

Recombinant Human Betacellulin Promotes the Neogenesis of β -Cells and Ameliorates Glucose Intolerance in Mice With Diabetes Induced by Selective Alloxan Perfusion

Koji Yamamoto, Jun-ichiro Miyagawa, Masako Waguri, Reiko Sasada, Koichi Igarashi, Ming Li, Takao Nammo, Makoto Moriwaki, Akihisa Imagawa, Kazuya Yamagata, Hiromu Nakajima, Mitsuyoshi Namba, Yoshihiro Tochino, Toshiaki Hanafusa, and Yuji Matsuzawa

Betacellulin (BTC), a member of the epidermal growth factor family, is expressed predominantly in the human pancreas and induces the differentiation of a pancreatic acinar cell line (AR42J) into insulin-secreting cells, suggesting that BTC has a physiologically important role in the endocrine pancreas. In this study, we examined the in vivo effect of recombinant human BTC (rhBTC) on glucose intolerance and pancreatic morphology using a new mouse model with glucose intolerance induced by selective alloxan perfusion. RhBTC (1 μ g/g body wt) or saline was injected subcutaneously every day from the day after alloxan treatment. The intraperitoneal glucose tolerance test revealed no difference between rhBTC-treated and rhBTC-untreated glucose-intolerant mice at 2–4 weeks. However, glucose tolerance was significantly improved and body weight was significantly increased in rhBTC-treated mice compared with untreated mice at 8 weeks. Islet-like cell clusters, consisting mainly of β -cells, were increased in the pancreas and were localized in contact with the ductal lining cells and sometimes with acinar cells. In conclusion, administration of rhBTC improved glucose tolerance in this mouse model by increasing β -cell volume, primarily through accelerated neogenesis from ductal lining cells. *Diabetes* 49:2021–2027, 2000

Pancreatic β -cells are thought to be terminally differentiated cells with little ability to regenerate. However, proliferation of preexisting β -cells and differentiation of β -cells from precursor cells, mainly residing in the pancreatic duct lining, have been demonstrated in some animal models (1–5). Recently, we developed a new mouse model of diabetes induced by selective perfusion of alloxan (100 μ g/g body wt) during the clamping of the superior mesenteric artery (1). In this model, glucose intolerance spontaneously resolves after one year because of the proliferation of surviving β -cells in the non-perfused segment of the pancreas and subsequently because of the differentiation of β -cells from ductal epithelial cells in the alloxan-perfused segment. In these mice, β -cells are completely destroyed in the alloxan-perfused segment (~80% of the total pancreatic β -cell mass), whereas β -cells in the non-perfused segment are spared. Therefore, this diabetic model allows us to clarify the process of β -cell neogenesis from the pancreatic ductal epithelium after pure loss of β -cells without any influence of damage to exocrine tissue, which usually occurs in other regenerating models such as duct ligation and subtotal pancreatectomy.

Betacellulin (BTC), a member of the epidermal growth factor (EGF) family, was initially detected in the conditioned medium of a mouse pancreatic β -cell tumor, β TC-3 (6). BTC is a glycoprotein with a molecular weight of 32 kDa that is composed of 80 amino acid residues and is generated by the cleavage of a 178-amino acid membrane-anchored precursor, pro-BTC. BTC is known to bind and activate the EGF receptor (EGFR/erbB-1) and erbB-4, and it induces tyrosine phosphorylation of erbB-2, which couples with the EGFR or erbB-4 (7–10), although the existence of a specific BTC receptor has also been suggested (11). Recently, BTC was demonstrated to be predominantly expressed in the human pancreas and small intestine and was shown to induce differentiation of a pancreatic exocrine cell line (AR42J) into insulin-secreting cells (12–14). In a study of pancreatic duodenal homeobox-containing transcription factor (PDX)-1-transfected glucagon-secreting α TC1 cells, insulin and glucokinase genes were induced by exposure to BTC (15). This suggests that BTC may have a physiologically important role in the differentiation and/or functioning of the endocrine pancreas, although its physiological significance remains unclear.

From the Department of Internal Medicine and Molecular Science (Ko.Y., J.M., M.W., M.L., T.N., M.M., A.I., Ka.Y., H.N., M.N., Y.T., T.H., Y.H.), Graduate School of Medicine, Osaka University, Suita; and the Discovery Research Laboratory II, Discovery Research Division, Takeda Chemical Industries (R.S., K.I.), Tsukuba, Japan.

Address correspondence and reprint requests to Jun-ichiro Miyagawa, MD, PhD, Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, 2-2 B5 Yamadaoka, Suita 565-0871, Japan. E-mail: miyagawa@imed2.med.osaka-u.ac.jp. Ko.Y. is currently affiliated with the Department of General Medicine, Osaka University Hospital, Suita. M.W. is currently affiliated with the Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan.

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ABC, avidin-biotin complex; BrdU, 5-bromo-2-deoxyuridine; BrdULI, BrdU labeling index; BTC, betacellulin; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DCK, duct cell-specific cytokeratin; EGF, epidermal growth factor; EGFR, EGF receptor; EIA, enzyme immunoassay; FITC, fluorescein isothiocyanate; ICC, islet-like cell cluster; IPF, insulin promoter factor; IPGTT, intraperitoneal glucose tolerance test; PBS, phosphate-buffered saline; PDX, pancreatic duodenal homeobox-containing transcription factor; rhBTC, recombinant human BTC.

