prolong Antifade solution

(Molecular Probes, Inc.) and were dried at room temperature before examination.

A laser scanning confocal microscope, model LSM 510 (Carl Zeiss, Thornwood, NY), fitted with an Axiovert 100M microscope (Carl Zeiss) was used with a 63 × 1.4NA pan Apochromat objective (Carl Zeiss). Excitation on the laser scanning confocal microscope was with a 15 mW argon ion laser running at 75% power emitting at 488 nm, a 1.0-mW helium/neon laser emitting at 543 nm, and a 5.0 mW helium/neon laser emitting at 633 nm. Emissions were collected using a 505- to 530-nm band pass filter to collect Alexa green emissions, a 560- to 615-nm band pass filter to collect Alexa red emissions and a 650-nm long pass filter to collect Cy5 and To-Pro3 emissions. Typically, 0.7-μm vertical steps were used with a vertical optical resolution of <1.0 μm.

Processing of semithin sections

Severely hyperglycemic 1 d post-SZ mice (bg > 500 mg/dl) were anesthetized and the pancreas perfused through the common pancreatic duct with fixative solution. This procedure produced a distention of the pancreas that facilitated the dissection of tissue surrounding the duct, which is rich in islets. The tissue was embedded in epon and consecutive semithin sections (2 μm thick) were stained with thionin. Although this technique gave clear cellular localization, it precluded the survey of a large number of pancreatic islets. We examined a total of 10 islets from two 1 d post-SZ mice.

Islet cultures

Mice were injected a solution of 200 mg/kg SZ prepared before each individual injection. Studies in a test group of animals indicated that this procedure assured the development of hyperglycemia (bg >350 mg/dl) in 100% of the injected mice (n = 30). Mice were anesthetized with sodium pentobarbital 15 min after the injection, the pancreas was perfused through bile duct with a 5 ml of a collagenase solution (Worthington Biochemical Corp., Lakewood, NJ), 2 mg/ml in HBSS (Invitrogen, Carlsbad, CA), placed in a Petri dish in a stationary 37 C water bath for 15 min, dissociated with a 5 ml pipette, centrifuged, the pellet resuspended in 10 ml HBSS + 100 μl DNase [(Worthington Biochemical Corp.) 1 mg/ml in HBSS: glycerol 1:1 vol/vol] and the islets handpicked under a dissecting microscope. Islets were transferred to a 13-mm round Thermanox coverslips (Nunc, Inc., Naperville, IL) placed in a 35-mm plastic Petri dish and embedded in 20 μl of Matrigel (BD Biosciences, Bedford, MA) diluted 1:1 with RPMI 1640 containing 5.6 mM glucose. The dishes were placed in a CO_2 incubator at 37 C for 30 min to allow the Matrigel to gel. Then, each dish received 2 ml of culture media (RPMI 1640 containing 5.6 mM glucose, 10% heat inactivated FCS (Invitrogen), 1% penicillin-streptomicin (10,000 U/ml) and 15 mM HEPES buffer), and the dishes were incubated overnight. The following day the cultures were fixed, processed for immunostaining and examined by confocal microscopy.

Determination β cell relative volume per islet and per tissue

To determine β cell relative volume per islet and per tissue each gland was sectioned throughout its length to avoid bias due to regional variations in islet distribution. The relative volume of β cells per islet was determined in sections immunostained for insulin by the point sampling method (24) using a 300 point ocular grid according to the formula: F = h/n in which h was the number of "hits" over β cells and n was the number of points scored over islets (24). The same formula was used to calculate the relative β cell volume per tissue. In this case, h was the number of hits over β cells and n the number of points over exocrine tissue (24). Tissues were examined with a Nikon Microphot SA microscope equipped with Nomarski optics and using a 10× ocular and 40× objective.

Statistical analysis

All values are shown as mean ± SE. For comparison between two groups, the unpaired *t* test (two tail) was used. A P value < 0.05 was considered significant.

Results

Ablation of β cells by SZ treatment

First, we sought to determine whether SZ eliminated almost all β cells in islets of acutely hyperglycemic 1-d post-SZ/200 mice (bg > 350 mg/dl). Sections of pancreas stained with C-peptide and examined by confocal microscopy revealed the presence of 2.1± 0.3 (n = 12) stained cells per islet (Fig. 1A). These cells were located in the periphery of the islet, whereas the islet core lacked stained cells but contained cell debris and scattered nuclei (Fig. 1A). The islet rim is demarcated in Fig. 1B in a consecutive section that was immunostained with a cocktail of antisera against SOM, PP, and GLU. Examination of serial optical sections from 12 randomly selected islets from three 1 d post SZ/200 mice confirmed the presence of 1−2 C-peptide$^+$ cells (not shown).

In contrast to islets of mice with acute hyperglycemia, islets of 1SZ/200 mice with mild hyperglycemia (140–300 mg/dl) contained up to 20 cells per islet (not shown). This finding agrees with the detection of scattered surviving β cells in mildly hyperglycemic Chinese hamsters during the first 24 h following injection of SZ (25). The fact that β cells remained in islets of animals with less severe hyperglycemia following SZ injection lead us to focus our studies on mice with blood glucose levels of 350 mg/dl or higher at 1 d post SZ.

Because it is known that chronic hyperglycemia induces β cells degranulation and decreased levels of insulin, Glut-2 and Pdx-1 (14–17), it could be argued that acute hyperglycemia, such as that in 1 SZ mice, had similar effects. If so, β cells would become degranulated at 1SZ, thus evading detection by immunohistochemical staining. Following the reestablishment of normoglycemia by exogenous insulin injection, these cells would have restored the intracellular insulin concentration to normal levels and would stain again for insulin.

To address this issue, we sought to determine whether β cells became degranulated during acute hyperglycemia. First, we searched for cell markers that continued to be expressed at high levels by islet cells of hyperglycemic mice and, therefore, would stain degranulated β cells. One of the potentially useful markers was AADC, a neuronal enzyme localized in the cytoplasm of the all the endocrine cells of the islet (26, 27). We compared islets of obese mice with islets of 1d post-SZ/200 mice. In contrast to controls (Fig. 2, A and B), islets of 2-month-old obese mice (n = 3; blood glucose levels between 250 and 270 mg/dl) were lightly stained with insulin and PDX-1 (Figs. 2, C and D). This staining pattern is characteristic of islets of chronically diabetic animals (Ref. 28 and references therein). Islets of ob mice, however, were darkly labeled with AADC (Fig. 2E). Similar levels of AADC staining were observed in islets of controls (not shown), indicating that AADC levels did not decrease with hyperglycemia. In agreement with the immunofluorescent observations mentioned above, examination of sections in which the bound insulin antibody was visualized with DAB revealed that the core of islets of hyperglycemic 1 d post-SZ/200 mice (blood glucose levels > 350 mg/dl) lacked β cells but contained insulin-stained cell debris (Fig. 2F) and was surrounded by PDX-1$^+$ cells (Fig. 2G). Importantly, in these mice, AADC$^+$ cells were found in the periphery but not in

