Effect on Regeneration of Pancreatic β-Cells in Neonatal Streptozotocin-Treated Rats

Lei Li, Zhaohong Yi, Masaharu Seno, and Itaru Kojima

Activin A and betacellulin (BTC) are thought to regulate differentiation of pancreatic β-cells during development and regeneration of β-cells in adults. In the present study, we used neonatal rats treated with streptozotocin (STZ) to investigate the effects of activin A and BTC on regeneration of pancreatic β-cells. One-day-old Sprague-Dawley rats were injected with STZ (85 µg/g) and then administered for 7 days with activin A and/or BTC. Treatment with activin A and BTC significantly reduced the plasma glucose concentration and the plasma glucose response to intraperitoneal glucose loading. The pancreatic insulin content and β-cell mass in rats treated with activin A and BTC were significantly increased compared with the control group on day 8 and at 2 months. Treatment with activin A and BTC significantly increased the DNA synthesis in preexisting β-cells, ductal cells, and δ-cells. The number of islet cell-like clusters (ICCs) and islets was significantly increased by treatment with activin A and BTC. In addition, the number of insulin/somatostatin-positive cells and pancreatic duodenal homeobox-1/somatostatin-positive cells was significantly increased. These results indicate that, in neonatal STZ-treated rats, a combination of activin A and BTC promoted regeneration of pancreatic β-cells and improved glucose metabolism in adults. Diabetes 53:608–615, 2004

Diabetes is characterized by absolute or relative deficiency of insulin secretion from pancreatic β-cells, and the β-cell mass is critical in the pathophysiology of diabetes (1,2). Although pancreatic stem cells have not been fully characterized (3), there are several lines of evidence showing that pancreatic stem cells exist in adults and differentiate into β-cells (β-cell neogenesis) in response to an increased demand for insulin (4–7). In pathological conditions, however, β-cell neogenesis is not sufficient to compensate for the needs for insulin. Therefore, investigation of the factors promoting β-cell neogenesis has raised great interest in the past few years (8).

Betacellulin (BTC) belongs to the epidermal growth factor family and is isolated from conditioned medium of insulinoma cells (9). The expression of BTC is predominantly found in the pancreas and the intestine. Specifically, immunoreactive BTC is found in endocrine precursor cells of the fetal pancreas and in insulin-secreting cells of patients with nesidioblastosis (10). Regarding its action, BTC converts amylase-secreting pancreatic AR42J cells into insulin-producing cells (11) and also has a mitogenic effect in human undifferentiated pancreatic epithelial cells (12). These results suggest that BTC plays an important role in regulating growth and/or differentiation of endocrine precursor cells of the pancreas. In this regard, we and others (13–15) have shown that BTC improves glucose metabolism by promoting β-cell regeneration in diabetic animals. Hence, BTC is a potentially intriguing growth factor in treating diabetes.

Activin A, a member of the transforming growth factor-β (TGF-β) superfamily, regulates growth and differentiation of many types of cells (16) and also regulates pancreatic development and endocrine determination (17,18). In vitro, activin A converts AR42J cells into pancreatic polypeptide-producing endocrine cells (11). This is achieved by inducing the expression of neurogenin 3 (19), a critical transcription factor in regulating differentiation of endocrine cells (20,21). Activin A also induces differentiation of human fetal pancreatic endocrine cells (12). Recently, we showed that the expression of activin A was upregulated in the pancreatic duct during pancreatic regeneration (22). Thus, it is possible that activin A regulates neogenesis of β-cells in vivo.

Treatment of neonatal rats with streptozotocin (STZ) provides a useful model for investigating β-cell regeneration (23–26). It has been reported that β-cell regeneration occurs through both increasing the replication of preexisting β-cells and neogenesis from the precursor cells located in or by the pancreatic duct. Because of the limited β-cell regeneration in this model, however, adult rats exhibit decreased β-cell mass and develop type 2 diabetes (27–28). In the present study, using neonatal STZ-treated rats, we investigated the effect of administration of activin A and BTC on β-cell regeneration. The results show that treatment with activin A and BTC during the neonatal
period improves the glucose metabolism in adults by promoting β-cell regeneration.

