at 340 and 380 nm, and fluorescence emitted at 510 nm was captured by a charge-coupled device (CCD) camera (Micro Max 5-MHz System; Roper Industries, Trenton, NJ). Fluorescence signals at 340-nm (F340) and 380-nm (F380) were detected every 20 s. Results are expressed as the ratios (F340/F380).

Measurement of insulin release from electrically permeabilized islets. Cultured islets were preincubated with KRBB with 2.8 mM glucose and 0.2% BSA for 30 min. The islets were washed twice in cold potassium aspartate buffer (KA buffer) containing 140 mM KA, 7 mM $MgSO_4$, 2.5 mM EGTA, 30 mM Hepes (pH 7.0), and 0.5% BSA, with $CaCl_2$ added to a Ca^{2+} concentration of 30 nM. The islets were then permeabilized by high voltage discharge (four exposures, each of 450 μs duration, to an electrical field of 4.0 kV/cm) in KA buffer and washed once with the same buffer. Groups of electrically permeabilized islets were then batch-incubated for 30 min at 37 °C in 0.4 ml KA buffer with various concentrations of Ca^{2+} and ATP. At the end of the incubation period, permeabilized islets were pelleted by centrifugation (15000*g*, 180 s), and aliquots of the buffer were sampled. The amount of immunoreactive insulin was determined by RIA as described above.

Statistical analysis. Results are expressed as means ± SE. Statistical significance was evaluated by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results and discussion

One intact allele in the pax6 gene is sufficient for maintenance of adult pancreatic islet architecture

In fetal pancreas in the homozygous state ($rSey^2/rSey^2$), insulin-positive cells are remarkably reduced and the alignment of the endocrine cells is not preserved (Fig. 2A). Especially, few or no glucagon-positive cells were found in the pancreas, and glucagon content could not be detected in RIA study of pancreas extract (data not shown). Recently, it has been reported that PAX6 is important especially for the endocrine cells to obtain final differentiation, rather than to proliferate [13]. Pax6 knockout mice have been shown to completely lack glucagon-producing cells in fetal pancreas [6]. In contrast, in the homozygous (Sey^{NEU}/Sey^{NEU}) mice, in which the PAX6 protein has a paired domain and homeodomain but lacks the transactivation domain (Fig. 1), the number of α-cells is reduced but is still present in the late fetal stage [9]. In the homozygous Sey mice [19] and $rSey^2$ rats (this study), in which the PAX6 protein has a paired domain but lacks a homeodomain and transactivation domain (Fig. 1), few or no glucagon-positive cells were detected in the later fetal stage in the homozygous state. These findings suggest that homeodomain is especially important in the formation of the pancreatic α-cells. In contrast to the homozygous fetal pancreatic islets, immunohistochemical evaluation of pancreata from adult heterozygote rats ($rSey^2/+$) revealed normal islet morphology with insulin-positive β-cells located in the center of the islet (Fig. 2B and C), and glucagon-positive α-cells (Fig. 2B and C), somatostatin-positive δ-cells (data not shown), and pancreatic polypeptide-positive PP-cells (data not shown) located in the periphery of the islets. Pancreatic insulin and glucagon contents in $rSey^2/+$ were the same as in wild-type (data not shown). Because of a recent report showing that expression of the glucose transporter GLUT2 was down-regulated in the pancreas of conditional inactivation of pax6 model mice [13], we examined GLUT2 expression in $rSey^2/+$ pancreatic islets. Adult $rSey^2/+$