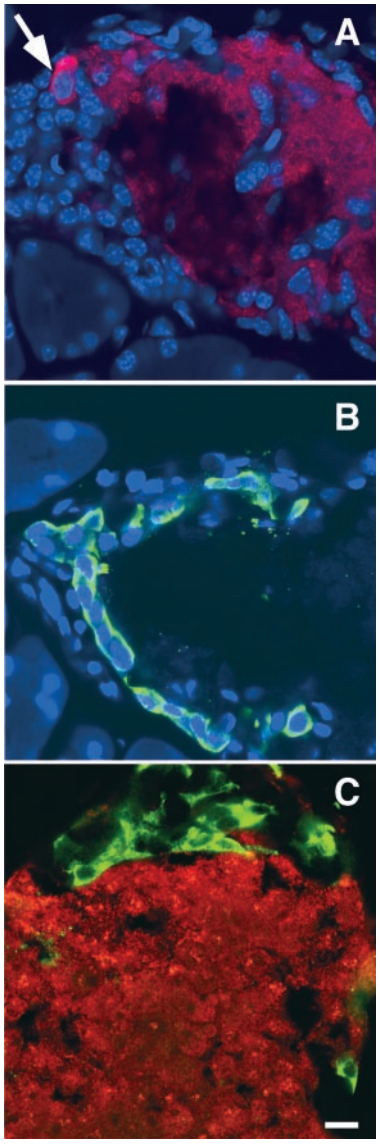

FIG. 1. Islets of 1 d post-SZ mice contained 1–2 IN^+ cells. A and B, Islet of 1 d post-SZ/200 mice examined by confocal microscopy. A, Insulin staining visualized with a Alexa-fluor 595 goat antiguinea pig (*red* fluorescence) and a Topro-Cy5 dye that labels DNA (*blue* fluorescence). Note the presence of one IN^+ cell in the periphery (*arrow*) and *red*-stained cell debris. B, Photomicrograph of a consecutive section illustrates the periphery of the islet. Nuclei are stained with Topro 3-Cy5 dye (*blue* fluorescence) and the cytoplasm with a cocktail of antibodies to SOM (produced in rat), GLU (mouse), and PP (mouse) and visualized with Alexa fluor 488 goat antimouse and Alexa fluor 488 goat antirat IgG (*green* fluorescence). The core of the islet is indicated with an X. C, Photomicrograph of an islet isolated 15 min after the mouse received SZ. The islet was maintained for 10 h in culture and was stained for insulin (*red*) and somatostatin (*green*) and examined by confocal microscopy. Note that the core of the islet lacks IN^+ cells but is filled with cell debris. *Bar*, 15 μm.

the center of the islets (Fig. 2H), confirming that most, if not all β cells, the endocrine cells present this location, were deleted by the toxin.

Second, because the presence of stained cell debris could hinder the detection of damaged cells in the islet core, semithin epon sections of 1 d post SZ islets stained with thionin, a general cell stain, were examined. No cells were found in the islet core with a discernable nucleus and cytoplasm that could account for "damaged" β cells (not shown). Third, we examined SZ-treated islets that were never exposed to hyperglycemic stimuli. To do this, mice were injected with 200 mg/KG streptozotocin, killed 15 min later, and the islets isolated and placed in culture. The mice were normoglycemic at the time of death and the islets were maintained in media containing 5.6 mM glucose, which is considered within the normal physiological range. The following day all islets showed a necrotic core. Examination of immunostained islets by confocal microscopy (10 islets/pancreas, n = 3) showed the presence of 0–2 IN^+ cells per islet (Fig. 1C). Thus, a dose of 200 mg/kg eliminated almost all the β cells whether or not the islets were exposed, as *in vivo*, to hyperglycemia. Taken together, these observations demonstrate that β cells would have been detected by immunostaining if they were alive just before fixation of the tissue. Therefore, we conclude that the almost total absence of β cells in islets of 1SZ/200 was due to the fact that they were eliminated by the toxin.

Reappearance of morphological normal islets in normoglycemic SZ + IN mice

To ascertain whether insulin-induced normoglycemia promoted β cell neogenesis, pancreas from 2 d post-SZ mice were examined. One group consisted of mice that were injected with 200 mg/kg SZ at d 0, with eluent at 1 d post SZ and killed the following day. These mice were hyperglycemic at 1 and 2 d post SZ. A second group of SZ-treated mice were injected with insulin at 1 d post SZ and attained normoglycemia (bg levels between 40 and 120 mg/dl) at 2 d post SZ. Pancreas of hyperglycemic 2 d post-SZ mice had islets that were almost completely devoid of β cells and still contained peroxidase stained cellular debris (Fig. 3B). In contrast to the hyperglycemic mice, more than half of the normoglycemic 2 d post SZ + IN mice (25 out of 40) had islets that contained 5–60 insulin cells per islet section (Fig. 3, C and D). Some of those islets contained IN^+ cells only in the periphery while, in other islets, IN^+ cells were also located in the core. The core of these islets lacked cellular debris, which was replaced by a bed of fibroblast-like unstained cells (Fig. 3D).

The relative number of β cells per islet, as revealed by insulin staining, increased with time and at 6 d post SZ, they filled the islet core (Fig. 3E). The β cells stained with antibodies to either insulin (Fig. 3) or C-peptide (not shown) indicating that the newly formed β cells were able to process proinsulin to completion. The core of insulin-containing cells was partially surrounded by endocrine nonβ cells, as determined by SOM staining (Fig. 3F). At this time the islets were irregularly shaped, suggesting that they had not fully recovered from the SZ-induced injury. While in most instances islets of 6 d post-SZ mice contained a significant number of β cells, in only 5 out of 16 cases the islets were completely repopulated by β cells (Fig. 3E). This indicates that the success of recovery was not uniform among normoglycemic mice, although each pancreas had similar degree of islet reformation. Because SZ damages many tissues in addition to the pancreas (29), it is probable that, in most SZ-treated