**RESEARCH DESIGN AND METHODS**

Pregnant Sprague-Dawley rats (17 days of pregnancy) were obtained from Japan SLIC, Inc. (Shizuoka, Japan). The pregnant rats were caged individually with free access to standard diet and water and were checked at 0900 and 1700 daily for delivery of pups. One-day-old neonates received a single intraperitoneal injection of 85 μg/g body wt of streptozocin (STZ) (Wako, Japan) freshly dissolved in 0.05 mmol/l citrate buffer (pH 4.5). The number of newborns per litter was kept between 9 and 13. The pups were left with their mothers until 4 weeks old. All neonates were tested 1 day after the STZ treatment and the blood glucose level was measured using an insulin assay kit (Morinaga, Yokohama, Japan) (29).

Blood samples from snipped tails were collected in heparinized 0.1% BSA (pH 5.5) (11) or control buffer were subcutaneously injected once a week from 1 week after the STZ injection. Five experimental groups were studied: the normal group, STZ group (STZ-injected rats treated with control buffer), STZ/A group (STZ-injected rats treated with activin A), STZ/B group (STZ-rats treated with BTC), and STZ/A+B group (STZ-injected rats treated with activin A and BTC). One day after the STZ injection, the blood glucose concentration was measured and the animals from various litters were randomly placed in five groups. Then, 200 ng/g body wt of recombinant human insulin in PBS (pH 7.4) and 100 ng/g body wt of recombinant human activin A in 10 mmol/l acetic anhydride containing 0.1% BSA (pH 5.5) (11) (control buffer) were subcutaneously injected once a day from day 1 to day 7 according to the experimental groups. The basal blood glucose concentration and the body weight were measured daily between 1400 and 1600 for the first week and then once a week for up to 8 weeks. The plasma insulin concentration was measured on day 8 and week 8 using an insulin assay kit (Moringa, Yokohama, Japan) with rat insulin as standard. Blood samples were obtained by decapitation on day 8 and from tail vein on week 8. Six weeks after the STZ treatment, an intraperitoneal glucose tolerance test (IPGGT) (2 g/kg body wt) was done after 14 h of fasting. Blood samples from snipped tails were collected in heparinized hematocrit tubes at different time points and assayed for blood glucose, and the remainder was stored at −20°C for insulin assay. At 8 weeks of age, rats were killed by decapitation. The experimental protocol was approved by the Animal Care Committee of Gunma University.

**Tissue processing.** On day 4 and day 8, the animals were injected intraperitoneally with 1 ml of bromodeoxyuridine (BrdU) (labeling reagent per 100 g wt of recombinant human insulin per 100 g body wt (cell proliferation kit; Amersham Pharmacia Biotech, Little Chalfont, U.K.) and decapitated after 3 h. The pancreas was excised, weighed, and homogenized in cold acid-ethanol, and the pancreatic insulin content and the plasma glucose concentration in STZ-treated rats was slightly but significantly elevated in STZ-treated rats (at 6 weeks of age: normal rat 133.2 ± 3.2 [n = 6] versus STZ rats 152.4 ± 3.5 [n = 11]; P < 0.005). The mortality rate caused by STZ in this study was not different from that in normal rats (data not shown).

The plasma insulin concentration at 8 weeks of age and the pancreatic insulin content and the β-cell mass on day 8 and 8 weeks of age in STZ-treated rats were severely reduced compared with those of normal rats (P < 0.001) (Tables 1 and 2). However, the β-cell size, body weight, and pancreatic weight of the STZ-treated rats were not different from those of normal rats (Tables 1 and 2).

The basal blood glucose concentration was measured and the area in these sections was measured. The data were shown as the number of ICCs or islets per micrometer squared of the pancreatic area. At least three different sections were analyzed per animal (four animals per group).

**β-Cell neogenesis.** Apoptotic cells were detected by a terminal deoxynucleotidyl transferase (TUNEL) method using an apoptosis in situ detection kit (Wako Jun-yaku, Tokyo, Japan) (29).

**Statistical analysis.** Results were expressed as means ± SE. For comparisons between two groups, the unpaired t test was used. For multiple comparisons, one-way ANOVA was used. A P value <0.05 was considered statistically significant.

