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Pancreatic beta-cell replication and amelioration of surgical diabetes by Reg protein

(*Reg* gene/diabetes mellitus/islets of Langerhans/growth factor)

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ABSTRACT **We previously isolated from a rat regenerating islet cDNA library a gene named *Reg*, which is expressed in regenerating islets but is not expressed in normal islets. Here we examined the effect of rat Reg protein on pancreatic beta-cell replication using both 90% depancreatized rats and isolated islets. The depancreatized rats that received i.p. administration of recombinant rat Reg protein (1 mg/kg per day) for 2 months showed amelioration of the surgical diabetes, as evidenced by a significant decrease in blood glucose with an increased beta-cell mass in the residual pancreas. In isolated rat islets, Reg protein (18–180 nM: 0.3–3 μg/ml) significantly increased [^{3}H]thymidine incorporation into the nuclei of beta cells. These results indicate that Reg protein is a growth factor for pancreatic beta cells and also suggest that the administration of Reg protein could be used as another therapeutic approach for diabetes mellitus.**

Pancreatic islets of Langerhans are the only organs of insulin production, but they have a limited capacity for regeneration, which predisposes to the development of diabetes mellitus. In 1984, we found that the administration of poly(ADP-ribose) synthetase inhibitors, such as nicotinamide, to 90% depancreatized rats induced the regeneration of pancreatic islets (1–3). In screening the regenerating islet-derived cDNA library, we identified a gene, *Reg* (i.e., regenerating gene), which is expressed in regenerating islets but is not expressed in normal islets (4). The rat Reg cDNA had a single open reading frame that encoded a 165-amino acid protein with a 21-amino acid signal peptide. We also isolated the human REG cDNA and gene, which encoded a 166-amino acid protein that showed a high degree of homology with the rat Reg protein (4–6). Although the Reg protein has been suggested to be involved in beta-cell regeneration or growth (4–11), no direct evidence for its role in stimulating the growth of pancreatic beta cells had yet been obtained. Here we demonstrate that recombinant rat Reg protein ameliorates the diabetes of 90% depancreatized rats with beta-cell proliferation and that stimulation by Reg protein causes DNA synthesis in islet beta cells in culture.

MATERIALS AND METHODS

Animal Experiment. Thirty-seven male Wistar rats (200–220 g of weight) were 90% depancreatized (1, 2, 4) and maintained on standard rat chow. Recombinant rat Reg protein, produced in *Saccharomyces cerevisiae*, purified (12) and dissolved at a concentration of 1 mg/ml in 50 mM acetic acid, was injected i.p. at a dose of 1 mg/kg of body weight into 20 rats every day. Seventeen control rats were injected i.p. with 50 mM of acetic acid without Reg protein after the 90% pancreatectomy. In both groups the injections were continued until the 60th postoperative day. Rats were fasted overnight before blood sampling, and the plasma glucose level was measured by the glucose oxidase method, using Glucose Auto and Stat GA-1110 (Kyoto Daiichi Instrument, Kyoto). The statistical significance of differences between rats injected with Reg protein (n = 20) and those injected with acetic acid (n = 17) was analyzed by using Student's t test. The residual pancreatic tissues were removed 2 months after the partial pancreatectomy and fixed in Bouins' solution. Hydrated 5-μm sections of paraffin-embedded pancreatic tissues were stained for insulin by the labeled streptavidin-biotin method, using an LSAB kit (Dakopatts, Glostrup, Denmark). After being stained, the relative volumes of beta cells were measured by the point-counting method (1, 13).