In the present study, we investigated the *in vivo* effect of recombinant human BTC (rhBTC) on impaired glucose tolerance and pancreatic β -cell neogenesis in mice with diabetes induced by selective perfusion of alloxan.

RESEARCH DESIGN AND METHODS

Animals. Male Jcl:ICR mice (8 weeks old) were used in this study. The mice were anesthetized with pentobarbital (Nembutal, 50 μ g/g body wt) and the superior mesenteric artery, which supplies the duodenal segment of the pancreas, was clamped. Then alloxan (5,6-dioxouracil monohydrate; 0.1 mg/g body wt) dissolved in sterile saline (154 mmol/l NaCl dissolved in distilled water) in the experimental group or saline alone in the control group was injected via the caudal vein. Within 10 min after injection, the arterial clamp was removed and the abdomen was closed (1). Then the mice were maintained with free access to water and standard mouse diet in a specific pathogen-free environment, and the body weight was measured every week.

Purification of rhBTC and measurement of the serum level in mice. rhBTC was purified from *Escherichia coli* (12). To determine the serum concentration of rhBTC, we established a BTC enzyme immunoassay (EIA) system. Whole blood was obtained from the hearts of control ICR mice at 1, 2, 6, 12, and 24 h ($n = 5$ at each time) after injection of rhBTC (1 μ g/g body wt) dissolved in saline (100 μ g/ml). Then a standard curve was created for the assay, using a mouse monoclonal anti-human BTC antibody (5E5) as the primary antibody and a horseradish peroxidase-labeled mouse monoclonal anti-human BTC antibody (2B2) as the secondary antibody. The detection limit of this assay was 300 pg/ml (16).

Treatment of mice with rhBTC. During the period from 17:00 to 20:00, the mice were injected subcutaneously with rhBTC (1 μ g/g body wt) dissolved in sterile saline (100 μ g/ml) from the day after selective alloxan or saline perfusion. Glucose-intolerant mice without rhBTC treatment were injected with the same volume of saline.

We evaluated the effect of rhBTC treatment on glucose intolerance using the intraperitoneal glucose tolerance test (IPGTT) (2 mg/g body wt), which was performed before and at 2, 4, and 8 week after alloxan perfusion. Blood was obtained from the retro-orbital plexus after fasting for 10 h. The blood glucose concentration was measured by the glucose oxidase method in samples obtained at 0, 60, and 120 min, using a Fuji DRI-Chem 1000 (Fuji Medical System, Tokyo).

Measurement of the pancreatic insulin content. Four mice each from the control and rhBTC-treated and untreated glucose-intolerant groups were used for measuring the pancreatic insulin content. The whole pancreas was excised and divided into alloxan-perfused and nonperfused segments at 8 weeks after perfusion. Extraction of insulin from the pancreas was performed according to Kenny's method (17), and the insulin content was measured using an insulin EIA test (Glazyme; Sanyo-kasei, Kyoto, Japan). Rat insulin (provided by Lilly Research Laboratories, Eli Lilly, Indianapolis, IN) was used to create a standard curve.

Analysis of early insulin secretion. At 8 weeks after alloxan perfusion, mice were fasted for 6 h and glucose (1 mg/g body wt) was injected intravenously via the tail vein. Three minutes after injection, blood was obtained from the retro-orbital plexus and the plasma insulin concentration was measured using the insulin EIA described above.

Staining of islets. To evaluate the total islet number and volume in the pancreas of the BTC-treated ($n = 6$) and untreated ($n = 6$) mice at 12 weeks after alloxan perfusion, we stained the islets with diphenylthiocarbazon (dithizone; Wako Pure Chemicals, Osaka, Japan), as described previously (1).

Immunohistochemistry and morphometric analysis. To examine morphological changes and mitotic activity in major organs of the mice, including the pancreas, liver, small intestine, kidney, and skeletal muscle (biceps femoris), BTC-treated and untreated mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU; Sigma, St. Louis, MO) at a dose of 100 μ g/g body wt at 8 and 12 weeks after alloxan perfusion. Six h after injection of BrdU, the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (100 μ g/g body wt), and their tissues were excised, fixed with 10% neutrally buffered formalin at 4°C for 5 h, and embedded in paraffin. Tissues were also obtained from BTC-treated and untreated mice without BrdU labeling at 8 week after perfusion of alloxan. Tissue sections (~5- μ m thick), which had been removed from the paraffin, were stained with hematoxylin and eosin and were examined under a microscope before immunostaining. All immunostaining procedures were carried out at room temperature unless otherwise stated, and sections were washed three times with 0.01 mol/l phosphate-buffered saline (PBS) (pH 7.4) before each incubation step, except for incubation with the primary antibody. All antibodies were diluted in PBS containing 1% bovine serum albumin.