**RESULTS**

**Characteristics of neonatal STZ-treated rats.** After STZ treatment, neonatal rats exhibited diabetes. Their blood glucose levels were >250 mg/dl 1 day after the STZ treatment (day 1) and peaked on day 2 with a peak value >350 mg/dl. Thereafter, the blood glucose concentration gradually decreased (Fig. 1A). After 6 weeks of age, the blood glucose concentration was slightly but significantly elevated in STZ-treated rats (at 6 weeks of age: normal rat 133.2 ± 3.2 [n = 6] versus STZ rats 152.4 ± 3.5 [n = 11]; P < 0.005). The mortality rate caused by STZ in this study was not different from that in normal rats (data not shown).

The plasma insulin concentration at 8 weeks of age and the pancreatic insulin content and the β-cell mass on day 8 and 8 weeks of age in STZ-treated rats were severely reduced compared with those of normal rats (P < 0.001) (Tables 1 and 2). However, the β-cell size, body weight, and pancreatic weight of the STZ-treated rats were not different from those of normal rats (Tables 1 and 2).

The IPGGT performed at 6 weeks of age showed that STZ-treated rats developed diabetes. The peak value of the plasma glucose concentration in STZ-treated rats was >350 mg/dl and remained high at 60 min (Fig. 1B). The
plasma insulin levels in STZ-treated rats were markedly decreased at 30 min compared with those in normal rats (Fig. 1B).

The replication of β-cells and ductal cells was determined by insulin/BrdU double immunostaining and CK/BrdU double immunostaining. In STZ-treated rats, the number of insulin/BrdU double-positive cells (STZ-treated 6.5 ± 0.35% vs. normal 4.4 ± 0.15% [n = 4]; P < 0.002) and CK/BrdU double-positive cells (STZ-treated 6.4 ± 0.3% vs. normal 4.1 ± 0.2% [n = 4]; P < 0.001) was significantly increased compared with that of normal rats on day 4.

The islets in STZ-treated rats were smaller than those in normal rats (Fig. 2). In STZ-treated rats, large islets that

### Table 1
Characteristics of normal, STZ, STZ/A, STZ/B, and STZ/A+B rats on day 8

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>STZ</th>
<th>STZ/A</th>
<th>STZ/B</th>
<th>STZ/A+B</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>14.9 ± 0.6 (10)</td>
<td>13.7 ± 0.8 (16)</td>
<td>14.5 ± 0.7 (13)</td>
<td>14.8 ± 0.6 (14)</td>
<td>13.9 ± 0.7 (15)</td>
</tr>
<tr>
<td>Pancreas weight (g)</td>
<td>36.5 ± 1.8 (4)</td>
<td>39.4 ± 1.8 (5)</td>
<td>41 ± 1.0 (9)</td>
<td>39.2 ± 0.5 (5)</td>
<td>44.8 ± 1.8 (4)</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>114.1 ± 2.6 (6)</td>
<td>153.3 ± 28.4 (16)</td>
<td>123 ± 5.8 (13)</td>
<td>118.8 ± 6.7 (14)</td>
<td>112.6 ± 5.6 (15)</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>0.386 ± 0.008 (4)</td>
<td>0.342 ± 0.014 (5)</td>
<td>0.380 ± 0.041 (4)</td>
<td>0.360 ± 0.026 (5)</td>
<td>0.435 ± 0.049 (4)</td>
</tr>
<tr>
<td>Insulin content</td>
<td>µg/pancreas</td>
<td>24.6 ± 2.2 (4)</td>
<td>3.46 ± 0.45 (5)</td>
<td>4.45 ± 0.2 (4)</td>
<td>4.48 ± 0.47 (5)</td>
</tr>
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<td></td>
<td>µg · pancreas</td>
<td>690.2 ± 44.1 (4)</td>
<td>88.3 ± 14.3 (5)</td>
<td>113.8 ± 2.6 (4)</td>
<td>138.0 ± 14.4 (5)†</td>
</tr>
<tr>
<td>β-Cell mass</td>
<td>mg/pancreas</td>
<td>0.92 ± 0.06 (4)</td>
<td>0.23 ± 0.04 (4)</td>
<td>0.256 ± 0.016 (4)</td>
<td>0.344 ± 0.034 (4)†</td>
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<td></td>
<td>mg/g · pancreas</td>
<td>25.0 ± 1.25 (4)</td>
<td>5.89 ± 0.91 (4)</td>
<td>6.24 ± 0.26 (4)</td>
<td>8.80 ± 1.06 (4)†</td>
</tr>
<tr>
<td>β-Cell size (µm²)</td>
<td>92.3 ± 4.8 (4)</td>
<td>97.1 ± 6.3 (4)</td>
<td>98.5 ± 4.2 (4)</td>
<td>96.4 ± 3.8 (4)</td>
<td>95.5 ± 5.4 (4)</td>
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</table>