***In Vitro* Experiment.** Pancreatic islets were isolated from male Wistar rats (body weight 200–250 g) by using density separation on a dextran gradient and cultured free-floating in RPMI 1640 medium/10% fetal calf serum/penicillin G at 100 μg/ml/streptomycin at 100 μg/ml for 48 hr to allow recovery from the isolation procedure (14). After this initial period, islets were transferred to 24-well culture dishes in groups of 50 islets. The islets were cultured in RPMI 1640 medium/2.7 mM D-glucose/2% fetal calf serum/penicillin G at 100 μg/ml/streptomycin at 100 μg/ml in the presence of increased concentrations of Reg protein for 72 hr. During the last 24 hr, the islets were cultured in the above medium to which [*methyl*-^{3}H]thymidine at 10 μCi/ml (Amersham; 1 Ci = 37 GBq) had been added. To estimate the amount of [^{3}H]thymidine incorporated into newly synthesized DNA, the islets were washed as described (14) after the culture period and sonicated in 10 mM Tris·HCl/5 mM EDTA. DNA was precipitated by the addition of 7% ice-cold trichloroacetic acid and trapped by filtration on a glass-fiber disc (Whatman GF/C). The discs were dried, and radioactivity was counted after the addition of scintillation fluid (Packard Ultima Gold F). The DNA content of the islets was measured by a flurometric DNA assay using Hoechst 33258. To estimate labeling indices for insulin-positive cells, the [^{3}H]thymidine-labeled islets were fixed in 4% paraformaldehyde for 20 min and embedded in OCT compound. Ten-micrometer sections were cut with a cryostat and immunostained with anti-porcine insulin guinea pig antiserum (DAKO, 1:500) and Vectastain ABC-GO kit (Vector Laboratories). Autoradiography of the immunostained sections was performed by using Konica NR-M2 emulsion.

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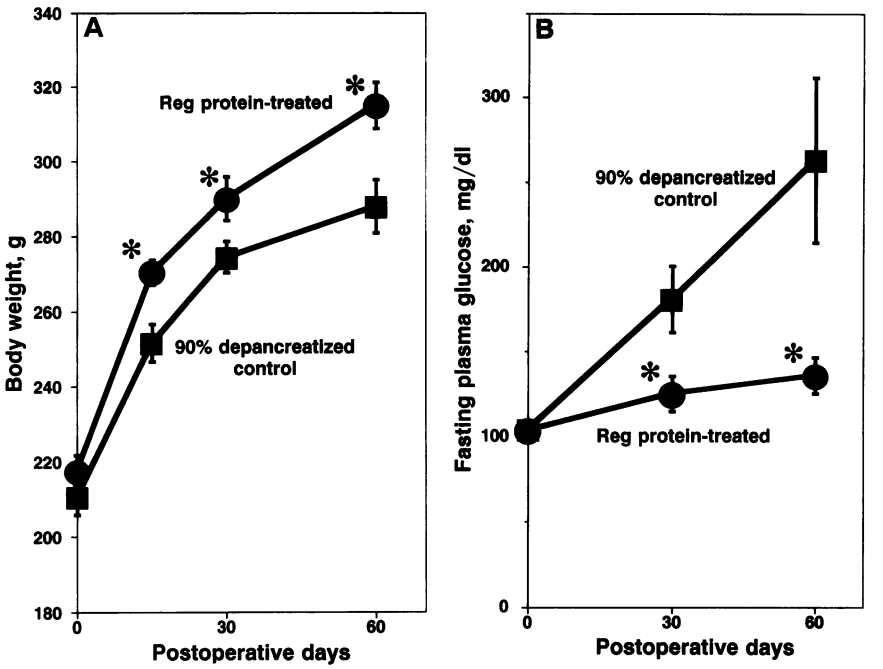


FIG. 1. The 90% depancreatized rats that received daily i.p. injections of recombinant Reg protein at 1 mg/kg of body weight showed an amelioration of the diabetes. Values represent means ± SEM of samples from control rats (n = 17) (■) and samples from Reg protein-treated rats (n = 20) (●). Results of Student's t test comparing values of Reg protein-treated rats as compared with the control rats. *, $P < 0.05$ versus control rats. (*A*) Changes of body weight. (*B*) Changes of fasting plasma glucose levels.

RESULTS AND DISCUSSION

In the present study, we injected 90% depancreatized rats i.p. with recombinant rat Reg protein (1 mg/kg per day) or the solvent (control rats). The food intake of neither group changed during the injections. On the 30th and 60th postoperative day, the fasting plasma glucose levels of the rats receiving Reg protein were significantly lower than those of 90% depancreatized control rats, and the rats treated with Reg protein also showed increased body weight (Fig. 1) with markedly decreased glucosurea. These results indicate that the administration of Reg protein ameliorates surgically induced diabetes.

