

Activin A and Betacellulin

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

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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 μ g/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, how-

ever, 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

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BTC, betacellulin; ICC, islet cell-like cluster; IPGTT, intraperitoneal glucose tolerance test; PDX, pancreatic duodenal homeobox; STZ, streptozotocin; TGF- β , transforming growth factor- β TUNNEL, terminal deoxynucleotidyl transferase.

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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 SLC, 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 μ g/g body wt of streptozotocin (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 (day 1) for blood glucose using Accu-Chek Active (Roche Diagnostics, Heiderberg, Germany). The animals were included in the study only if their blood glucose concentration was between 200 and 350 mg/dl on day 1. Animals whose blood glucose was >350 mg/dl on day 1 developed severe diabetes, and the blood glucose concentrations in these animals were >600 mg/dl on day 2 or 3. The mortality rate of these rats was >50% during the first 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 BTC 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) or control buffer were subcutaneously injected once a day from day 1 to day 7 according to the experimental groups.

The casual 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 (Morinaga, 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 (IPGTT) (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 body wt (cell proliferation kit; Amersham Pharmacia Biotech, Little Chalfont, U.K.) and decapitated after 3 h. The pancreas was excised, weighed, and divided into two parts. One portion from the splenic segment was fixed in 4% paraformaldehyde/PBS overnight at 4°C and processed for paraffin embedding. Four series from each pancreas was cut at intervals of 100 μ m in neonate and 300 μ m in adults for immunostaining and histomorphometry. Half pancreas from the other portion was homogenized in cold acid-ethanol, heated for 5 min in 70°C water bath, centrifuged, and the supernatant was stored at -20°C until insulin assay.

Sources of antibodies. Sources of the primary antibodies and the dilutions were as follows: guinea pig anti-porcine insulin, 1:1,000 (a generous gift from Dr. T. Matozaki of Gunma University); rabbit anti-human somatostatin, 1:500 (Dako, Carpinteria, CA); rabbit anti-pancreatic duodenal homeobox-1 (PDX-1) 1:3,000 (14); monoclonal mouse anti-BrdU, 1:100 (Amersham, U.K.); rabbit anti-bovine keratin for wide-spectrum screening (CKwss), 1:1,000 (Dako); and rabbit anti-glucose transporter 2 (GLUT2), 1:50 (Biogenesis Ltd, Poole, U.K.).

Sources of the second antibodies and the dilutions were as follows: goat Alexa Fluor 568 conjugated anti-guinea pig IgG, 1:1,000; goat Alexa Fluor 488 conjugated anti-guinea pig IgG, 1:500; goat Alexa Fluor 568 conjugated anti-mouse IgG, 1:1,000; goat Alexa Fluor 488 conjugated anti-mouse IgG, 1:500; goat Alexa Fluor 568 conjugated anti-rabbit IgG, 1:1,000; and goat Alexa Fluor 488 conjugated anti-rabbit IgG, 1:500 (Molecular Probes, Eugene, OR).

Immunohistochemistry. The sections were deparaffinized and rehydrated, incubated in a microwave oven for BrdU-staining, and digested with protein K (Dako) for CK-staining, washed with PBS, and blocked with DAKO protein block solution. The sections were incubated for 1 h at room temperature with a mixture of primary antibody, washed with PBS, and incubated for 45 min at room temperature with a mixture of the second antibody. The counterstaining was done with 4',6-diamidino-2-phenylindol-HCl (DAPI) (Boehringer Mannheim, Mannheim, Germany). Finally, the sections were mounted with PermaFluor Aqueous Mounting Medium (IMMUNON Thermo, Shandon, PA).

Double staining of PDX-1 and somatostatin was performed as described previously (15).

Measurement of the β -cell mass and size. Quantitative evaluation of the β -cell area was performed on insulin-stained sections using image analysis software (National Institutes of Health image) by means of an AX70 Epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a PXL 1400 cooled-charge-coupled device camera system (Photometrics, Tucson, AZ) operated with IP Lab Spectrum software (Signal Analysis, Vienna, VA). At least 40 random fields (magnification $\times 200$) from one section (three sections from different series per block) were measured for the area of insulin-positive cells and these fields. The ratio of the β -cell area was calculated by dividing the area of all insulin-positive cells in one block by the total area of these fields. The β -cell mass was calculated by multiplying the pancreas weight by the ratio of the β -cell area.