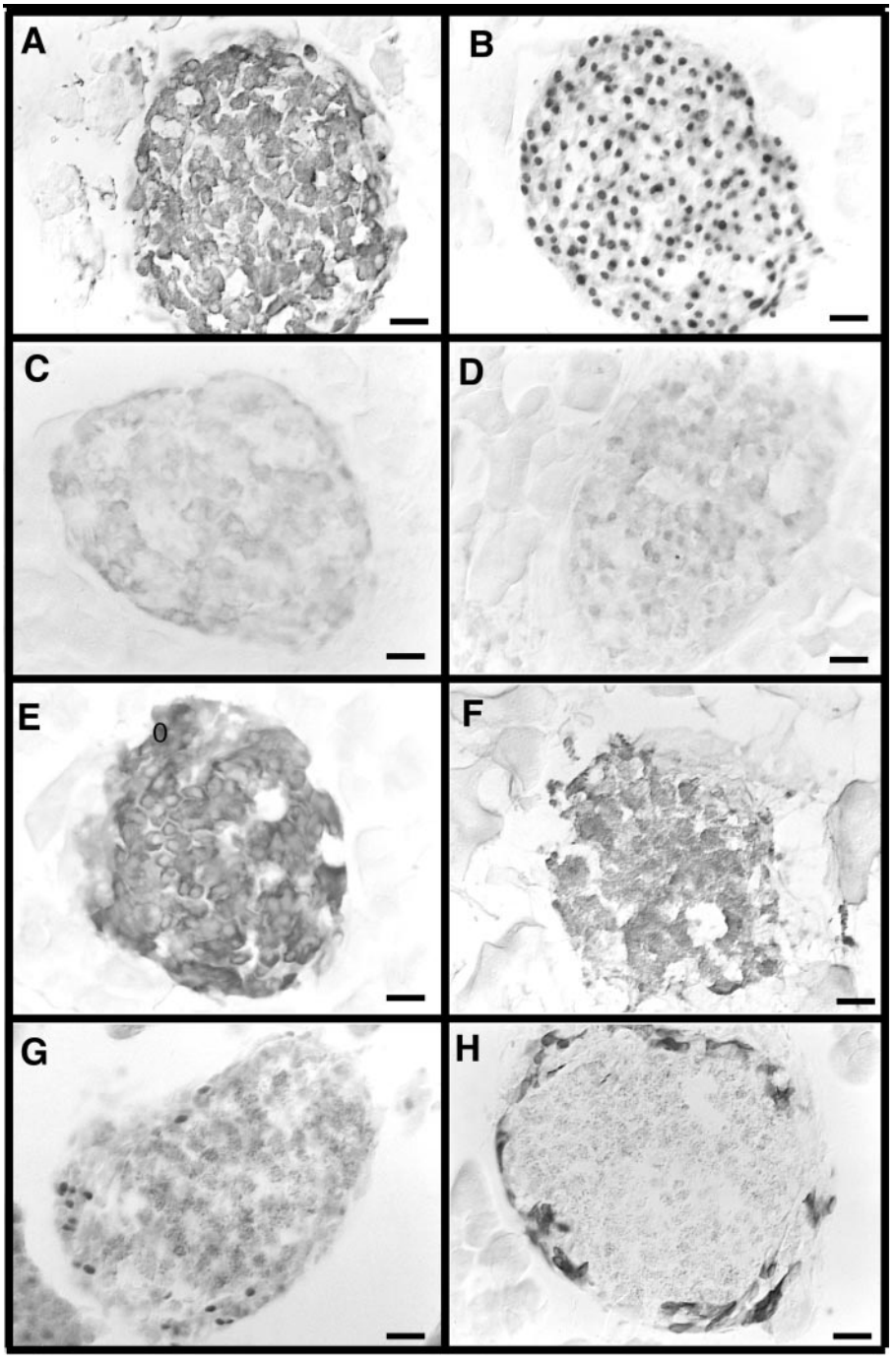

FIG. 2. Absence of endocrine cells in the core of islets of 1 d post-SZ mice. A and B, Islets of control mice and C and D of obese mice. Islets in A and C were stained with insulin. Islets in B and D were stained with PDX-1. In contrast to controls, islets of obese mice show decreased levels of insulin and PDX-1. E, Islet of ob mice stained with AADC. Note the presence of darkly stained cells. Figs. F, G and H correspond to islets of 1 d post-SZ/200 mice processed with insulin, PDX-1, and AADC, respectively. Note that islets lack IN^+ stained cells and that its core is filled with cell debris (F) and that its rim contains $PDX\text{-}1^+$ cell nuclei (G) and cells expressing AADC (H). Importantly, the core of the islet shown in H lacks $AADC^+$ cells, a characteristic of all pancreatic islets of 1 d post SZ/200 mice. *Bar*, 30 μm.

mice, its deleterious effect was only partially reversed by the exogenous insulin treatment.

Morphometric analysis confirmed that, in addition to promoting rapid islet recovery from injury, the restoration of euglycemia induced an increase in the relative volume of β cells per islet area. The effect of insulin induced normoglycemia was already evident at 2 d post SZ (24 h after injection of the hormone) because the relative volume of β cells per islet was almost 20-fold higher in normoglycemic mice than in hyperglycemic 2 d post-SZ mice (Fig. 4, Ref. 11).

The volume of β cells per islet increased with time and in 6 d post SZ + IN mice, it reached almost 40% of control values (Fig. 4). The relative β cell volume per tissue also increased from 2% of control values at 2 d post SZ to 40% of control values at 6 d post SZ (Fig. 5). These findings indicates that insulin-induced normoglycemia promoted an early burst in β cell regeneration but that the number of newly formed β cells per islet did not reached control levels during the time period of this study.

Next, we determined whether the increase in β cell number at 2 d post SZ was due to β cell proliferation. Examination of islets of normoglycemic post SZ + IN mice revealed that

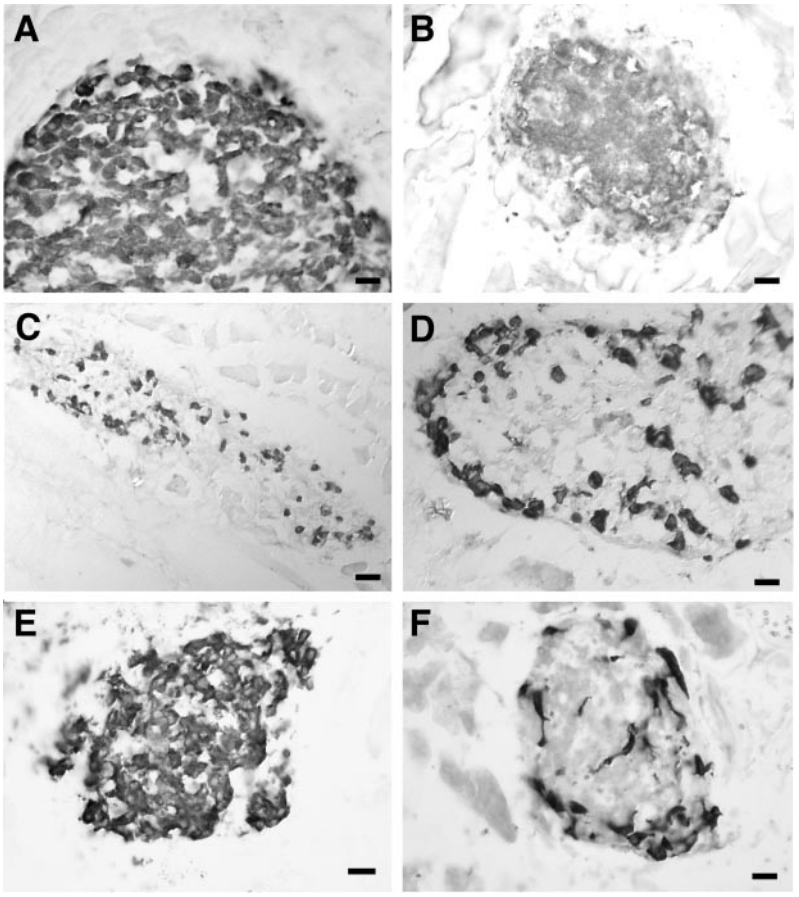

FIG. 3. Euglycemia was beneficial to islet cell regeneration. A–E, Islets stained for insulin. A, Islet of control mouse. B, Islet of a hyperglycemic 2 d post SZ mouse. Most, if not all of the stained material in the islet is cell debris. *Bar*, 30 μm. C and D, Photomicrograph of islets of a normoglycemic 2 d post SZ + IN mouse. Note the presence of many stained β cells. *Bar* in C, 60 μm. *Bar* in D, 30 μm. E, Islet of 6 d post SZ normoglycemic mice. The pancreas contained other islets of larger size, similar to that shown in C, but that were completely filled by β cells. *Bar*, 30 μm. F, Islet of normoglycemic 6 d post SZ + IN mouse shown in E, stained for somatostatin. Note that some ∂ cells are located in the core of the islet and that the stained cells do not form a uniformly shaped rim, suggesting an islet in the process of reformation. *Bar*, 30 μm.