We subsequently examined morphologically the remaining pancreas that had been stained for insulin. We found no tumors, including insulinomas, in any of the rats used in the experiments. As shown in Fig. 2*A*, normal islets of untreated, nondepancreatized rats are oval in shape, and the diameters range from 100 μm to 200 μm. After 2 months, almost all the islets of 90% depancreatized control rats were destroyed, and the insulin-positive cells were markedly decreased in number; fibrotic degeneration and degranulation were frequently

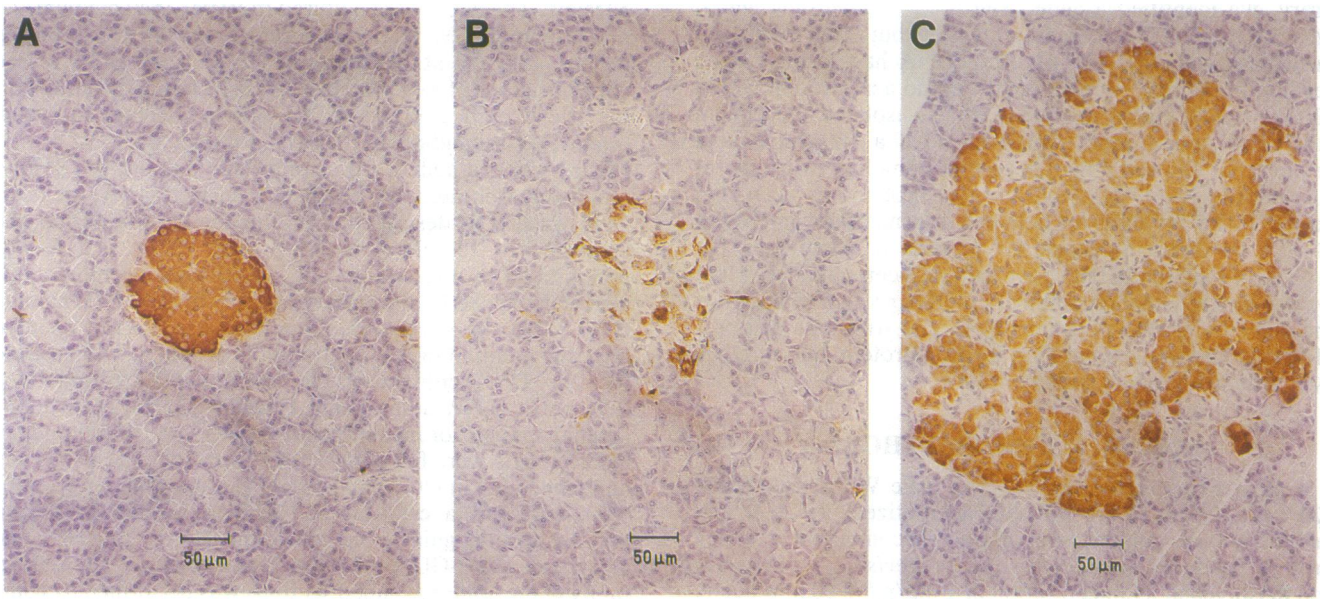


FIG. 2. Amelioration of diabetes coincided with beta-cell regeneration. (*A*) Islets from a normal untreated rat appear oval in shape, and the diameters ranged from 100 μm to 200 μm. (*B*) Islets from a control 90% depancreatized rat show severe degranulation of beta cells and fibrotic degeneration in the stroma. (*C*) In contrast, islets from a 90% depancreatized rat that received Reg-protein treatment show increases in diameter. The enlarged islets are densely and almost entirely stained for insulin. Pancreata were removed from the rats as indicated and fixed in Bouins' solution; 5-μm sections were then stained for insulin. Representative illustrations after treatment are shown.