To analyze the effect of exogenous rhBTC on the pancreas, we made three sets of eight paraffin sections from each pancreas for immunostaining and morphometric analysis. Each set was cut at intervals of 500 μ m and included two pairs of mirror sections and four consecutive sections. The first pair of mirror sections was used for double immunostaining for four islet hormones and for duct cell-specific cytokeratin (DCK) to detect islets and islet-like cell clusters (ICCs) that had formed from duct lining cells (1). Then the number of islets per square millimeter in the whole pancreatic section was calculated. These sections received double immunostaining for islet hormones and DCK. One of the mirror sections was treated with an antibody cocktail containing anti-glucagon (diluted to 1:500) (Linco Research, St. Charles, MO), anti-somatostatin (1:500) (Dako, Glostrup, Denmark), and anti-pancreatic polypeptide (1:500) (Dako) antibodies. Then the sections were incubated with biotinylated goat anti-rabbit IgG antibody (1:500) and developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Zymed Laboratories, San Francisco, CA) using the avidin-biotin complex (ABC) method. After blocking residual avidin and biotin from the primary immunohistochemical reaction with a biotin blocking system (Dako), immunostaining for DCK by indirect immunofluorescent method was performed using rabbit anticytokeratin antibody (Nichirei, Tokyo) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:500). The other mirror section was first immunostained for insulin by incubation with guinea pig anti-porcine insulin antibody (1:2000) (Dako) and with biotinylated goat anti-guinea pig IgG (Dako) using the ABC method, and positive staining was visualized with DAB. Secondary immunostaining for DCK was carried out as described above.

One of the second pair of mirror sections was used to stain either glucagon or somatostatin plus insulin, whereas insulin promoter factor 1/PDX-1 (IPF1/PDX-1) and DCK were double immunostained in the other section (1,18). The sections were immunostained for glucagon or somatostatin by incubation with a polyclonal rabbit anti-glucagon antibody (Linco Research) or a polyclonal rabbit anti-somatostatin antibody (Dako), respectively. Sections were then incubated with biotinylated anti-rabbit IgG (Vector Laboratories), and the positive reaction was detected by incubation with avidin-conjugated fluorescein isothiocyanate (avidin-FITC) diluted to 1:500 in PBS. The same sections were then immunostained for insulin by incubation with guinea pig anti-porcine insulin antibody (1:500) and with rhodamine-conjugated goat anti-guinea pig IgG antibody (1:200) (Chemicon, Temecula, CA). For detecting IPF1/PDX-1 and DCK in the other section, incubation was done with rabbit anti-IPF1/PDX-1 antibody (1:200) by the ABC method and visualization was done with DAB. The same sections were then immunostained for DCK with anti-cytokeratin by the ABC method, and positive staining was visualized by incubation with avidin-FITC.

Employing the next three consecutive sections, we performed double immunostaining for BrdU and insulin, BrdU and DCK, and BrdU and pancreatic amylase using the ABC method to examine the proliferative activity of β -cells, DCK⁺ ductal cells, and amylase⁺ acinar cells. The details of the method were described previously (1). The indirect immuno-alkaline phosphatase technique was performed with a BrdU Labeling and Detection Kit II (Boehringer Mannheim Biochemica, Mannheim, Germany). Before the second immunostaining was done for insulin, DCK, or amylase, sections were blocked with avidin-biotin blocking solution (Dako). For insulin immunostaining, sections were incubated with guinea pig anti-porcine insulin antibody (1:500), followed by biotinylated goat anti-guinea pig IgG (1:200). To detect DCK, sections were treated as described above. To detect pancreatic amylase, sections were immunostained by incubation with an anti-pancreatic amylase antibody (Sigma) (1:500) for 30 min, followed by incubation with biotinylated anti-rabbit IgG for 30 min. The reaction for insulin, DCK, or pancreatic amylase was visualized by incubation for 30 min with streptavidin-AMCA (9-amino-6-chloro-2-methoxyacridine) (Vector Laboratories) diluted to 1:200 in PBS.

Using the final sections from each set, immunohistochemistry for EGFR and erbB-4 was performed to detect BTC receptors in the pancreas. The ABC method was applied to detect putative BTC receptors of the erbB receptor family, (i.e., EGFR and erbB4) using incubation with anti-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-erbB-4 (Santa Cruz) antiserum (1:200) for 30 min, followed by incubation with biotinylated goat anti-rabbit IgG (1:200) for 30 min. Positive reactions were visualized with DAB.

After immunostaining, sections were counterstained with hematoxylin or methyl green for immunohistochemistry and with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) for fluorescent immunocytology and then were mounted with aqueous mounting medium (PermaFluor; Immunon, Pittsburgh, PA).

Histomorphometric analysis was done using sections cut at intervals of ~500 μ m and immunostained as described above. The BrdU labeling index (BrdULI) and the relative numbers of ICCs per section were determined from light microscopic images (Provis AX 80 equipped with an HDTV system and a color-chilled 3 charged coupling device camera, Olympus, Tokyo) using an image analysis system (Macscope version 2.55; Mitani, Fukui, Japan).

TABLE 1

Body weight of control mice, alloxan-perfused diabetic mice without rhBTC treatment, and alloxan-perfused diabetic mice with rhBTC treatment

Time after rhBTC treatment	Control	<i>n</i>	Alloxan without rhBTC	<i>n</i>	Alloxan with rhBTC	<i>n</i>
Before (g)	33.7 ± 0.6	11	33.2 ± 0.4	26	33.9 ± 0.3	14
2 weeks (g)	38.0 ± 0.9	11	34.5 ± 0.6*	26	35.4 ± 0.6*	14
4 weeks (g)	39.9 ± 0.9	11	36.8 ± 0.5*	26	37.1 ± 0.4*	14
8 weeks (g)	42.5 ± 1.2	10	39.9 ± 0.6*	18	42.4 ± 0.4	12

Data are means ± SE. **P* < 0.01 vs. control at the same point.