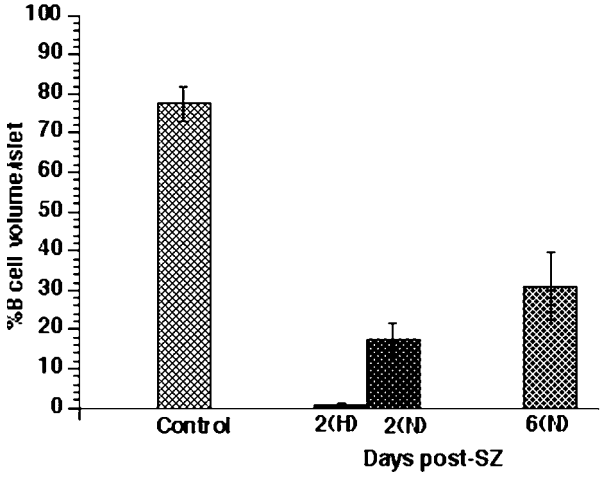

FIG. 4. Insulin injection promoted an increase in β cell volume per islet. Mice examined were injected with SZ at d 1 and IN at d 2. *Bar graphs* indicate results for controls, and SZ-treated mice examined at d 2 and 6 d after SZ. Fifty randomly selected islets per pancreas were examined, 5 mice per group. At least 5000 points were scored for each group. The number of β cells per islet area is expressed as mean ± SEM. $P > 0.001$ when 2 SZ(N) was compared with 6 SZ. H, Hyperglycemic; N, normoglycemic.

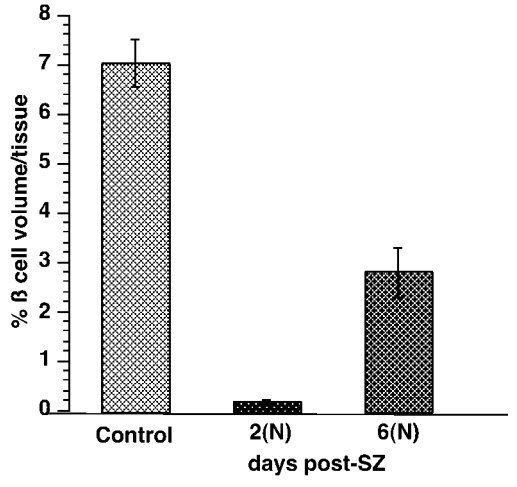

FIG. 5. Relative β cell volume per tissue increased during IN-treatment. The relative β cell volume was measured in randomly selected sections from three mice per experimental group. *Bar graphs* indicate results for controls and 2 and 6 d post SZ. Relative β cell volume of hyperglycemic 2 d post SZ (2×10^{-4}) was not included in the graph. Absolute islet mass in controls is obtained by multiplying the relative β cell volume by the weight of the pancreas (0.15 g ± 0.01, n = 5). Weight of pancreas from experimental animals was not determined. Over 5000 points were scored for each experimental group. Values are expressed as mean ± SEM. $P < 0.001$ *vs.* controls.

IN+ cells did not proliferate (Fig. 6A), indicating that the increased β cell mass found during the first 4 d after SZ was not due to proliferation of the insulin containing cells present at those stages. Islets of 2 d post-SZ mice contained a significant number of proliferating nonβ cells. Some of these proliferating cells were macrophages (Fig. 6B). Other proliferating cell types were the α (not shown) and ∂ (Fig. 6C) cells

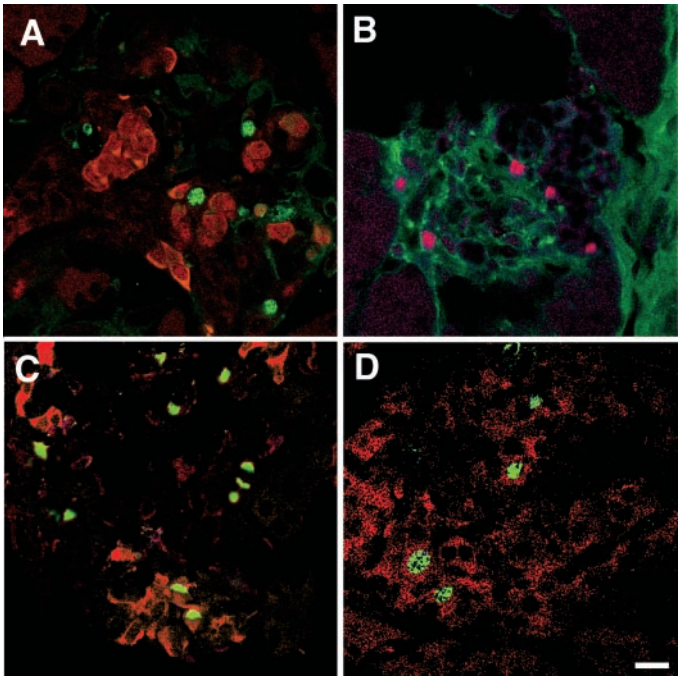

FIG. 6. Identity of proliferating cells. All photomicrographs are from pancreas of normoglycemic 2 d post SZ + IN mice. A, IN (*red*) cells did not incorporate BrdU (*green*), indicating that these cells did not actively proliferate. B–D, Cells that actively proliferate and were easily detected in sections by confocal microscopy. B, Macrophages, labeled with the marker 4–80 (*green*) incorporated BrdU (*red*); C, Some SOM^+ cells (*red*) contained Ki67p labeled nuclei (*red*); D, Photomicrograph illustrates the presence of Glut-2^+ cells (*red*) that had incorporated BrdU (*green*). The granular Glut-2 labeling is due to the use of a higher dilution of the secondary antibody. *Bar*, 15 μm.

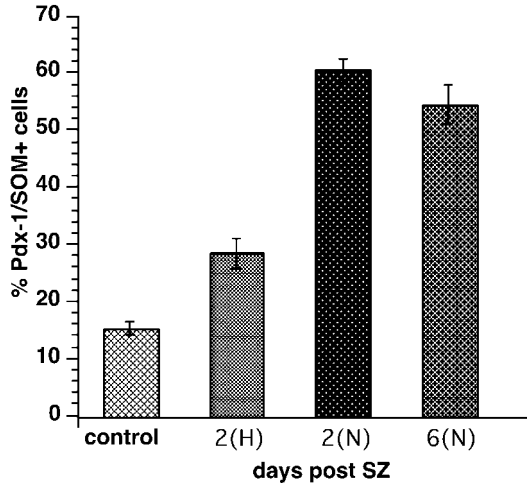

FIG. 7. The percentage of PDX-1/SOM^+ cells increased in normoglycemic SZ + IN-treated islets. The percentage of PDX-1/SOM^+ cells at different times after injection of SZ and IN is compared between controls, 2 and 6 d post-SZ mice. At least three pancreas per stage were examined in controls and in normoglycemic 2 and in 6 d post-SZ mice. These tissues did not show hemorrhage or necrosis. Total number of cells scored for the PDX-1/SOM^+ combination in islets of control and hyperglycemic 2 d post-SZ mice was 1234 from 15 islets and 2353 from 42 islets, respectively. Total number of cells scored for the PDX-1/SOM^+ combination for normoglycemic mice at 2 and 6 d post SZ were 1924 from 30 islets and 1,419 from 19 islets. The number of cells expressing PDX-1 and SOM is expressed as the mean ± SEM of the cells immunoreactive for SOM. In this figure, the number of days after SZ injection = x days post SZ. Insulin was injected 24 h after the injection of SZ. *, $P < 0.001$ *vs.* controls. H, Hyperglycemic; N, normoglycemic.

and cells located in the periphery of the islet that expressed Glut-2 (Fig. 6D).