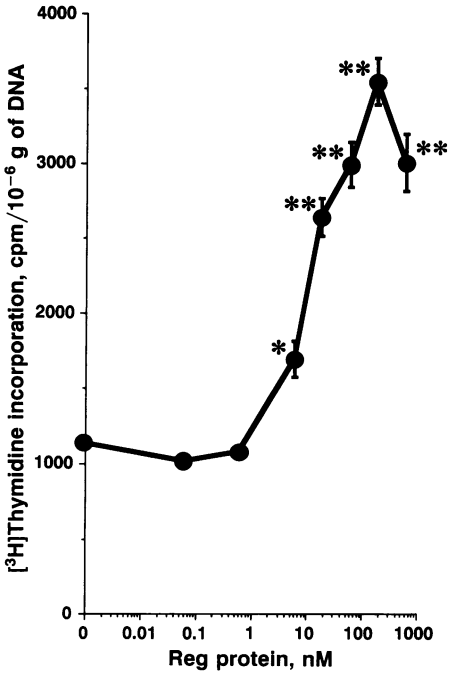
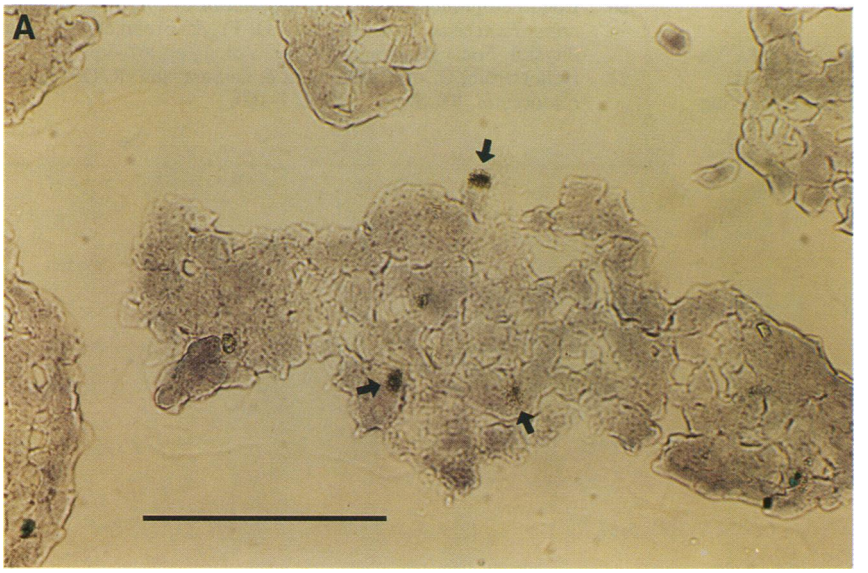


FIG. 3. Reg protein stimulates [^{3}H]thymidine incorporation into islet DNA. Recombinant rat Reg protein was dissolved in 50 mM acetic acid as solvent; the final concentration of the acetic acid in the culture was 0.55 mM. Acetic acid alone at the same final concentration did not affect [^{3}H]thymidine incorporation. Data are expressed as radioactivity of [^{3}H]thymidine incorporation per μg of islet DNA. Values represent means ± SEMs (n = 4). Asterisks indicate difference from control without Reg protein (*, $P < 0.05$; **, $P < 0.001$ in ANOVA).

encountered (Fig. 2*B*). In contrast, almost all the islets of the remaining pancreas in Reg protein-treated rats were enlarged (Fig. 2*C*), and the enlarged islets were densely and almost entirely stained for insulin, while the exocrine tissues of the remaining pancreas showed no significant histopathological changes (Fig. 2 *A–C*). The beta-cell mass in the remaining pancreas was ≈2.5-fold greater in the Reg protein-treated (1.17 ± 0.56 mm^3, n = 8) than in the normal untreated, nondepancreatized rats (0.47 ± 0.16 mm^3, n = 6). These results indicate that Reg protein stimulates the regeneration and/or growth of pancreatic beta cells, thereby ameliorating the diabetes.

We next examined the effect of Reg protein on [^{3}H]thymidine incorporation into beta cells by using isolated rat islets. As shown in Fig. 3, Reg protein stimulated [^{3}H]thymidine incorporation in a dose-dependent manner; there was a 2.8-fold increase in the presence of 180 nM (3 μg/ml) Reg protein. In the same conditions, bovine serum albumin (up to 15 μM) did not affect the incorporation (data not shown). As shown in Fig. 4*A*, almost all the autoradiographically labeled cells were insulin positive, and the grains were localized in the nuclei of beta cells. There was a 2.4-fold increase in the labeling index of beta cells (0.19 ± 0.02 versus 0.08 ± 0.02, $P < 0.05$) when islets were incubated with 60 nM (1 μg/ml) Reg protein (Fig. 4*B*). These results clearly indicate that Reg protein itself stimulates beta-cell replication in the pancreatic islets of Langerhans.