Data are means ± SE (n). Neonatal rats were treated with STZ and then activin A and/or BTC were administered for 7 days. Various parameters were measured on day 8. Animals were in the nonfasting state. *P < 0.05; †P < 0.01 vs. STZ group.
are usually observed in normal rats were absent. Insulin immunoreactivity was weak in β-cells of the islets of the STZ-treated rats. No difference in GLUT2-staining of β-cells was observed between normal and STZ-treated rats (Fig. 2).

**Effect of BTC and activin A on blood glucose and IPGTT.** Treatment with a combination of activin A and BTC significantly decreased the blood glucose in neonatal STZ-rats at the early time points (Fig. 1A). The effect of activin A and BTC was persistent until adult age (Table 2). The body weight, pancreatic weight, β-cell size, and plasma insulin concentration were not changed by either of the treatments (Tables 1 and 2).

A glucose tolerance test was performed at 6 weeks of age to assess the long-term effect of activin A and BTC. Compared with the STZ groups, the blood glucose in the STZ/A+B group was significantly lower after 30, 60, and 120 min, although the plasma insulin concentration was not significantly increased compared with those in the STZ group (Fig. 1B). There was no significant difference in glucose tolerance between the STZ/A or STZ/B group and STZ group.

**Effect of BTC and activin A on the insulin content and the β-cell mass.** Treatment with activin A and BTC improved the glucose metabolism and IPGTT. We then examined the pancreatic insulin content and the β-cell mass in the experimental groups on day 8 and at 2 months of age. The results are shown in Tables 1 and 2. The pancreatic insulin content in the STZ/A+B group increased 70% at 8 days of age and >30% at 2 months of age compared with that in the STZ group (P < 0.01) (Tables 1 and 2). Similarly, the β-cell mass also significantly increased 77% on day 8 and 60% at 2 months of age in the STZ/A+B group (P < 0.05). The treatment with activin A and BTC did not change the β-cell size (Table 2). The pancreatic insulin content and the β-cell mass in the STZ/B group were also significantly increased (P < 0.05). On the other hand, the pancreatic insulin content and the β-cell mass in the STZ/A group was not significantly changed compared with that of the STZ group.

**Effect of BTC and activin A on the regeneration of β-cells.** The above results showed that treatment with activin A and BTC increased the β-cell mass without affecting the β-cell size. These factors thus increased the number of β-cells. The β-cell number is determined by a balance between the generation of β-cells and β-cell death. When we assessed apoptosis by TUNEL method, the frequency of apoptotic β-cells was very low (data not shown). We therefore investigated the effect of BTC and activin A on the regeneration of β-cells in STZ-treated neonatal rats.

There are at least three pathways for β-cell regeneration: replication of preexisting β-cells, neogenesis from the precursors located in the duct, and transdifferentiation of non-β-cells in islets. In the STZ-treated group, replications of β-cells were significantly higher than those in normal rats. The treatment with A+B or BTC alone significantly increased the replication of β-cells (Fig. 3). Though it is difficult to estimate the neogenesis of β-cells from ductal cells, treatment with activin A and BTC alone significantly increased the proliferation of ductal cells and the number of ICCs compared with STZ group rats (Fig. 4). The number of islets in STZ/A+B and STZ/B significantly increased, although there was no difference between STZ and normal rats (Fig. 4D).