The β -cell size was determined on an insulin-stained section by evaluating the mean cross-sectional area of the individual β -cell. The area of the β -cell in islet was measured as described above, and the number of β -cell nuclei in the islet was counted. Ten islets per animal were measured. The mean cross-sectional area of the individual β -cell was calculated by dividing the total β -cell area in the 10 islets by the number of β -cell nuclei in these islets.

Measurement of replication of β -, δ -, and ductal cells. BrdU/insulin-positive cells were analyzed on BrdU/insulin double-stained sections as a marker of replication of preexisting β -cells. The results were expressed as the percentage of BrdU-positive β -cells. The replication of δ -cells was analyzed by BrdU and somatostatin double-staining. The results were expressed as the percentage of BrdU-positive δ -cells. At least 500 β - and δ -cells were counted per animal, and four animals per group were examined. BrdU/CK-positive cells and CK-positive cells were counted on the BrdU/CKwss double-stained sections. At least 1,000 ductal cells per animal (four animals per group) were analyzed. The results were expressed as the percentage of BrdU-positive ductal cells.

β -Cell neogenesis and the number of islets. To assess the β -cell neogenesis, we quantified the number of islet cell-like clusters (ICCs) (less than five cells across). The number of ICCs and islets was counted in the section stained with anti-insulin antibody, 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 apoptosis. 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 \pm 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.

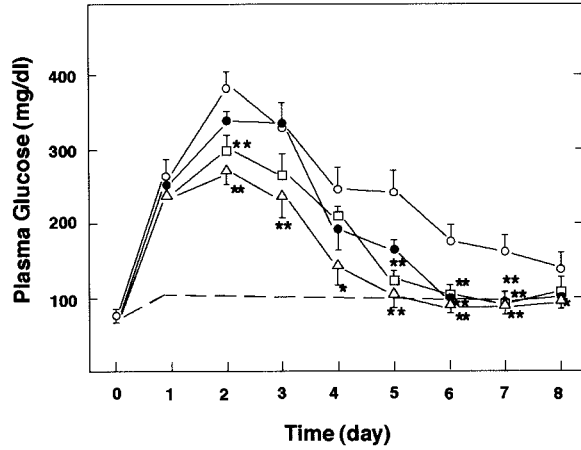
RESULTS

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 IPGTT 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

A



B

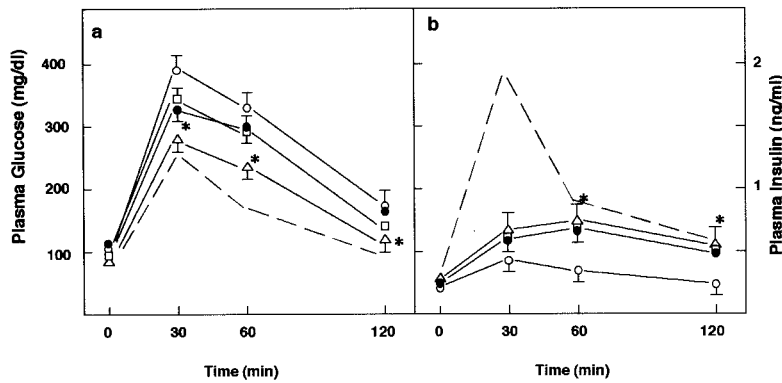


FIG. 1. Changes in the plasma glucose concentration and results of glucose tolerance test in STZ-treated rats. **A:** STZ (85 $\mu\text{g/g}$) was injected on day 0 (neonate of 1-day-old) and daily injection of activin A and/or BTC was started 1 day later. The plasma glucose concentration was measured. Values are the means \pm SE. \circ , STZ group ($n = 16$); \bullet , STZ/A group ($n = 13$); \square , STZ/B group ($n = 13$); \triangle , STZ/A+B group ($n = 15$). —, normal SD rats. * $P < 0.05$; ** $P < 0.01$ vs. the STZ group. **B:** Glucose tolerance test was performed on 6-week-old rats as described in RESEARCH DESIGN AND METHODS. **a:** Changes in the plasma glucose concentration. **b:** Changes in the plasma insulin concentration. Values are the means \pm SE. \circ , STZ group ($n = 11$); \bullet , STZ/A group ($n = 9$); \square , STZ/B group ($n = 9$); \triangle , STZ/A+B group ($n = 11$); —, normal SD rats ($n = 6$). * $P < 0.05$ vs. STZ group.