The present study demonstrated that Reg protein can ameliorate diabetes in partially depancreatized rats. Morphologic and immunocytochemical examination of the remaining pancreas of Reg protein-treated rats showed a marked increase in the beta-cell population. Therefore, it is reasonable to assume that Reg protein induces pancreatic beta-cell regeneration, thereby improving diabetes caused by partial pancreatectomy. The present study also demonstrated that Reg protein directly stimulated islet beta-cell replication *in vitro*. These results strongly suggest that Reg protein is a growth factor for pancreatic beta cells. This concept is reconcilable with the previous observations that the *Reg* gene was expressed in regenerating islets of poly(ADP-ribose) synthetase inhibitor-treated depancreatized rats and in hyperplastic islets of aurothioglucose-treated mice (4, 9). Furthermore, Reg protein, the product of the *Reg* gene, was



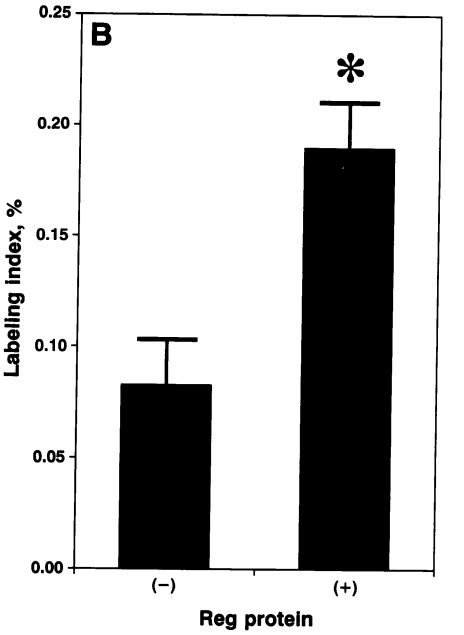


FIG. 4. Reg protein stimulates [^{3}H]thymidine incorporation in islet beta cells. (*A*) A section stained for insulin demonstrates that [^{3}H]thymidine-incorporated grains are localized in the beta-cell nuclei of islets. Insulin-positive cells are stained dark violet. Arrows indicate grains. (Bar = 100 μm.) (*B*) Labeling index of insulin-positive cells after the addition of 60 nM Reg protein. The labeling indices were estimated by counting a total of 19,000–71,000 insulin-positive cells in each well and determining the proportion that were labeled. Data are expressed as percentage of labeled insulin-positive cells. Values represent means ± SEMs (n = 3 wells). Asterisk indicates difference from control without Reg protein (*, $P < 0.05$ in Student's t test).

expressed in regenerating islets after partial pancreatectomy throughout several postoperative weeks (7), during which time the beta cells showed high [^{3}H]thymidine incorporation (2). This concept also fits the recent evidence of a close association between *Reg* gene expression and islet cell replication *in vitro* (14). In the light of the present results and the close association between *Reg* gene expression and regeneration or growth of islet beta cells *in vivo* and *in vitro* (1–4, 7–11, 14), Reg protein would appear to act on pancreatic beta cells as an autocrine growth factor.

Reg protein is also expressed in normal pancreatic acinar cells (5–9). Pancreatic stone protein (15, 16), isolated from human pancreatic stones and juice, and pancreatic thread protein (17), isolated from human pancreas, are just different names for the same protein derived from the *REG* gene (5, 9). Although pancreatic stone protein was reported to inhibit the formation and precipitation of crystals of $CaCO_3$ *in vitro* (18), the physiological significance of pancreatic stone protein in acinar cells has not yet been clarified. However, the expression of Reg mRNA in acinar cells was remarkably elevated during beta-cell regeneration induced by partial pancreatectomy (Y.S., M.U., H.Y., and H.O., unpublished results) and by removal of s.c. transplanted insulinomas (19). These findings suggest that the Reg protein expressed in the acinar cells can act on islets or their precursor cells in a paracrine manner. This paracrine action may be necessary to maintain beta-cell mass even in the normal pancreas.

A low capacity for beta-cell regeneration has been suggested as a predisposing factor for the development of human diabetes (20, 21). We have already isolated human REG cDNA (4), expressed it in yeast, and purified the human protein (12). As it is possible that this protein could be used as another treatment for human diabetes to stimulate the regeneration of pancreatic beta cells, more detailed studies concerning the effects of Reg protein on exocrine function, anabolic efficiency, food intake, and other mechanisms will surely soon follow.

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