Possible sources of new β cells

Next, we sought to determine whether presumptive β precursor cells found in the pancreas during development reappeared in islets during regeneration. One of these islet cell types were SOM cells that expressed Pdx-1 and presumably initiated IN expression. SOM^+/Pdx-1^+ cells were abundant in embryos (Refs. 30, 31 and Fig. 12, E–G) and during regeneration (11) in hyperglycemic mice. The present study confirmed the appearance of Pdx-1/SOM cells in SZ + IN mice. The percentage of Pdx-1/SOM^+ cells in normoglycemic 2 d post SZ (60%, Fig. 7) was higher than in 2 d post-SZ mice that did not receive the hormone and remained hyperglycemic (30%, this study and Ref. 11). The percentage of Pdx-1/SOM^+ cells in normoglycemic SZ + IN mice did not decrease with time, remaining at similar high levels at 6 d post SZ (Fig. 7). In contrast, no difference was found in the percentage of Pdx-1/GLU^+ and Pdx-1/PP^+ cells between experimental animals and untreated controls (not shown).

Triple label immunofluorescence labeling of pancreatic islets of normoglycemic 2 d SZ + IN mice indicated the presence of PDX-1^+cells coexpressing SOM and IN (Fig. 8). While some islets contained several SOM/IN+ cells (Figs. 8, A and B) in addition to the cells containing only SOM, other islets in the same pancreas were populated mainly by monospecific β and ∂ cells (not shown). SOM/IN^+ cells were not detected in islets of hyperglycemic 1 and 2 d post-SZ/200 mice or controls (data not shown). No cells expressing GLU/IN (Fig. 8, C and D) or PP/IN (not shown) were seen at this or other stages after SZ or in controls. These observations indicate that cells coexpressing SOM and IN appeared 3 d earlier in normoglycemic 2 d post SZ + IN mice than in animals that did not receive IN treatment (11). In addition, islets of normoglycemic 2 and 6 SZ + IN mice also contained a significant number of cells expressing only IN, a cell type that was scarce in islets of hyperglycemic of 5 d post-SZ mice (11).

Because it has been reported that islet precursor cells expressed Glut-2 (8), we also sought to determine whether Glut-2^+ cells appeared in islets following SZ treatment. No cells expressing Glut-2 were found in islets at 1 d post SZ (Fig. 9B). The fact that in controls Glut-2 expression is restricted to β cells (Figs. 9A and 10, A–C), this observation confirmed the toxin-mediated elimination of the original β cell population. Cells expressing Glut-2 in the cytoplasm reappeared in the periphery of islets of normoglycemic 2 d post-SZ mice (Fig. 9C). At 4 d post SZ, many islet cells distributed throughout the islet expressed Glut-2 either in the cytoplasm or the cell membrane (Fig. 10H), whereas at 6 d post SZ, most islet cells displayed membrane staining (Fig. 9D). These observations suggested a translocation of the transporter from the cytoplasm to the cell membrane during cell maturation. In contrast to euglycemic mice, islets of hyperglycemic 2 d post-SZ mice (no insulin treatment) did not have Glut-2^+

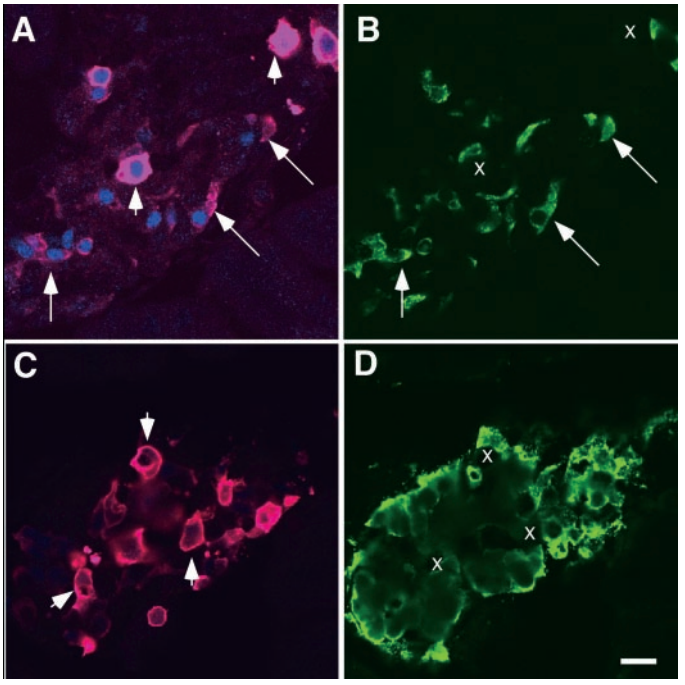

FIG. 8. Monospecific IN cells differentiate following restoration of normoglycemia. Histological sections of 2 d post SZ + IN pancreas were examined by confocal microscopy. A and B, Triple immunofluorescence labeling performed to localized IN (produced in guinea pig, *red*), SOM (rat, *green*) and PDX-1 (rabbit, *blue*) in the same section. A, Localization of PDX-1 and IN; B, localization of SOM. In section illustrated in A and B note the presence of cells expressing IN and SOM (*arrows*) and cells that contains only IN (*arrowheads*). Location of monospecific IN cells of A (*arrowheads*) is indicated with a cross in Fig B. Note that several cells are IN/SOM$^+$ and that the intensity of staining is higher in monospecific IN cells than in doubly labeled cells. Most of the SOM/IN$^+$ and IN$^+$ cells shown in this field contain PDX-1 in the nucleus. C and D, Islet of 2 d post SZ mouse immunostained with IN (produced in guinea pig, *red*) and glucagon (mouse monoclonal, *green*). The location of IN cells present in C is indicated with an *X* in D. Note the absence of cells coexpressing both hormones. *Bar*, 15 μm.

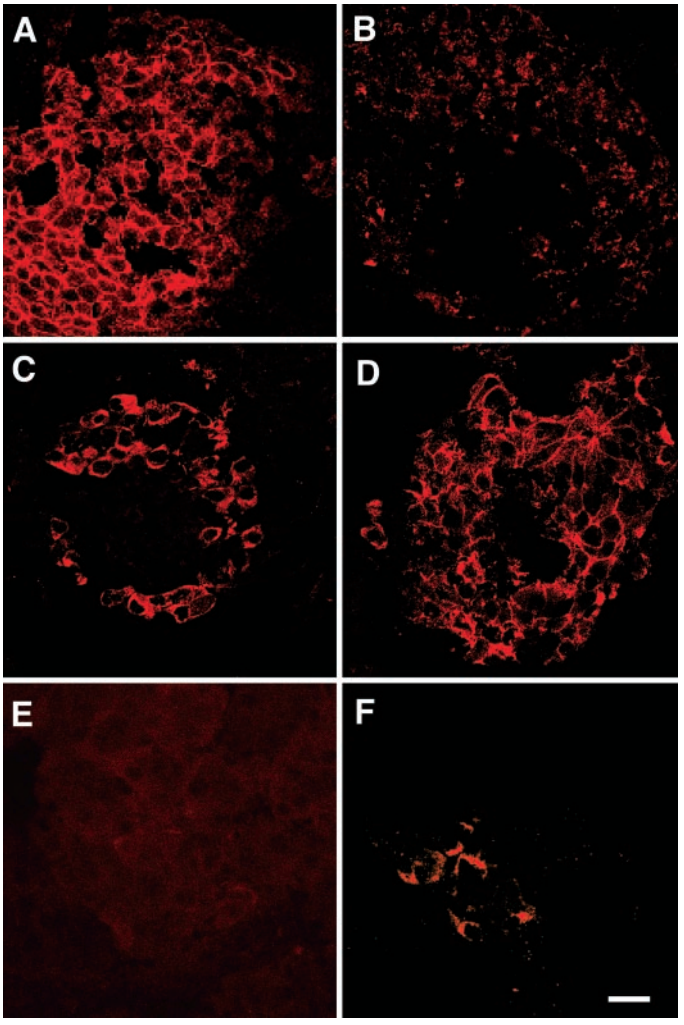

FIG. 9. Glut-2 expression reappears in SZ-treated islets. A, Glut-2 expression in a representative islet of controls. B, Islet of 1 d post SZ. Note the presence of dispersed stained debris. C, Islet of normoglycemic 2 d post SZ + IN mouse illustrate the presence of Glut-2$^+$ cells in the periphery of the islet. D, Islet of 6 d post SZ + IN mouse documents the presence of a large number of Glut-2$^+$ cells. The distribution of these cells, however, is more irregular than in controls. In contrast to cells in C, the staining is mostly localized to the cell membrane. E, Photomicrograph of a representative islet of hyperglycemic 2 d post SZ day mouse illustrates the lack of Glut-2$^+$ cells. F, Islet of a hyperglycemic 6 d post SZ mouse contains few Glut-2$^+$ cells that show cytoplasmic staining. *Bar*, 20 μm.