It was shown that β-cell regeneration occurred in adult STZ-treated mice through transdifferentiation of δ-cells to β-cells (6,30). We then examined whether regeneration

TABLE 2
Characteristics of normal, STZ, STZ/A, STZ/B, and STZ/A+B rats are 2 months

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>STZ</th>
<th>STZ/A</th>
<th>STZ/B</th>
<th>STZ/A+B</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>236.8 ± 11.6 (6)</td>
<td>225.2 ± 12.5 (11)</td>
<td>236.0 ± 9.7 (9)</td>
<td>238.4 ± 15.1 (9)</td>
<td>228.9 ± 11.7 (11)</td>
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<tr>
<td>Pancreas weight (g)</td>
<td>0.869 ± 0.024 (6)</td>
<td>0.856 ± 0.022 (11)</td>
<td>0.898 ± 0.027 (9)</td>
<td>0.923 ± 0.043 (9)</td>
<td>0.914 ± 0.038 (11)</td>
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<td>Plasma glucose (mg/dl)</td>
<td>135.6 ± 4.4 (6)</td>
<td>163.5 ± 4.0 (11)</td>
<td>154 ± 4.8 (9)</td>
<td>151 ± 4.1 (9)</td>
<td>148.5 ± 3.4 (11)*</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>2.40 ± 0.43 (6)</td>
<td>0.94 ± 0.14 (8)</td>
<td>1.10 ± 0.18 (7)</td>
<td>1.138 ± 0.25 (7)</td>
<td>1.02 ± 0.14 (8)</td>
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<tr>
<td>Insulin content</td>
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<tr>
<td>μg/pancreas</td>
<td>103.3 ± 7.5 (6)</td>
<td>41.2 ± 18.1 (11)</td>
<td>45.8 ± 3.7 (9)</td>
<td>51.6 ± 4.0 (9)*</td>
<td>64.8 ± 2.1 (11)†</td>
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<tr>
<td>μg/g·pancreas</td>
<td>118.8 ± 8.1 (6)</td>
<td>46.9 ± 2.8 (11)</td>
<td>51.1 ± 4.1 (9)</td>
<td>56.1 ± 4.4 (9)</td>
<td>60.0 ± 3.5 (11)†</td>
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<tr>
<td>β-Cell mass</td>
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<tr>
<td>mg/pancreas</td>
<td>8.11 ± 0.81 (4)</td>
<td>2.87 ± 0.31 (4)</td>
<td>3.55 ± 0.36 (4)</td>
<td>4.53 ± 0.41 (4)*</td>
<td>5.89 ± 0.35 (4)†</td>
</tr>
<tr>
<td>mg/g·pancreas</td>
<td>9.67 ± 0.86 (4)</td>
<td>3.37 ± 0.31 (4)</td>
<td>3.85 ± 0.28 (4)</td>
<td>4.87 ± 0.29 (4)*</td>
<td>5.93 ± 0.33 (4)*</td>
</tr>
<tr>
<td>β-Cell size (μm²)</td>
<td>192.6 ± 4.5 (4)</td>
<td>202.2 ± 5.6 (4)</td>
<td>199.6 ± 3.5 (4)</td>
<td>195.3 ± 2.7 (4)</td>
<td>197.7 ± 3.8 (4)</td>
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</table>

Data are means ± SE (n). Neonatal rats were treated with STZ and then activin A and/or BTC were administered for 7 days. Various parameters were measured 2 months later. Animals were in the nonfasting state. *P < 0.05; †P < 0.01 vs. STZ group.

FIG. 2. Morphology of typical islets in normal and STZ-treated rats. Pancreatic sections obtained from neonatal STZ-treated (STZ) and nontreated (N) rats at 2 months of age were stained with anti-insulin (red) and anti-GLUT2 antibodies (green). Nuclei were stained with DAPI (blue) (original magnification ×200).

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through this route occurred in STZ-treated neonatal rats. We investigated the changes in δ-cells in STZ-treated neonatal rats. Replication of δ-cells was significantly increased compared with that of normal rats on day 4 (STZ-treated 4.4 ± 0.4% vs. normal 2.8 ± 0.3% [n = 4]; P < 0.02). Treatment with activin A and BTC or BTC alone further promoted the replication of δ-cells (Fig. 5B). In addition, the number of PDX-1/somatostatin double-positive cells was markedly increased in STZ-treated neonatal rats (STZ-treated 35.2 ± 3.2% vs. normal 26.9 ± 2.1% [n = 4]; P < 0.05). Treatment with activin A and BTC or BTC alone further increased the number of PDX-1/somatostatin double-positive cells compared with STZ group rats (Fig. 6B). In STZ-treated rats, insulin/somatostatin double-positive cells were observed (Fig. 6D). The number of insulin/somatostatin double-positive cells was significantly increased in rats treated with activin A and BTC (Fig. 6E).

