explore the effect of ghrelin on the islet architecture and β cell mass. There were no obvious abnormalities in the intra islet cytoarchitecture and cell number of insulin, glucagon, somatostatin and PP cells in the islets of the RIP-G Tg (Fig.5A, B, C, D). The intensity of staining of these four islet hormones in the islets of the RIP-G Tg was not apparently different from those of non transgenic littermates. The ratio of the β cell area to whole pancreas was not changed significantly (Fig.5I). We also studied the tissue sections of RGP-G Tg and found no significant differences (Fig.5E, F, G, H, J).

Expression of insulin mRNA and insulin content. Since RIP-G Tg showed suppression of insulin secretion, we examined pancreatic mRNA expression and peptide content of insulin in RIP-G Tg and their nontransgenic littermates by Northern blot analysis and RIA. The insulin mRNA in RIP-G Tg did not differ from those of their nontransgenic littermates (Fig.6A, B). No significant differences of insulin contents were observed between RIP-G Tg and their nontransgenic littermates (Fig.6C).

PDX-1 and GLUT2 immunoreactivity. We examined the immunoreactivity of PDX-1, and GLUT2 in RIP-G Tg. The staining intensities of PDX-1 and GLUT2 in the RIP-G Tg (Fig.7A, C) were not apparently different from those in the nontransgenic littermates (Fig.7B, D).

Batch incubation of islets. The insulin secretion from isolated islet of RIP-G Tg by

batch incubation was indistinguishable from that of nontransgenic littermates, in 3.3 or 8.7 or 16.7 mM glucose conditions (Fig. 9).

Lipid metabolism. Plasma total cholesterol level of RIP-G Tg tended to be lower than those of nontransgenic littermates, but it did not reach statistical significance (total cholesterol;85.4±6.9 vs. 79.4±7.5 mg/dl, n=6, NS). Plasma triglyceride level of RIP-G Tg tended to be lower than that of nontransgenic littermates, but it did not reach statistical significance (154.5±11.0 vs. 136.9±10.3 mg/dl, n=6, NS). Free fatty acid level and HDL-cholesterol level of RIP-G Tg were not significantly different from those of non transgenic littermates free fatty acid; 0.44±0.05 vs. 0.48±0.07 mEq/L, n=6, NS, HDL-cholesterol; 46.1±2.3 vs. 44.9±3.4 mg/dl, n=6, NS).

Expression of GHS-R mRNA. To rule out possible down-regulation of GHS-R due to chronic exposure to high-level ghrelin, we measured the expression level of GHS-R mRNA in pancreas and pituitary by real time quantitative RT-PCR. There were no significant differences in GHS-R mRNA levels between RIP-G Tg and their nontransgenic littermates either in pancreas (Fig.8A) or in pituitary (Fig.8B).

Discussion

In wild-type mouse, no ghrelin-like immunoreactivity was detected in most of the islets. C-terminal ghrelin-like immunoreactivity was observed in the periphery of minor proportion of islets of wild type mice, which is consistent with the previous report (24). By the serial section analysis, most of the ghrelin producing cells also showed glucagon-like immunoreactivity. These findings indicate that ghrelin was expressed in minor proportion of mouse pancreatic alpha cells. Expression of ghrelin was not detected in pancreatic β cells of wild type mice.

In the present study we developed RIP-G Tg, in which pancreatic ghrelin concentration measured by C-RIA was approximately 1,000 times higher than that of nontransgenic littermates. By immunohistochemistry using anti-C-terminal ghrelin [13-28] antiserum we detected C-terminal ghrelin-like immunoreactivity in almost whole area of islets. Therefore, since ghrelin was not detected in β cells of control mice by immunohistochemistry, ghrelin transgene driven by RIP was considered to be expressed in β cells.

We also found about 3 times higher expression level of ghrelin mRNA in the brain of RIP-G Tg compared to that of nontransgenic littermates, which could not be detected by immunohistochemistry. Although small amount of ghrelin has been

reported to be expressed in brain, which can be detected by immunohistochemistry only after colchicine treatment (1), there have been controversies on whether this small amount of ghrelin in the brain has biological role. Since the food intake of RIP-G Tg was not different from that of nontransgenic littermates, the ghrelin produced by transgene in the brain seems not to show bioactive effect of n-octanoylated ghrelin.