cells (Fig. 9E). In hyperglycemic mice, Glut-2$^+$ cells were first seen at 6 d post SZ (Fig. 9F). However, the number of Glut-2$^+$ cells was significantly lower in hyperglycemic than in normoglycemic animals at that stage and the transporter had a diffuse, cytoplasmic localization. These observations indicated that the reestablishment of normal blood glucose levels accelerated the time of appearance of Glut-2$^+$ cells in islets and promoted the maturation of these cells.

Next we sought to identify the cells expressing Glut-2. In normoglycemic mice at 2 d post SZ + IN, Glut-2 cells coexpressed IN (Fig. 10, D–F) and the number of Glut-2/IN$^+$ cells increased with time (Fig. 10, G–I). Islets of hyperglycemic 6 d post SZ + IN mice contained IN$^+$ cells and some of these cells coexpressed Glut-2 (Fig. 10, J–L). However, in these cells the transporter had a cytoplasmic localization, indicating that they were not functionally mature.

At 2 d post SZ, islets also contained a significant number of Glut-2/GLU$^+$ cells and cells that expressed only Glut-2 (Fig. 11). At this and later stages, Glut-2 was localized to the cytoplasm of the α cells. Glut-2 expression by GLU cells persisted in islets of pancreas with suboptimal regeneration even at 6 d post SZ, but was transient in pancreas with fully reformed islets (not shown). This observation indicated that the inhibition of Glut-2 expression by GLU was correlated with the success of the regeneration process. No Glut-2 cells expressed SOM or the macrophage marker 4–80 (not shown). We did not test for coexpression of Glut-2 and PP. As expected β, but not α cells of controls expressed Glut-2 (Fig. 11, D–F).

The Glut-2/Glu cells could be unique to regenerating islets or they could also populate developing islets of embryos. We determined that pancreas of e-17 embryos contained Glut-2$^+$ cells expressing GLU (Fig. 12, A–C) in addition to the Glut-2/

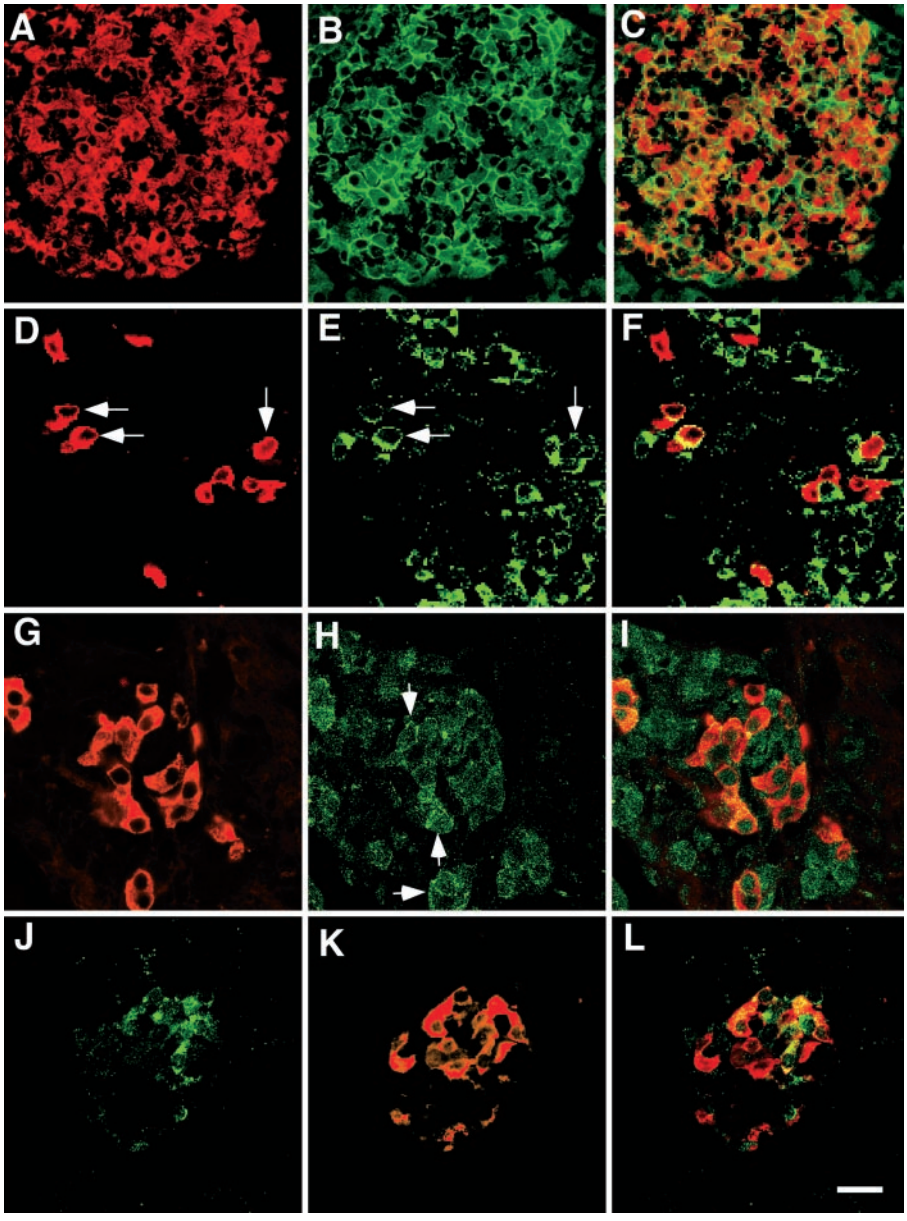

FIG. 10. Reappearance of Glut-2/IN$^+$ cells during regeneration. A–C, Control islets costained with IN (produced in guinea pig, A) and Glut-2 (rabbit, B). C illustrates A + B. D–F, Islet of normoglycemic 2 d post SZ mouse costained with IN (D)and Glut-2 (E). F illustrates D + E. Note the presence of cells (illustrated with *arrows*) that contain both Glut-2 and IN. These cells are *yellow* in F. G–I, Photomicrographs of an islet of a normoglycemic 4 d post SZ + IN mouse costained with IN (G) and Glut-2 (H). *Arrows* indicate some cells with Glut-2 staining in the cell membranes. I illustrates G + H. J–L, Islet of hyperglycemic 6 d post SZ mouse stained with Glut-2 (J) and IN (K). L illustrates J + K. Some IN$^+$ cells have diffuse Glu-2 staining. *Bar*, 20 μm.