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

$6.5 \pm 0.35\%$ vs. normal $4.4 \pm 0.15\%$ [$n = 4$]; $P < 0.002$) and CK/BrdU double-positive cells (STZ-treated $6.4 \pm 0.3\%$ [$n = 4$] vs. normal $4.1 \pm 0.2\%$ [$n = 4$]; $P < 0.001$) 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

	Normal	STZ	STZ/A	STZ/B	STZ/A+B
Body weight (g)	14.9 \pm 0.6 (10)	13.7 \pm 0.8 (16)	14.5 \pm 0.7 (13)	14.8 \pm 0.6 (14)	13.9 \pm 0.7 (15)
Pancreas weight (g)	36.5 \pm 1.8 (4)	39.4 \pm 1.8 (5)	41 \pm 1.0 (9)	39.2 \pm 0.5 (5)	44.8 \pm 1.8 (4)
Plasma glucose (mg/dl)	114.1 \pm 2.6 (6)	153.3 \pm 28.4 (16)	123 \pm 5.8 (13)	118.8 \pm 6.7 (14)	112.6 \pm 5.6 (15)
Plasma insulin (ng/ml)	0.386 \pm 0.008 (4)	0.342 \pm 0.014 (5)	0.380 \pm 0.041 (4)	0.360 \pm 0.026 (5)	0.435 \pm 0.049 (4)
Insulin content					
$\mu\text{g/pancreas}$	24.6 \pm 2.2 (4)	3.46 \pm 0.45 (5)	4.45 \pm 0.2 (4)	4.48 \pm 0.47 (5)	6.06 \pm 0.28 (4)*
$\mu\text{g} \cdot \text{pancreas}$	690.2 \pm 44.1 (4)	88.3 \pm 14.3 (5)	113.8 \pm 2.6 (4)	138.0 \pm 14.4 (5)†	136.4 \pm 7.4 (4)†
β -Cell mass					
mg/pancreas	0.92 \pm 0.06 (4)	0.23 \pm 0.04 (4)	0.256 \pm 0.016 (4)	0.344 \pm 0.034 (4)†	0.408 \pm 0.046 (4)†
mg/g \cdot pancreas	25.0 \pm 1.25 (4)	5.89 \pm 0.91 (4)	6.24 \pm 0.26 (4)	8.80 \pm 1.06 (4)†	9.14 \pm 1.06 (4)†
β -Cell size (μm^2)	92.3 \pm 4.8 (4)	97.1 \pm 6.3 (4)	98.5 \pm 4.2 (4)	96.4 \pm 3.8 (4)	95.6 \pm 5.4 (4)

Data are means \pm 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.

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

	Normal	STZ	STZ/A	STZ/B	STZ/A+B
Body weight (g)	236.8 ± 11.6 (6)	225.2 ± 12.5 (11)	236.0 ± 9.7 (9)	238.4 ± 15.1 (9)	228.9 ± 11.7 (11)
Pancreas weight (g)	0.869 ± 0.024 (6)	0.856 ± 0.022 (11)	0.898 ± 0.027 (9)	0.923 ± 0.043 (9)	0.914 ± 0.038 (11)
Plasma glucose (mg/dl)	135.6 ± 4.4 (6)	163.5 ± 4.0 (11)	154 ± 4.8 (9)	151.1 ± 4.1 (9)	148.5 ± 3.4 (11)*
Plasma insulin (ng/ml)	2.40 ± 0.43 (6)	0.94 ± 0.14 (8)	1.10 ± 0.18 (7)	1.138 ± 0.25 (7)	1.02 ± 0.14 (8)
Insulin content					
μg/pancreas	103.3 ± 7.5 (6)	41.2 ± 1.8 (11)	45.8 ± 3.7 (9)	51.6 ± 4.0 (9)*	64.8 ± 2.1 (11)†
μg/g · pancreas	118.8 ± 8.1 (6)	46.9 ± 2.8 (11)	51.1 ± 4.1 (9)	56.1 ± 4.4 (9)	60.0 ± 3.5 (11)†
β-Cell mass					
mg/pancreas	8.11 ± 0.81 (4)	2.87 ± 0.31 (4)	3.55 ± 0.36 (4)	4.53 ± 0.41 (4)*	5.89 ± 0.35 (4)†
mg/g · pancreas	9.67 ± 0.86 (4)	3.37 ± 0.31 (4)	3.85 ± 0.28 (4)	4.87 ± 0.29 (4)*	5.93 ± 0.33 (4)*
β-Cell size (μm ²)	192.6 ± 4.5 (4)	202.2 ± 5.6 (4)	199.6 ± 3.5 (4)	195.3 ± 2.7 (4)	197.7 ± 3.8 (4)

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.