Statistical Analysis. Results are expressed as the means ± SE. Differences among the control, rhBTC-treated, and untreated alloxan-perfused mice were evaluated by Dunnett's multiple comparison test and *P* < 0.05 was accepted as statistically significant.

RESULTS

Body weight and IPGTT. Before and 2 and 4 weeks after perfusion with alloxan, there was no significant difference between the body weight of rhBTC-treated and untreated glucose-intolerant mice, and the weight of both groups was significantly lower than that of the control group. The weight of rhBTC-treated mice was significantly greater than that of untreated glucose-intolerant mice at 8 week and became similar to that of sham-operated control mice (Table 1).

The IPGTT showed no difference among the 3 groups before alloxan treatment. No significant difference was observed between treated and untreated mice at 2 and 4 weeks after rhBTC treatment, and the glucose intolerance of both groups was more severe than that of the control group. However, the blood glucose levels at 8 weeks in the rhBTC-treated glucose-intolerant mice were 6.2, 15.6, and 10.5 mmol/l, whereas the levels in the untreated glucose-intolerant mice were 7.9, 19.9, and 13.3 mmol/l at 0 (before), 60, and 120 min, respectively. On the other hand, the blood glucose levels in sham-operated control mice were 6.3, 11.0, and 7.5 mmol/l at 0 (before), 60, and 120 min, respectively. In the IPGTT at 8 weeks, the blood glucose levels at 0 (before) and 60 min were significantly lower in rhBTC-treated mice than in untreated mice, and the blood glucose level at 0 min (before) in rhBTC-treated mice was similar to that in sham-operated control nondiabetic mice (Table 2). Thus, the glucose intolerance of rhBTC-treated mice improved significantly compared with that of untreated glucose-intolerant mice.

Serum level of rhBTC. The serum level of rhBTC was 19.7 ± 2.8 ng/ml at 60 min and 5.4 ± 0.6 ng/ml at 120 min after

rhBTC injection. The levels before and 6, 12, and 24 h after injection were below the detection limit.

Insulin content. In the control group, the insulin content at 8 week was 53.5 ± 12.1 ng/mg wet wt in the nonperfused segment of the pancreas and 32.9 ± 2.6 ng/mg wet wt in the perfused segment. Among the alloxan-perfused mice, the insulin content of the nonperfused segment was 106.2 ± 21.4 ng/mg wet wt in rhBTC-treated mice, whereas that of untreated mice was 66.9 ± 12.5 ng/mg wet wt. The insulin content of the perfused segment was 2.3 ± 0.2 ng/mg wet wt in untreated mice and 3.0 ± 0.4 ng/mg wet wt in rhBTC-treated mice. The insulin content of both the alloxan-perfused and nonperfused pancreatic segments showed no significant difference between rhBTC-treated and untreated mice.

Early insulin secretion. The plasma insulin level at 3 min after glucose injection was 962 ± 108, 578 ± 56, and 442 ± 62 pg/ml in control, rhBTC-treated alloxan-perfused mice, and untreated alloxan-perfused mice, respectively. The insulin level in control mice was significantly higher than that in alloxan-perfused rhBTC-treated mice or untreated mice. The insulin level in rhBTC-treated alloxan-perfused mice tended to be higher than in untreated alloxan-perfused mice, but there was no significant difference.

Islet staining and histomorphometric analysis. Islets were stained in bright red color with dithizone. At 12 weeks after alloxan perfusion, the total islet volume was 1.34 ± 0.12 and 1.08 ± 0.03 mm³ (*P* < 0.05) in rhBTC-treated alloxan-perfused mice and untreated alloxan-perfused mice, respectively. There was no significant difference of whole pancreas volume between rhBTC-treated alloxan-perfused mice (54.3 ± 3.12 mm³) and untreated alloxan-perfused mice (52.6 ± 3.58 mm³). The total islet number was 351 ± 22 and 312 ± 18 (*P* < 0.05) in rhBTC-treated alloxan-perfused mice and untreated alloxan-perfused mice, respectively.

TABLE 2

Blood glucose levels in IPGTT of control mice, alloxan-perfused diabetic mice without rhBTC treatment, and alloxan-perfused diabetic mice with rhBTC treatment