IN cells reported by others (8). We also confirmed the presence of cells coexpressing SOM and IN at that stage of development (Ref. 32 and Fig. 12, E–G). These observations indicate that at least two sets of embryonal cell types reappear in islets following SZ-induced injury, *i.e.* the SOM/IN$^+$ and the Glut-2$^+$ cells. Because both SOM$^+$ and Glut-2$^+$ cells proliferate, it is likely they generate the newly differentiated IN$^+$ cells. It has recently been reported that adult rat and human islets contain nestin + precursor cells that differentiate into insulin-containing cells *in vitro* (33). It remains to be determined whether the Glut-2 and SOM/IN$^+$ cells of SZ-treated islets are generated by precursor cells expressing nestin.

Discussion

We previously reported that newly differentiated β cells appeared in pancreas of hyperglycemic SZ-treated mice 5 d after injection of the toxin (11). The number of islets per pancreas and of β cells per islets, however, was very low and further decreased with time. In this study, we sought to determine first, whether insulin-induced normoglycemia improved islet cell regeneration and second, the possible source of the newly differentiated β cells. We found that the reestablishment of normal glucose levels in SZ mice had the following effects: 1) faster removal of necrotic β cells and accelerated recovery of islets from SZ-induced injury; 2) appearance of presumptive β precursor cells, such as Pdx-1/SOM$^+$, SOM/IN$^+$ cells, and cells expressing Glut-2$^+$ in islets of normoglycemic SZ-treated mice; and 3) differentiation of monospecific β cells and of morphologically normal pancreatic islets.

One of the possible β cell precursors found in this model of neogenesis were the Glut-2$^+$ cells that appeared in islets

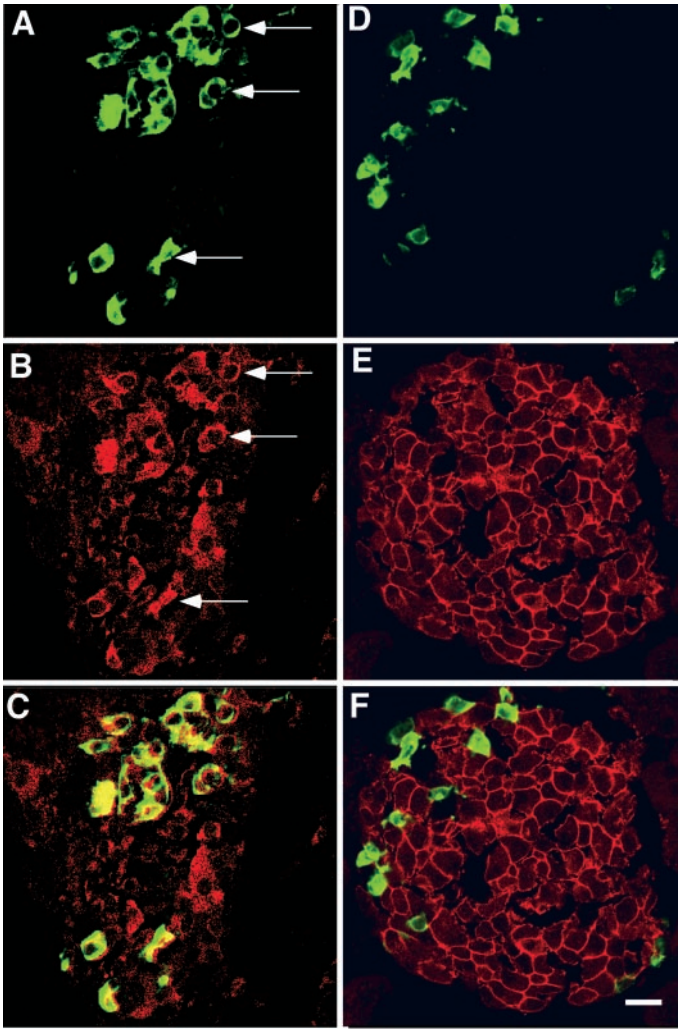

FIG. 11. A subset of Glut2 cells coexpress GLU. Photomicrographs A–C illustrate a representative islet from normoglycemic 2 d post SZ + IN mice stained for GLU (mouse monoclonal, A) and Glut-2 (B). C is A + B. Note the presence of cells coexpressing both markers. Some of these cells are indicated with *arrows* in A and B. Other cells contain only Glut-2. D and E, Islet from control mice stained for GLU (D) or Glut-2 (E). F is D + E. As expected, GLU cells of controls do not express GLUT-2. *Bar*, 15 μm.

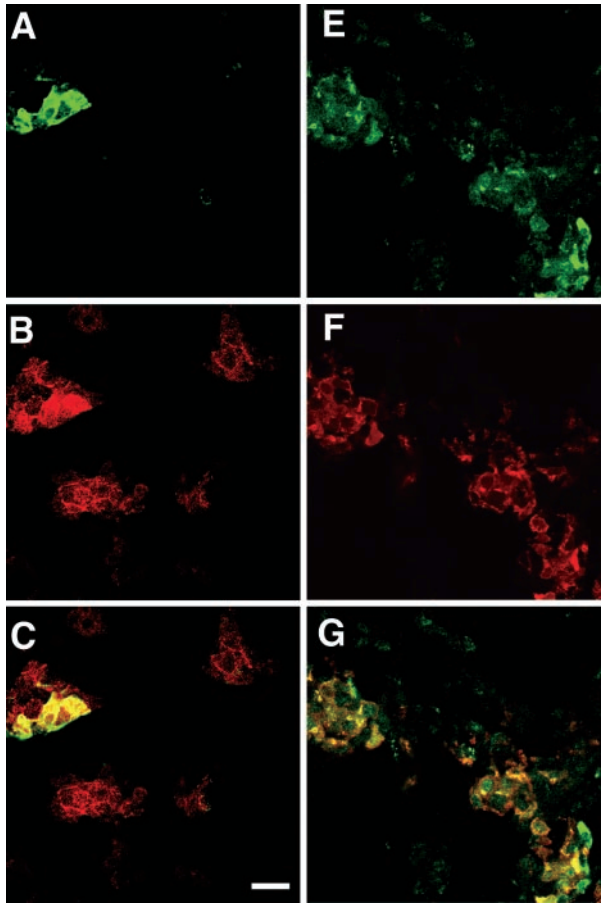

FIG. 12. Immature islet cell types in embryos: photomicrograph of embryonic pancreas at d 17 of gestation. A–C, Cells stained for GLU (A, *green*) or Glut-2 (B, *red*). C represents A + B. Note the presence of GLU cells coexpressing Glut-2 (*yellow*). E and F, Cells stained for SOM (E, *green*) or IN (F, *red*). G represents E + F. At this stage most cells coexpress IN and SOM. These bi-hormone cells have relatively low levels of SOM than monospecific cells expressing only SOM (*bright green*). *Bar*, 20 μm.

of SZ-treated normoglycemic mice. It has been previously reported that Glut-2 expression in β cells was regulated by glucose levels and that it decreased during hyperglycemia (17, 28, 34, 35). Because islets of hyperglycemic 1 d post SZ lacked Glut-2$^+$ cells, it could be argued that the absence of these cells could be due to the high circulating glucose levels. If this were true, β cells could re-express the transporter following the restoration of euglycemia. However, the cells expressing Glut-2 in normoglycemic 2 d post-SZ mice are located in the periphery of the islets, the location of the nonβ cells rather than in the core of the islet, supporting the view that this was a novel population of cells. Indeed, one unexpected finding in our study was that a significant number of Glut-2 cells present at 2 d post-SZ mice coexpressed GLU, a cell type unique to embryos and to islets of SZ mice. The fact that Glut-2 expression appeared sooner and was more abundant in islets of normoglycemic than hyperglycemic SZ-treated mice indicate that differences in glucose levels affected the initiation of Glut-2 expression. Expression of Glut-2 by GLU cells was transient and it disappeared at 6 d post SZ in mice with optimal regeneration but persisted in mice in which regeneration was less successful. Because mice with different degrees of regeneration were euglycemic, the fate of Glut-2 expression in nonβ cells was related not only to variations in glucose levels but also to other unidentified factors affecting the outcome of the process of regeneration.