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 69% 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 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 or 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

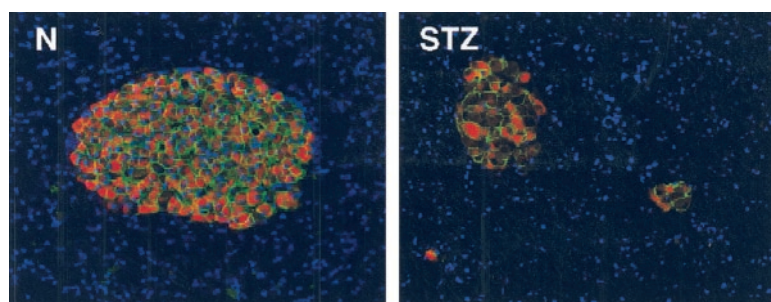


FIG. 2. Morphology of typical islets in normal and STZ-treated rats. Pancreatic sections obtained from neonatal STZ-treated (STZ) and nontreated (N) rats at 2 months of age were stained with anti-insulin (red) and anti-GLUT2 antibodies (green). Nuclei were stained with DAPI (blue) (original magnification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

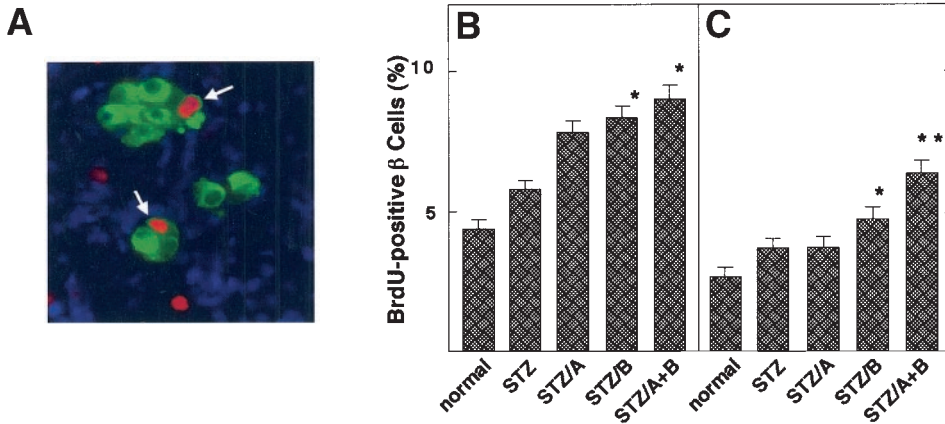


FIG. 3. Effects of activin A and BTC on the replication of insulin-positive cells in STZ-treated neonatal rats. **A:** Double immunostaining for insulin (green) and BrdU (red) in STZ/A+B rats on day 4 (original magnification $\times 400$). The arrows show cells positive for insulin and BrdU. **B:** Effects of activin A and BTC on day 4. The numbers of BrdU/insulin-positive cells and insulin positive-cells were measured. The results were expressed as the percent of BrdU-positive β -cell. Values are the mean \pm SE ($n = 4$). **C:** Effect of activin A and BTC on day 8. Values are the means \pm SE ($n = 4$). * $P < 0.05$; ** $P < 0.01$ vs. the STZ group.

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 $4.4 \pm 0.4\%$ vs. normal $2.8 \pm 0.3\%$ [$n = 4$]; $P < 0.02$). 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 $35.2 \pm 3.2\%$ vs. normal $26.9 \pm 2.1\%$ [$n = 4$]; $P < 0.05$). 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.