	Before			2 weeks			4 weeks			8 weeks		
	Control group	Alloxan without BTC group	Alloxan with BTC group	Control group	Alloxan without BTC group	Alloxan with BTC group	Control group	Alloxan without BTC group	Alloxan with BTC group	Control group	Alloxan without BTC group	Alloxan with BTC group
<i>n</i>	10	18	10	12	23	13	11	24	11	10	14	12
0 min (mmol/l)	5.1 ± 0.2	5.3 ± 0.2	5.2 ± 0.4	6.7 ± 0.5	7.7 ± 0.3	8.0 ± 0.5	5.8 ± 0.5	6.5 ± 0.4	7.3 ± 0.7	6.3 ± 0.4	7.9 ± 0.5*	6.2 ± 0.3*†
60 min (mmol/l)	8.0 ± 0.7	7.8 ± 0.5	7.3 ± 0.6	11.9 ± 0.5	20.4 ± 0.8*	21.2 ± 1.1*	11.4 ± 0.9	20.5 ± 0.5*	18.6 ± 1.2*	11.0 ± 0.8	19.9 ± 1.2*	15.6 ± 1.4*†
120 min (mmol/l)	6.2 ± 0.4	6.2 ± 0.2	5.9 ± 0.4	8.7 ± 0.7	14.6 ± 0.8*	15.9 ± 1.4*	7.6 ± 0.6	14.6 ± 1.0*	14.0 ± 1.4*	7.5 ± 0.5	13.3 ± 1.3*	10.5 ± 1.3

Data are means ± SE. **P* < 0.01 vs. control at the same point; †*P* < 0.01 vs. alloxan without BTC at the same point.

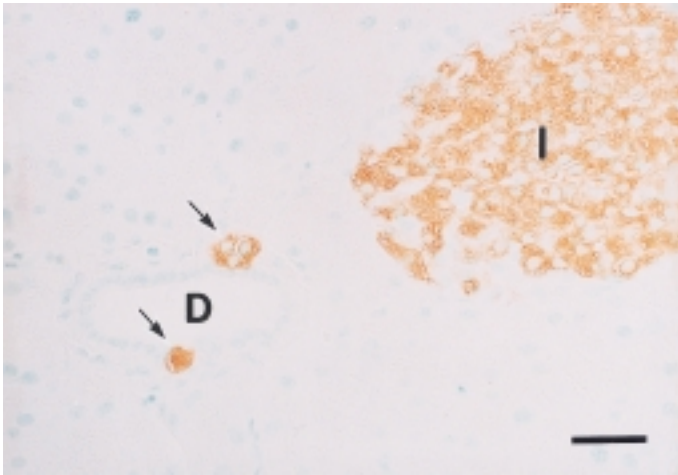


FIG. 1. Neogenesis of β -cells from ductal epithelium in the alloxan-perfused segment of a glucose-intolerant mouse at 8 weeks after the start of rhBTC treatment. *A*, β -Cells were observed among ICCs (\rightarrow) in contact with the ductal lining in the nonperfused segment. *D*, duct lumen; *I*, preexisting islet. Counterstaining: methylgreen. (— = 50 μ m, original magnification $\times 240$)

Immunohistochemistry and histomorphometric analysis.

In this glucose-intolerant model, we observed insulin⁺ cells near the ducts in the alloxan-perfused segment without rhBTC treatment. After rhBTC treatment, such neogenesis could be detected in both the alloxan-perfused and nonperfused segments. As shown in Fig. 1, single β -cells and small islet-like cell clusters (ICCs), including β -cells, were observed arising from the ductal lining cells. Moreover, in the pancreases of sham-operated mice treated with rhBTC, newly formed ICCs, or several insulin⁺ cells closely associated with duct epithelium could be also recognized (Fig. 2*A, B*, and *C*), although we could not perform morphometric evaluation, insulin content, or IPGTT using enough animals in this group to be statistically significant. Immunostaining for insulin and double immunostaining for IPF1/PDX-1 and DCK revealed insulin⁺ cells and newly formed ICCs, including

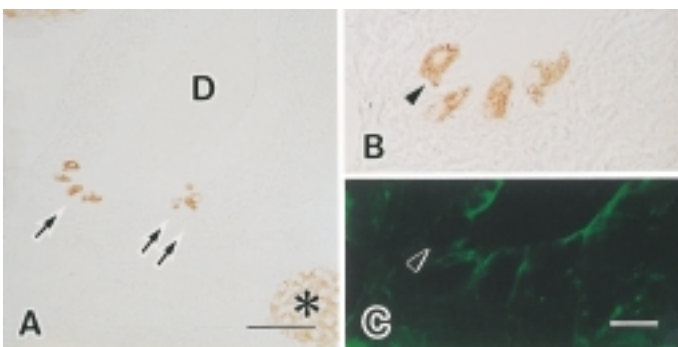


FIG. 2. Double immunostaining for insulin and duct cell-specific cytochrome in sham-operated, alloxan nonperfused mice treated with rhBTC for 8 weeks. Several insulin⁺ cells closely associated with ductal epithelium were also recognized (\rightarrow) (*A*). At higher magnification, a group of insulin⁺ cells, as indicated by the arrows, was localized among cytochrome⁺ duct cells (*B* and *C*). *D*, duct lumen; *preexisting islet. (*A*: — = 50 μ m, original magnification $\times 160$) (*B* and *C*: — = 20 μ m, $\times 260$)

β -cells, adjacent to DCK⁺ duct cells in the nonperfused segment (Fig. 3*A, B*, and *C*). IPF1/PDX-1 was positive in the ductal cells and the β -cells adjacent to the ducts (Fig. 3*B*). There were IPF1/PDX-1 and insulin double⁺ cells, as well as IPF1/PDX-1⁺ and insulin⁻ cells (Fig. 3*A* and *B*). Histomorphometric analysis demonstrated that the relative number of new ICCs in the alloxan-perfused segments was significantly increased compared with that in the control mice, and the relative number of ICCs was further increased by rhBTC treatment. However, there was no difference in the relative number of ICCs in the alloxan-perfused segment between the rhBTC-treated and untreated groups. On the other hand, the relative number of ICCs in the nonperfused segment was significantly increased in the mice with rhBTC treatment when compared with alloxan-perfused mice without rhBTC treatment and control nondiabetic mice (Fig. 4*A* and *B*).