**DISCUSSION**

Newborn rats treated with STZ at birth have been widely used to study the regeneration of pancreatic β-cells (23–26). In the present study, we used this model to investigate the effect of activin A and BTC on regeneration of pancreatic β-cells. We injected activin A subcutaneously at a daily dose of 100 ng/g for 7 days. This dose of activin A was shown to promote bone formation in rats (31). At this dose, activin A slightly decreased the plasma glucose concentration on days 6 and 7, but the effect was minimal. Higher doses may have been more effective but we could not examine the possibility because of the limited amount of activin A available. Nevertheless, activin A significantly enhanced the effect of BTC on both glucose metabolism and β-cell regeneration. For example, activin A augmented the BTC effect on the insulin content on day 8 and week 8. Also, a combination of activin A and BTC but not BTC alone increased the number of BrdU-positive ductal cells at day 4 and the number of insulin/somatostatin double-positive cells. As in AR42J cells (11), activin A and BTC thus acted coordinately and induced regeneration of β-cells. As in the case with glucagon-like peptide-1 and its long-acting agonist exendin (26), a combination of activin A and BTC is effective in improving diabetes in neonatal STZ-treated rats.
In this study, we observed that treatment with activin A and BTC was effective in improving glucose metabolism in newborn rats treated with STZ. Although we could not quantify the route by which these factors promoted β-cell regeneration, the treatment increased the β-cell mass and the insulin content (Tables 1 and 2). As shown in Fig. 3, treatment with activin A and BTC promoted replication of preexisting β-cells. In addition, replication of ductal cells and the number of ICCs were increased by treatment with activin A and BTC (Fig. 4). Collectively, treatment with activin A and BTC also promoted neogenesis from precursor cells located in the pancreatic duct. These data are in accordance with the previous report on the effect of BTC on β-cell regeneration (13–15).

There was no significant increase in the number of ICCs on days 4 and 8 in rats treated with STZ compared with that of normal rats (Fig. 4C). In agreement with this finding, the number of islets in 2-month-old rats treated with STZ was the same as that in normal rats of the same age (Fig. 4D). A similar result was previously reported by other research groups (28,32). These results suggest that neogenesis from the precursor cells located in or by the pancreatic duct in this model may not be accelerated compared with that in normal rats. Based on the data on the β-cell size and β-cell replication on days 4 and 8, we estimated the increase in the β-cell mass from day 4 to day 8 using the previously described method for evaluating the parameter of β-cell growth (32,33). In normal rats, we found that the predicted increase in β-cell mass through β-cell replication was 64%, which is slightly higher than the increase in the measured β-cell mass (52%) during this period. Of course, the neogenesis from the duct precursor may also contribute to the increase in the β-cell mass. There is a discrepancy between the measured β-cell mass and predicted β-cell mass. In fact, β-cell apoptosis may participate in the remodeling of the endocrine pancreas in neonatal rats (38), although the frequency of apoptosis β-cell was low in the present study. In STZ-treated neonatal rats, the number of islets in 2-month-old rats treated with STZ was the same as that in normal rats of the same age (Fig. 4D). A similar result was previously reported by other research groups (28,32). These results suggest that neogenesis from the precursor cells located in or by the pancreatic duct in this model may not be accelerated compared with that in normal rats. Based on the data on the β-cell size and β-cell replication on days 4 and 8, we estimated the increase in the β-cell mass from day 4 to day 8 using the previously described method for evaluating the parameter of β-cell growth (32,33). In normal rats, we found that the predicted increase in β-cell mass through β-cell replication was 64%, which is slightly higher than the increase in the measured β-cell mass (52%) during this period. Of course, the neogenesis from the duct precursor may also contribute to the increase in the β-cell mass. There is a discrepancy between the measured β-cell mass and predicted β-cell mass. In fact, β-cell apoptosis may participate in the remodeling of the endocrine pancreas in neonatal rats (38), although the frequency of apoptosis β-cell was low in the present study.

![Image](46x46 to 414x319)

**Fig. 5.** Effects of activin A and BTC on replication of β-cells. A: Double immunostaining for somatostatin (green) and BrdU (red) in STZ/A+B rats on day 4 (original magnification ×400). Nuclei were stained with DAPI (blue). B: Effect of activin A and BTC on the replication of β-cells on day 4. BrdU/somatostatin-positive cells and somatostatin-positive cells were counted on day 4. The results were shown as the percent of BrdU-positive β-cells. C: Effect of activin A and BTC on the replication of β-cell on day 8. Values are the means ± SE (n = 4). *P < 0.05 vs. the STZ group.