By immunohistochemistry using anti-ghrelin [1-11] antiserum that recognizes the n-octanoylated portion of ghrelin, ghrelin-like immunoreactivity was also demonstrated in nearly whole area of islets of RIP-G Tg, indicating the production of n-octanoylated-ghrelin in β cells. This finding indicates that the mechanism of acylation may exist not only in pancreatic α cells but also in β cells. This is reasonable since α and β cells are pancreatic endocrine cells derived from common precursor cells (40). Since N-RIA/C-RIA ratio of the pancreatic tissue ghrelin concentration of RIP-G Tg was much lower than that of the stomach (0.0053 % vs. 11.67 %, $P<0.01$), the ability of acylation in β cell might be lower than that of in ghrelin-producing cell in the stomach (X/A-like cell). It is possible that exocrine pancreatic enzymes might interfere with the results although these were inactivated by boiling before extraction. The other possibility is that because of the formalin fixation of ghrelin in the tissue section the epitope recognized by immunohistochemistry using

anti-ghrelin [1-11] antiserum might not be the exactly same that recognized by N-RIA or ELISA. Since the amount of n-octanoylated-ghrelin was so little that it could not be detected by RIA if any, we may as well consider that the phenotype of these transgenic mice is due to the effect of des-acyl ghrelin. Des-acyl ghrelin has been shown not to activate GHS-R (39). There have been several reports saying that des-acyl ghrelin has biological activities, such as promoting adipogenesis (41), inhibition of cell proliferation, inhibition of apoptosis (42) and counteracting the effect of n-octanoylated ghrelin (35).

We showed here that the ghrelin level in portal vein is significantly higher than that in retroorbital vein in wild type mouse. Ghrelin has been reported to be mainly synthesized in stomach and intestine. The step-up of plasma ghrelin level in gastric vein has been reported previously (43), but there has been no report showing the step-up of plasma ghrelin level in portal vein as compared to that in systemic circulation. The present study is the first report of the step-up of plasma ghrelin levels in portal vein. Moreover, the step-up of des-acyl ghrelin in RIP-G Tg was much higher than that in control littermates, indicating overproduction of des-acyl ghrelin by transgene in the pancreas.

The body weight, %body fat and food consumption of RIP-G Tg were not

significantly different from those of nontransgenic littermates. Recently, we and Asakawa et al. have reported the studies of β-actin promoter ghrelin transgenic mouse (44,45), in which plasma des-acyl ghrelin levels were 30 and 50 times higher than those of their nontransgenic littermates. These transgenic mice were reported to show small phenotype, although some discrepancy of interpretation regarding on etiology exists. Asakawa et al. reported that the triglyceride level of β-actin promoter ghrelin transgenic mouse was lower, but that cholesterol level and free fatty acid level were not changed compared to their nontransgenic littermates. The triglyceride levels of our RIP-G Tg only showed lower tendency compared to that of nontransgenic littermates. The lack of small phenotype and milder phenotype of lipid metabolism in RIP-G Tg may result from the fact that plasma des-acyl ghrelin level of RIP-G Tg was only 3.4 times higher than those of nontransgenic littermates.

The tissue sections of the pancreas of these transgenic mice showed no apparent disarrangement in the islet architecture and in β cell mass. There have been several reports on the transgenic mice overexpressing humoral factors in the β cells, such as parathyroid hormone-related peptide, hepatocyte growth factor, and insulin-like growth factor-I (46-49). Some of these transgenic mice showed islet hypertrophy or disarrangement of the endocrine cells in the islet (46-49). Our observation showed

that des-acyl ghrelin might have no apparent effects on the islet architecture and β cell mass.

In the present study plasma insulin levels after the 3.0 g/kg glucose injection were significantly lower in RIP-G Tg than those in nontransgenic littermates although there was no significant difference in plasma insulin levels between RIP-G Tg and nontransgenic littermates on the fasting state.

To rule out the decreased production of insulin caused by exogenous insulin promoter, we measured insulin mRNA level and content in the pancreata of our transgenic mice. The insulin mRNA level and content from the transgenic mice were not significantly different from those from nontransgenic littermates. Therefore, the insulin production might not be disturbed in these mice either in transcriptional or translational levels. The immunoreactivity of PDX-1, which is master regulator of the pancreas development and essential for insulin transcription, in RIP-G Tg β cell was not different from that in nontransgenic littermates' β cell. These results suggest that the suppression of glucose-stimulated insulin secretion in RIP-G Tg might not be due to the transcriptional disregulation of insulin caused by injection of exogenous insulin promoter.