Because BTC is a growth factor, we examined its effect on the mitotic activity of cells from the pancreas. At 8 weeks, BrdULI of DCK⁺ duct cells was significantly higher in the alloxan-perfused segment than in the saline-infused segment of the pancreas in control mice without rhBTC treatment (Fig. 5*A*). In this segment, BrdU incorporation was also higher after rhBTC treatment than in control mice, but the labeling index of the alloxan-perfused segment was not significantly altered by rhBTC treatment. In contrast, the BrdULI of ductal cells in the nonperfused segment of glucose-intolerant mice without rhBTC treatment was almost the same as in control mice, but the BrdULI of duct cells was significantly elevated to a similar level in the alloxan-perfused segment at 12 week after treatment.

The BrdULI of β -cells in both alloxan-perfused and nonperfused segments was not significantly different among the glucose-intolerant mice with or without rhBTC treatment and control mice (Fig. 5*B*). The BrdULI was not significantly

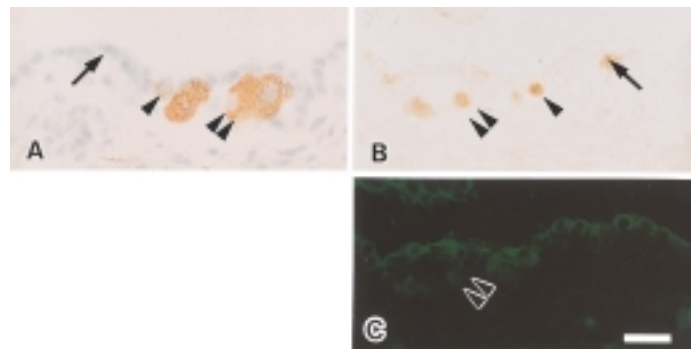


FIG. 3. Immunostaining for insulin and double immunostaining for IPF1/PDX-1 and duct cell-specific cytochrome in a set of mirror sections from the nonperfused pancreatic segment of a glucose-intolerant mouse at 8 week after the start of rhBTC treatment. In addition to ICCs containing insulin⁺ cells, cells with positive staining for insulin are closely associated with the ductal lining cells in *A*. In the replicate section to that shown in *A*, an IPF1/PDX-1⁺ cell was seen (\rightarrow in *B*), but it was negative for insulin (\rightarrow in *A*). A cell with both IPF1/PDX-1 and insulin was also observed near the ICCs (the cell indicated by \blacktriangleright in *A* and *B* is the same cell). Immunostaining for duct cell-specific cytochrome in the same replicate section (*C*) revealed that cells expressing insulin IPF1/PDX-1 and ICCs were localized in or directly associated with the ductal lining. Counterstaining: hematoxylin in section *A*, none in sections *B* and *C*. (— = 20 μ m, original magnification of *A, B*, and *C* $\times 380$)

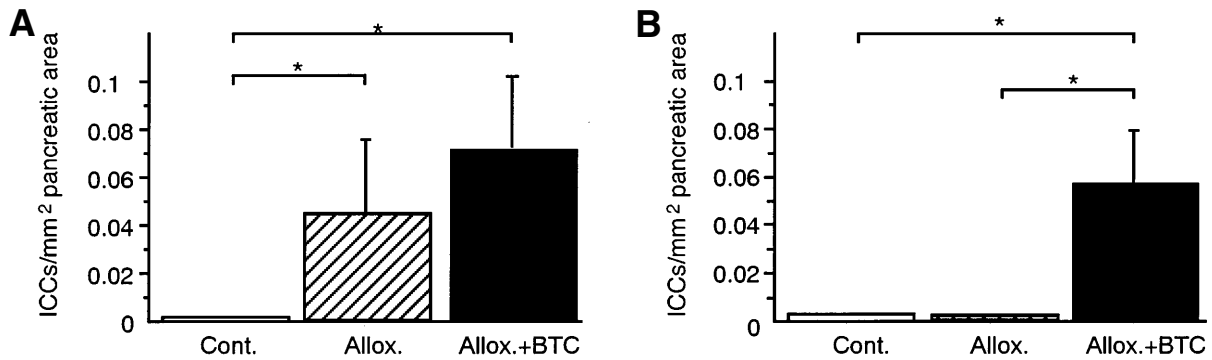


FIG. 4. Effect of rhBTC treatment on the relative number per square millimeter (pancreatic area) of ICCs in mice with diabetes induced by selective perfusion of alloxan at 8 weeks after the start of rhBTC injection; $n = 5$ for each group. **A:** The relative number of ICCs in the alloxan-perfused segments was significantly increased over that in control nondiabetic mice. In this segment, the relative number of ICCs in glucose-intolerant mice with and without rhBTC treatment was significantly increased over that in control nondiabetic mice, but there was no difference between alloxan-perfused glucose-intolerant mice treated with rhBTC and those without rhBTC ($*P < 0.05$). **B:** The relative number of ICCs in the nonperfused segments of glucose-intolerant mice with rhBTC treatment was significantly increased compared with the glucose-intolerant and control nondiabetic mice without rhBTC treatment ($*P < 0.05$).

different for amylase⁺ acinar cells in both segments irrespective of rhBTC-treatment (Fig. 5C).