![Image](46x46 to 414x319)

**Fig. 6.** Effect of activin A and BTC on the number of PDX-1-positive β-cells and insulin/somatostatin double-positive cells. A: Double immunostaining for somatostatin (red) and PDX-1 (brown) in STZ/A+B rats on day 4. Nuclei were stained with hematoxylin (blue). PDX-1/somatostatin double-positive cells were counted on day 4. The results were shown as the percent of PDX-1/somatostatin double-positive cells. B: Effect of activin A and BTC on the number of PDX-1-positive β-cells on day 4. PDX-1/somatostatin double-positive and somatostatin-positive cells were counted. The data were shown as the percent of PDX-1-positive β-cells. C: Effect of activin A and BTC on the number of PDX-1-positive β-cells on day 8. Values are the means ± SE (n = 4). *P < 0.05 vs. the STZ group. D: Double-staining for insulin (red) and somatostatin (green) in STZ/A+B rats on day 4. Nuclei were stained with DAPI (blue). The arrow shows a cell expressing both insulin and somatostatin. E: Changes in the number of insulin/somatostatin double-positive cells were counted. Values are the means ± SE (n = 4). *P < 0.05 vs. the STZ group.
tal rats, the increase in the measured β-cell mass was 259%, while the increase in the predicted β-cell mass through β-cell replication was 98%. Therefore, replication of preexisting β-cells cannot explain the drastic increase in the β-cell mass during the early time point in STZ-treated neonatal rats. Another possible pathway is thought to exist to increase the β-cell mass in this regeneration model. Recently, studies have shown that β-cell regeneration has an alternate route, namely differentiation of precursor cells located in islets (6,30). Also, islet neogenesis from intra-islet precursor cells of the diabetic pancreas has been reported (34). It is therefore necessary to investigate whether β-cell neogenesis from the precursor cells located in the islets occurs in neonatal STZ-treated rats. In STZ-treated neonatal rats, temporal increases in the replication of β-cells (day 8: 4.4 ± 0.4%, day 8: 2.6 ± 0.15% [n = 4]) and the number of PDX-1/somatostatin cells (day 4: 35.2 ± 1.0%, day 8: 28.7 ± 1.7% [n = 4]) were observed, and many somatostatin/insulin double-positive cells that rarely exist in normal rats appeared on day 4 (Fig. 6). PDX-1/somatostatin cells and insulin/somatostatin cells are presumptive β-cell precursors in STZ-treated mice and pancreatic development (6,30,35). Consequently, neogenesis from the precursor cells located in the islets at least partly contributed to the increase in the β-cell mass in this model. As shown in Figs. 5 and 6, administration of activin A and BTC significantly increased the number of BrdU/somatostatin-, PDX-1/somatostatin-, and insulin/somatostatin-positive cells. It is therefore likely that BTC and activin A promoted β-cell neogenesis from precursor cells located in the islets.

The treatment with activin A and BTC significantly increased the β-cell mass and the insulin content and decreased the plasma glucose concentration. Also, the plasma glucose response after glucose loading was significantly improved. However, rapid insulin response to glucose loading was still absent. The reason for the improved glucose response in the absence of rapid insulin secretion is not totally clear. One possible reason is that small increases in insulin secretion, albeit delayed, may be beneficial for whole-body glucose metabolism and thus improve glucose tolerance. Although the insulin content and the β-cell mass was increased by activin A and BTC, the insulin response was delayed. Similar results were previously reported by other research groups as well (26,28,36). The reason that the insulin-secreting ability of regenerating β-cell is low in response to glucose loading is not totally clear. One possible reason is that glucose-sensing mechanism is impaired in regenerating β-cells (28). This is partly due to glucotoxicity during neonatal period.

In summary, in STZ-treated neonatal rats, treatment with activin A and BTC coordinately promoted regeneration of β-cells, increased the β-cell mass and insulin content, and persistently improved glucose metabolism in adult age. The β-cell neogenesis from the precursor cells located in the islets may play an important role during the early phase of pancreatic regeneration in neonatal rats treated with STZ.

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