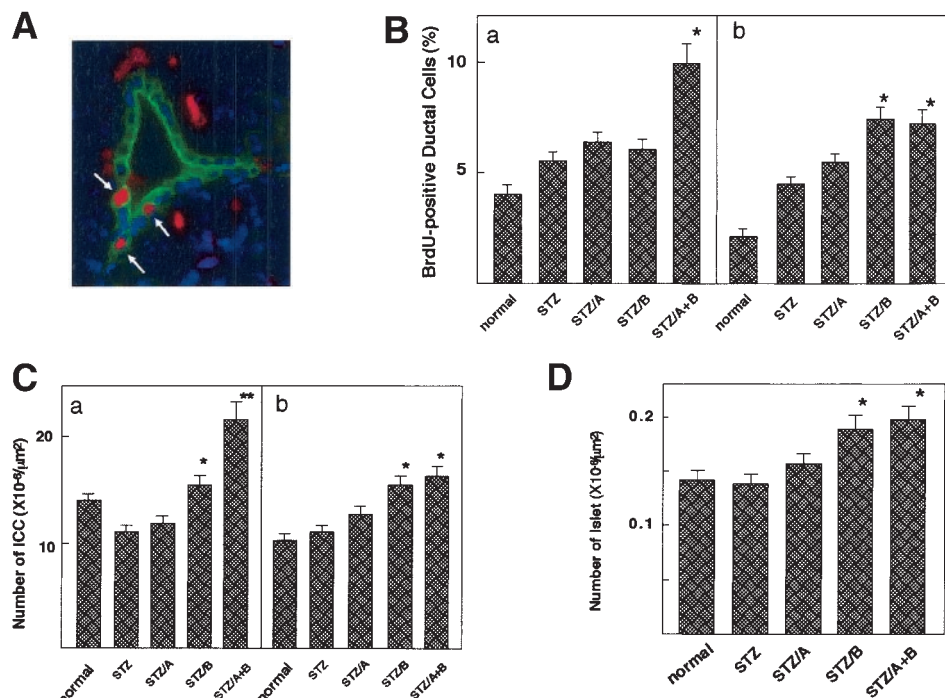


FIG. 4. Effects of activin A and BTC on replication of ductal cells and the number of ICCs and islets. **A:** Double immunostaining for CK (green) and BrdU (red) in STZ/A+B rats on day 4 (original magnification $\times 400$). Nuclei were stained by DAPI. Arrows show the cells positive for CK and BrdU. **B:** Effect of activin A and BTC on replication of ductal cells on day 4 (a) and day 8 (b). The numbers of BrdU/insulin-positive cells and CK-positive cells were counted. The results were expressed as the percent of BrdU-positive duct cells. Values are the means \pm SE ($n = 4$). * $P < 0.05$ vs. the STZ group. **C:** Effect of activin A and BTC on the number of ICCs. **a:** Effect of activin A and BTC on the number of ICCs on day 4. **b:** Effect of activin A and BTC on the number of ICCs on day 8. Data were shown as the number of ICCs per micrometer squared of the pancreatic area. Values are the means \pm SE ($n = 4$). * $P < 0.05$; ** $P < 0.01$ vs. the STZ group. **D:** The number of islets was counted in the section stained with insulin in 2-month-old rats. Data were expressed as the number of islets per micrometer squared of the pancreatic area. Values are the means \pm SE ($n = 4$). * $P < 0.05$ vs. the STZ group.

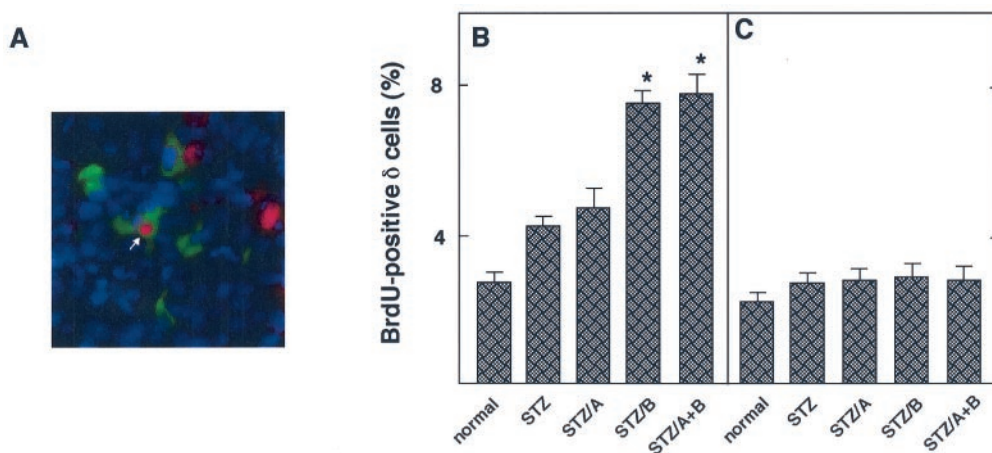


FIG. 5. Effects of activin A and BTC on replication of δ -cells. **A:** Double immunostaining for somatostatin (green) and BrdU (red) in STZ/A+B rats on day 4 (original magnification $\times 400$). Nuclei were stained with DAPI (blue). **B:** Effect of activin A and BTC on the replication of δ -cells 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 δ -cells. **C:** Effect of activin A and BTC on the replication of δ -cell on day 8. Values are the means \pm SE ($n = 4$). * $P < 0.05$ vs. the STZ group.