We also immunohistochemically examined the EGF receptor family, which binds BTC and mediates its biological effects on the pancreas. The EGF receptor (EGFR/erbB-1) was positive in both islet cells and ductal epithelial cells, but it was undetectable level in acinar cells. In contrast, erbB-4 was only expressed in ductal epithelial cells of the interlob-

ular excretory ducts as well as the intralobular and intercalated ducts (Fig. 6A, B, C, and D). Thus, rhBTC could bind to both islet and ductal cells but may bind to more than one erbB receptor protein.

DISCUSSION

In this glucose-intolerant mouse model, we have previously reported the proliferation of residual β -cells in the nonper-

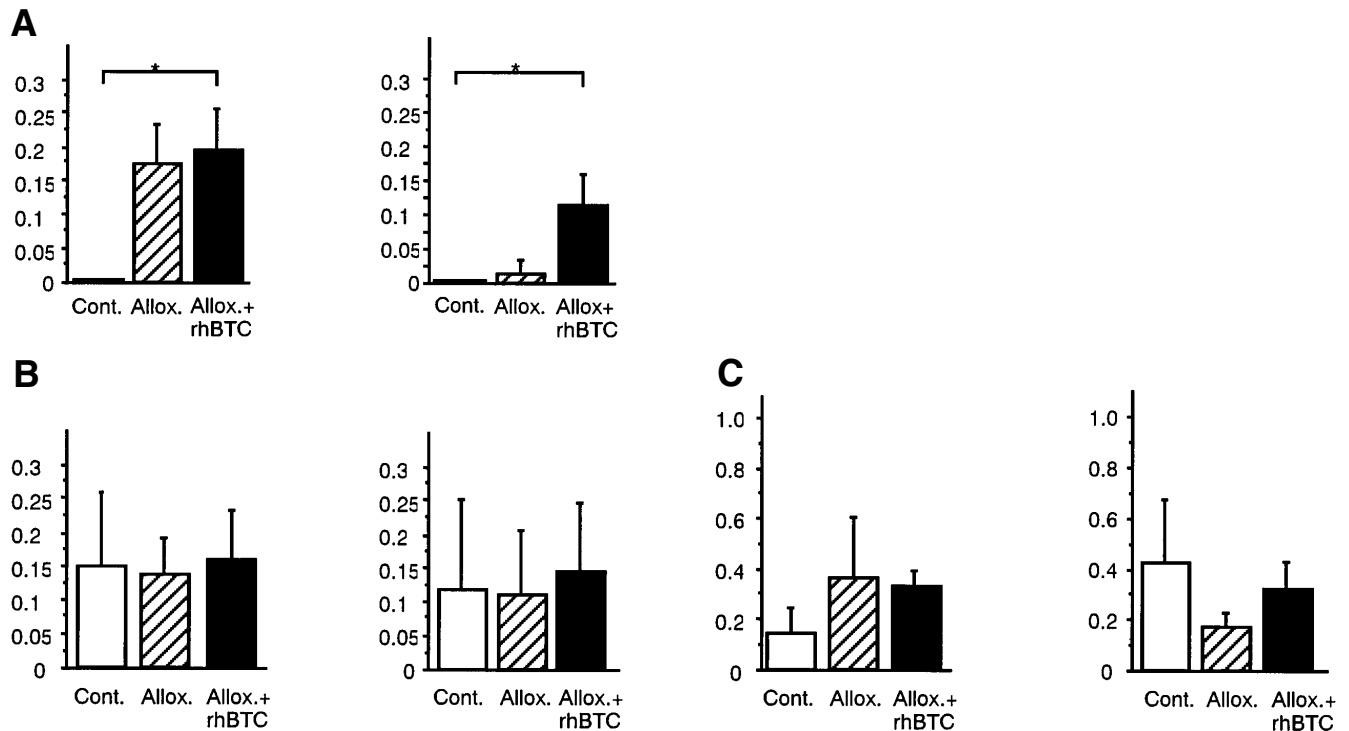


FIG. 5. BrdU LI of duct cells, β -cells, and acinar cells from glucose-intolerant mice with or without rhBTC treatment at 8 weeks after the start of rhBTC injection. The left graph of each panel indicates the data from the perfused segments, and the right graph of each panel indicates the data from the nonperfused segments. $n = 4$ for each group. **A:** In selectively alloxan-perfused glucose-intolerant mice without rhBTC treatment, the BrdU LI of duct cells was significantly higher in the perfused segment than in control nondiabetic mice. In glucose-intolerant mice treated with rhBTC, the BrdU LI was also higher compared to that in control nondiabetic mice and was similar to or higher than in alloxan-perfused glucose-intolerant mice without rhBTC treatment. In the nonperfused segment, the BrdU LI of duct cells from glucose-intolerant mice with rhBTC treatment was significantly elevated compared to that of glucose-intolerant mice without rhBTC treatment and control nondiabetic mice. **B and C:** The BrdU LI of β -cells and acinar cells in the perfused segment of glucose-intolerant mice was not significantly changed by rhBTC treatment (**B and C**), and also the BrdU LI of β -cells and acinar cells in the nonperfused segment was not altered significantly (**B and C**).