In addition to the Glut-2/GLU$^+$ cells, islets of normoglycemic post-SZ mice contain Glut-2$^+$/IN$^+$ cells and the number of these cells increased with time. Our results suggest that cells expressing only Glut-2 appeared in islets of normoglycemic 2 d post SZ +IN mice and that, as in embryos, these cells were β cell precursors and initiated IN synthesis. Because IN$^+$ cells did not proliferate during the initial stages of regeneration, it is likely that the temporal increase in β cell number was due to the augmentation in the number of Glut-2 cells that initiated IN expression. In contrast to IN cells,

Glut-2 cells proliferated actively and could have generated β cells without depleting the size of the precursor pool. In addition, GLU cells expressing the transporter could have converted into β cells. However, this is improbable because islets of SZ-treated mice lacked cells coexpressing IN and GLU. Glut-2/IN$^+$ cells appeared in islets of hyperglycemic 6 d post-SZ mice, but these cells were scarce and immature, suggesting that the presence of high circulating glucose levels prevented their maturation.

The restitution of euglycemia also promoted the differentiation of the PDX-1/SOM$^+$ cells, a presumptive β precursor cell type, into monospecific β cells. Thus, islets of normoglycemic 2 d post SZ + IN mice contained a high percentage of PDX-1/SOM$^+$ cells, and also had SOM/IN$^+$ cells and monospecific IN$^+$ cells. SOM/IN$^+$ cells were also found during development but not in islets of adult, untreated mice. Conceivably, the process of transformation of SOM$^+$ cells into insulin containing β cells in normoglycemic SZ mice occurred in at least three separate stages. The first step would be the activation of PDX-1 expression in SOM$^+$ cells and/or the stimulation of undifferentiated precursors present in or around islets to initiate PDX-1 and SOM expression. The second step would be the initiation of insulin expression by PDX-1/SOM$^+$ cells and, in the third stage of the conversion, SOM/IN cells down regulated SOM expression and differentiated into fully mature, Glut-2$^+$ β cells. We previously reported that cells expressing SOM and IN eventually appeared in islets of hyperglycemic mice at 5 d post SZ, but these islets contained very few cells expressing only insulin (11). These observations and our present findings strongly suggest that hyperglycemia inhibited the maturation of the two types of precursor cells that appeared in islet of SZ-treated mice, namely Glut-2 and SOM/IN cells, into functional β cells.

The previous discussion underscores the dramatic effect of the elimination of β cells by SZ treatment on the non-β cells of the islet. After exposure to SZ, both ∂ and α cells reverted to an immature phenotype characteristic of these cells during development. Thus, the ∂ cells initiated expression of Pdx-1 and IN while the α started expressing Glut-2. In addition, following SZ-induced injury, precursors present in islets presumably activated Glut-2 expression and differentiated into β cells. Although the signals responsible for these phenotypic changes are presently unknown, the cellular transformations did not occur until the islets were free of cellular debris. Thus, in contrast to islets of normoglycemic 2 d post SZ, which were mostly free of debris and showed regeneration, islets of hyperglycemic mice at that stage still contained many dead β cells and lacked newly differentiated IN$^+$ cells.

The removal of dead cells is likely to be accomplished by macrophages invading the islet following SZ treatment. Islets of both normoglycemic and hyperglycemic mice contained more macrophages than islets of controls (Refs. 35–38 and this study). However, high glucose levels decrease macrophage phagocytic activity and affects the pattern of cytokine secretion (39–41). Our results suggests that, in normoglycemic mice, macrophages would rapidly remove cell debris and then secrete molecules that activate the proliferation and differentiation of precursor cells while hyperglycemia would delay or eliminate these activities. Macrophage-derived cytokines could be involved in the observed activation of proliferation of several islet cell types at 2 d post SZ and in the increase in the number of Pdx-1/SOM$^+$ cells. The presence of a high percentage of Pdx-1/SOM cells in islets of nonobese diabetic mice (11), which are invaded by cells secreting proinflammatory cytokines (42), supports an involvement of hematopoietic cells in the regulation of Pdx-1 expression by SOM cells. Differences in macrophage function could also determine the appearance of Glut-2$^+$ precursor cells in islets of normoglycemic but not of hyperglycemic mice. In addition to circulating blood glucose levels, macrophages respond to a multitude of signals that regulate their function (43), a response that could be skewed in the SZ-treated euglycemic mice. This would explain the fact that some, but not all of the normoglycemic mice showed islet regeneration.

Contrary to our expectation, the percentage of SOM/Pdx-1$^+$ cells did not decrease in regenerated islets of 6 d post-SZ mice that appeared to have recovered from the SZ-induced injury. This suggests that the mechanism/s involved in the activation of Pdx-1 expression in SOM cells persist for a long time in regenerated islets. Alternatively, it is possible that Pdx-1/SOM$^+$ cells were unable to down-regulate Pdx-1. The fact that in embryos, a large percentage of ∂ cells express Pdx-1 when they first differentiate and that this percentage decreases during development (30, 31) indicates the presence of mechanisms inhibiting Pdx-1 expression in SOM$^+$ cells. Conceivably, these regulatory mechanisms were not active in the Pdx-1/SOM$^+$ cells that appeared in adult islets following injury. Finally, because the number of β cells in regenerated islets at 6 d post SZ was lower than in controls, it is also possible that the presumptive precursor cells were still being generated even at that stage, and the islets were primed to grow until they reached control values.

Our proposition that insulin therapy induced β cell neogenesis is in agreement with previous reports indicating that insulin administration to SZ-treated neonatal rats promoted the recruitment of new β cells from a precursor population (44). The question remains as to whether insulin directly promotes β cell regeneration or if it acts indirectly through its role in the normalization of blood glucose levels. Recent studies on mildly diabetic SZ-treated rats indicated that short-term hyperinsulinemia had no effect on β cell mass, whereas a 48 h glucose and insulin infusion sufficed to restore β cell number to control values (45). The findings reported by Bernard *et al.* (45) and others (46) support the view that both mild hyperglycemia and hyperinsulinemia were required for maximal β cell growth. In our model of acute hyperglycemic mice, the success of the regeneration process was dependant upon the restoration of normal glucose levels by exogenous insulin injection. However, due to the technique used to regulate blood sugar levels, we cannot rule out the possibility that the mice underwent periods of hyperglycemia and hyperinsulinemia, which could have had a positive effect on β cell regeneration. Taken together, these observations stress the need for further examination of the roles

of both insulin and glucose in the SZ and other models of β cell neogenesis.

In conclusion, we found that islets of adults contain β precursor cells and that the re-establishment of normoglycemia by exogenously administered insulin accelerates the differentiation of these cells into monospecific β cells and the reappearance of morphologically normal islets less than 1 wk after SZ treatment. Two types of β precursor cells were identified, the Glut-2$^+$ cells and cells coexpressing PDX-1/SOM. It remains to be determined whether the neoformed β cells have all the properties characteristic of mature insulin containing cells and are able to regulate circulating glucose levels within the physiologically normal range.

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