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). Consistent with these data, the number of islets in the STZ/A+B group was increased. 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 neona-

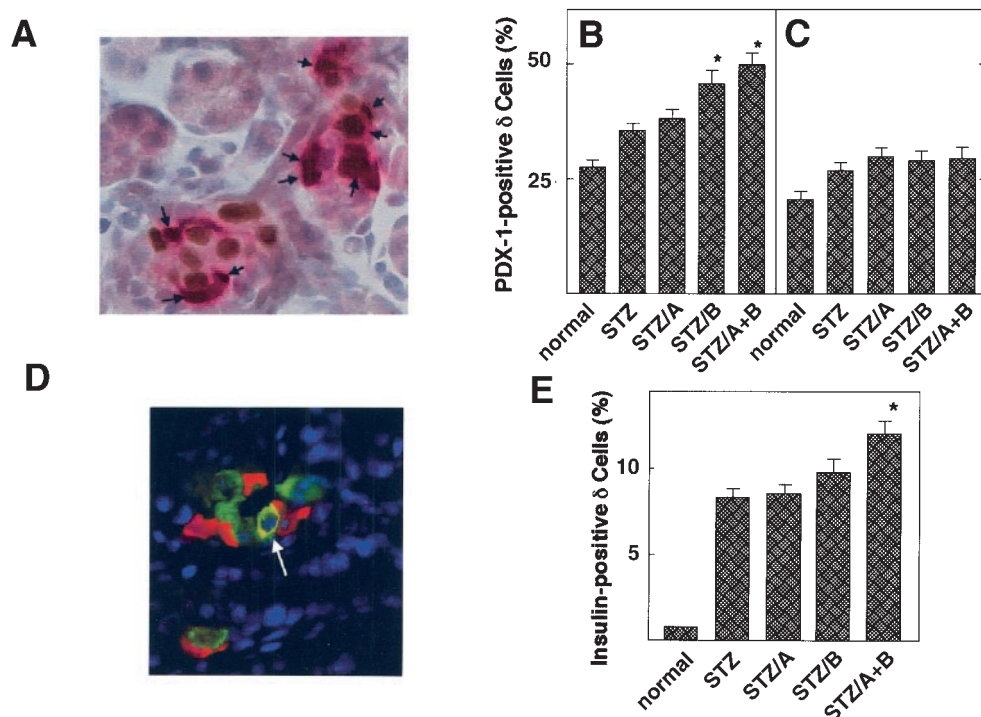


FIG. 6. Effect of activin A and BTC on the number of PDX-1-positive δ -cells and insulin/somatostatin double-positive cells. **A:** Double immunostaining for somatostatin (red) and PDX-1 (brown) in STZ/A+B rats on day 4. Nuclei were stained with hematoxylin (blue). PDX-1/somatostatin double-positive cells were indicated by the arrow. **B:** Effect of activin A and BTC on the number of PDX-1-positive δ -cells on day 4. PDX-1/somatostatin-positive cells and somatostatin-positive cells were counted. The data were shown as the percent of PDX-1-positive δ -cells. **C:** Effect of activin A and BTC on the number of PDX-1-positive δ -cells on day 8. Values are the means \pm 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 \pm 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 intraislet 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 δ -cells (day 4: $4.4 \pm 0.4\%$, day 8: $2.6 \pm 0.15\%$ [$n = 4$]) and the number of PDX-1/somatostatin cells (day 4: $35.2 \pm 1.6\%$, day 8: $28.7 \pm 1.7\%$ [$n = 4$]) 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 still not totally clear. One possible reason is that the β -cell mass was not restored because at least 75% of the normal β -cell mass is needed for maintaining normal glucose homeostasis (37). Another possible reason is that, in STZ-treated rats, there were no large islets that usually exist in normal rats (Fig. 2). It is possible that large islets may play an important role in insulin release in response to glucose loading. The treatment with activin A and BTC partly increased the size of islets, but the largest islets in STZ/A+B adult rats could not be comparable to that of normal rats of the same age. It is also possible that the 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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