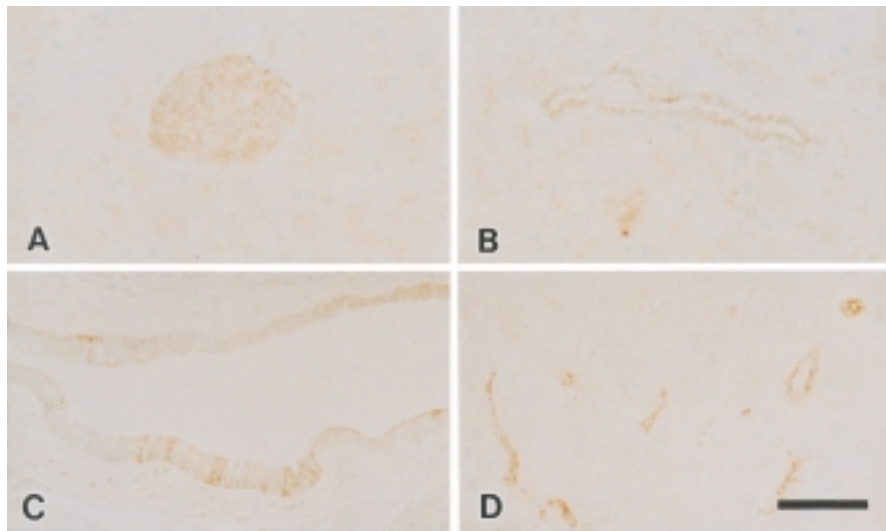


FIG. 6. Detection of EGFR and erbB-4, which can potentially bind with rhBTC, in pancreatic tissue from control nondiabetic mice. EGFR was mainly detected in islet cells (A) and ductal cells (B). On the other hand, erbB-4 was expressed in interlobular excretory duct cells (C) and in intralobular small duct cells or intercalated duct cells (D), but it was undetectable in both islet cells and acinar cells. (— = 50 μ m, original magnification $\times 240$)

fused segment and β -cell differentiation in the alloxan-perfused segment (1). It is thought that impaired glucose tolerance is gradually ameliorated by these changes. The present study demonstrated that rhBTC accelerated the improvement of glucose tolerance in these glucose-intolerant mice. In the nonperfused pancreatic segments, the BrdULI of ductal cells, but not β -cells or acinar cells, was elevated by treatment with rhBTC. Proliferation of ductal cells in the regenerating pancreas has been well documented to occur concomitantly with the process of β -cell neogenesis (3). These results indicate that subcutaneous rhBTC could stimulate the proliferation of ductal cells but not preexisting or newly formed β -cells. Previous *in vivo* studies have shown that nicotinamide and Reg protein can ameliorate glucose intolerance in surgical diabetes (19,20). These two factors are thought to stimulate pancreatic β -cell replication and may differ from BTC in their effect on β -cells.

Small ICCs were observed between ductal epithelial and acinar cells in both the alloxan-perfused and nonperfused segments of rhBTC-treated mice (1). The number of ICCs per square millimeter was increased in the nonperfused segment of rhBTC-treated mice when compared with the control and untreated mice. In the perfused segment, the number of ICCs per square millimeter was increased in both rhBTC-treated and untreated mice, but showed no significant difference between the two groups. In previous studies, we detected ICCs in the pancreases of glucose-intolerant mice without rhBTC treatment (1,21). However, ICCs in close contact with ductal epithelial cells were limited to the alloxan-perfused segment. In rhBTC-treated mice, however, ICCs were also found even in the normal pancreases and the nonperfused segments and were observed among the acinar cells. This might suggest that rhBTC treatment would be less effective in the nonperfused segment, but neogenesis of β -cells may still be potentiated by rhBTC. In the present study, rhBTC was also found to potentiate ductal cell proliferation. It is suggested that exogenous rhBTC promoted β -cell differentiation from nonendocrine precursor cells residing in the duct lining and probably also from

acinar or centroacinar cells, which led to the amelioration of glucose intolerance in this glucose-intolerant model.

The peak serum rhBTC level (~ 20 ng/ml) was the sufficient to induce the differentiation of AR42J cells into insulin-secreting cells *in vitro* (13). Possible receptors for BTC, EGFR, and erbB-4, were also detected on the duct cells of the pancreas (7–9). Because both EGFR and erbB4, to which rhBTC binds, were expressed in duct cells, it is considered that the serum concentration of rhBTC was high enough to induce a biological effect and that rhBTC may bind and activate EGFR and/or erbB-4 to stimulate β -cell neogenesis mainly among ductal or endocrine precursor cells residing in the duct lining.

In conclusion, rhBTC accelerated the improvement of glucose tolerance in our model, and this effect was considered to be the result of promoting β -cell differentiation and regeneration from ductal and/or acinar cells. Increasing the β -cell mass in the pancreas by the induction or promotion of neogenesis with a β -cell differentiation factor such as BTC may become a new therapeutic strategy for diabetic patients whose insulin secretion is severely impaired.

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