RIP-G Tg did not show decreased-insulin secretion in response to arginine.

Arginine is known to stimulate insulin secretion by the mechanism that are different from those used by glucose, although the detail remains controversial (50,51). However, it seems certain that arginine somehow evoked Ca^{2+} influx into the β cell and that leads to the exocytosis of insulin-containing vesicles (52,53). So at least, the decreased insulin secretion in RIP-G Tg might not be due to disorders in exocytosis process. Egido reported that ghrelin inhibits insulin secretion from rat pancreas in response to arginine in vitro (28), however, there has been no report on the effect of des-acyl ghrelin on arginine-induced insulin secretion.

The immunoreactivity of GLUT2, glucose transporter in the pancreatic β cell, in RIP-G Tg β cell was indistinguishable from that of in nontransgenic littermates' β cell. Although immunohistochemistry is not so suitable for quantitative analysis, at least no apparent decreased expression or disposition of GLUT2 in RIP-G Tg β cell exists. Chronic exposure to the high level of des-acyl ghrelin may not influence on GLUT2 expression.

We performed batch incubation study of RIP-G Tg islet. The insulin secretion from isolated islet of RIP-G Tg was indistinguishable from that of nontransgenic littermates. This finding indicates that insulin secretion was not affected by over expression of ghrelin transgene in vitro but was affected in vivo. The

difference of observations between in vitro and in vivo may be explained by dilution of ghrelin produced by transgene with incubation buffer. Alternatively, suppression of insulin secretion of RIP-G Tg was not due to the effect of des-acyl ghrelin on insulin secretion from β cell but on insulin sensitivity. Recently Gauna et al. reported that co-administration of des-acyl ghrelin and active ghrelin improves insulin sensitivity in humans (54), and that des-acyl ghrelin suppress glucose output from liver(55). Although insulin tolerance test did not show statistically significant difference in blood glucose levels between RIP-G Tg and their nontransgenic littermates, there were tendency that blood glucose levels of RIP-G Tg were lower. Moreover, plasma triglyceride levels of RIP-G Tg showed lower tendency. Taken together, these results may indicate that des-acyl ghrelin may improve insulin sensitivity of RIP-G Tg. The suppression of insulin secretion of RIP-G Tg is likely due to the effect of des-acyl ghrelin on insulin sensitivity.

To explore if chronic exposure to high-level des-acyl ghrelin may influence on the expression level of GHS-R, we investigated the mRNA level of GHS-R in the pancreas and pituitary of RIP-G Tg. No significant differences were found in GHS-R mRNA levels in pancreas or in pituitary between RIP-G Tg and their nontransgenic littermates. These finding indicates that chronic exposure to high-level des-acyl

ghrelin might not influence on GHS-R mRNA expression level.

We also developed RGP-G Tg. The pancreatic tissue ghrelin concentrations determined by C-RIA of RGP-G Tg were about 50 times higher than those of their nontransgenic littermates, indicating that ghrelin was overexpressed in RGP-G Tg. However, there was no obvious phenotype regarding insulin secretion and pancreatic morphology. Considering the observation that portal ghrelin levels were not elevated in RGP-G Tg compared to those in their nontransgenic littermates, the amount of secreted ghrelin from α cell may not outstrip the amount from stomach.

In summary we developed RIP-G Tg, in which pancreatic des-acyl ghrelin content was approximately 1,000 times higher than that in control littermates. We detected n-octanoylated-ghrelin-like immunoreactivity in pancreatic β cells by immunohistochemistry, indicating that the mechanism of acylation may exist not only in pancreatic α cells but also in β cells. The glucose-stimulated insulin secretion of RIP-G Tg was decreased. There was no abnormality about arginine-induced insulin secretion, pancreatic histology, pancreatic insulin mRNA levels and insulin content in the RIP-G Tg. Absence of insulin suppression in islet batch incubation study, lower tendency of blood glucose levels in ITT and lower tendency of plasma triglyceride level may indicate that the suppression of insulin secretion of RIP-G Tg is likely due to the

effect of des-acyl ghrelin on insulin sensitivity. Although we also developed RGP-G Tg with 50-fold increase of pancreatic des-acyl ghrelin content, we did not find obvious phenotype regarding insulin secretion and pancreatic morphology. The present study raises the possibility that des-acyl ghrelin may have influence on glucose metabolism.

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