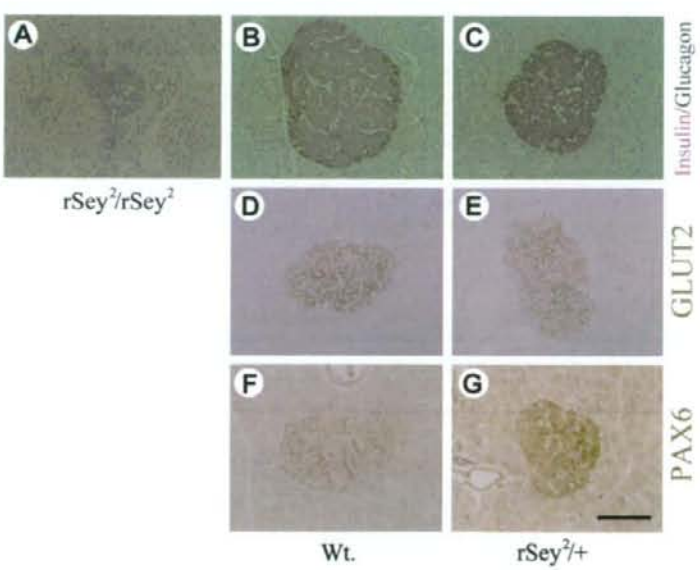

Fig. 2. One intact allele in the pax6 gene is sufficient for maintenance of adult pancreatic islet architecture. Immunohistochemical staining for islet protein was performed on paraffin-embedded sections of the pancreas. The sections were double-labeled for insulin (red) and glucagon (black) in the fetal (20.5E) homozygous state ($rSey^2/rSey^2$) (A) and adult wild-type (B), $rSey^2/+$ (C) rat pancreas or labeled for GLUT2 (D,E) and PAX6 (F,G) in the adult wild-type (D,F) and $rSey^2/+$ (E,G) rat pancreas. Bar 100 μm.

showed normal GLUT2 expression in the pancreatic endocrine cells (Fig. 2D and E). Thus, one allele of the wild-type pax6 gene is essential and sufficient to maintain morphologically normal pancreatic islets in adult. Anti-PAX6 antibody, which recognizes the C-terminus of PAX6 protein, was used to identify wild-type PAX6 protein derived from the wild-type pax6 allele, and rSey^2/+ was found to have similar PAX6 protein expression pattern in the nuclei of pancreatic islets (Fig. 2F and G).

Glucose-induced insulin secretion is preserved in heterozygous small eye rat strain (rSey^2/+)

Both male and female rSey^2/+ had similar body weight as wild-type rats (Fig. S1(A)). Pancreatic weight measured in males was similar in rSey^2/+ and wild-type (data not shown). Male rSey^2/+ showed normal fasting blood glucose levels, but had significantly higher fasting plasma insulin levels (Table 1). In the fed state, rSey^2/+ showed significantly lower blood glucose levels, and the plasma insulin level was similar in the two groups (Table 1). As these findings indicate relative hyperinsulinemia in rSey^2/+, we assessed the glucose-lowering effect of insulin by insulin tolerance test (ITT). rSey^2/+ and wild-type showed similar insulin sensitivity (Fig. S1(B)), indicating that the hyperinsulinemia is not derived from insulin resistance. In intraperitoneal glucose tolerance test (IPGTT), plasma glucose elevation elicited by glucose load in rSey^2/+ was similar to that of wild-type rats (Fig. S1(C)). Insulin secretion during IPGTT was also similar (Fig. S1(D)). Thus, it is possible that insulin secretion in response to secretagogues other than glucose is enhanced, resulting in hyperinsulinemia in rSey^2/+ rats. Glucagon is not a candidate as plasma glucagon levels were similar in the fasted and fed state (Table 1).

Insulin secretion induced by arginine is augmented in rSey^2/+ perfused pancreas

To determine which secretagogues contribute to the enhanced insulin release of rSey^2/+ in vivo, pancreatic perfusion was performed. rSey^2/+ rats showed the same biphasic insulin release from isolated perfused pancreas in response to stepwise increases in glucose concentration from 5.5 to 16.7 mM. However, insulin release in response to 10 mM arginine at the basal glucose level was significantly increased in rSey^2/+ rats (Fig. 3A). The integrated response to 10 mM arginine in the presence of 5.5 mM glucose was 1062 ± 285 ng of insulin in wild-type ($n = 5$) versus 2068 ± 131 ng of insulin in rSey^2/+ rats ($n = 5$) ($P < 0.05$) (Fig. 3B). On the other hand, rSey^2/+ rats showed similar glucagon release from the perfused pancreas in response to 10 mM arginine in the presence of 5.5 mM glucose (data not shown). This finding demonstrates that increased insulin response to 10 mM arginine in rSey^2/+ is not due to simultaneous enhancement of glucagon release.

Insulin secretory response to membrane-depolarizing stimuli in rSey^2/+ pancreatic islets

As one of the mechanisms by which arginine potentiates insulin release is direct depolarization of the pancreatic β-cell membrane, we examined insulin secretion evoked by membrane-depolarizing insulin secretagogues other than arginine. In batch incubation experiments, insulin release from isolated pancreatic islets in response to glucose stimulation was similar in rSey^2/+ (16.7 mM glucose: rSey^2/+ 0.82 ± 0.065 ng/islet/30 min ($n = 4$)) and wild-type rats (0.97 ± 0.087 ng/islet/30 min ($n = 4$)) ($P = 0.22$). However, insulin secretory responses to 10 mM arginine, 30 mM K^+, or 100 μM tolbutamide were significantly increased in rSey^2/+ pancreatic islets (10 mM arginine in the presence of 5.5 mM glucose: rSey^2/+ 0.55 ± 0.028 ng/islet/30 min ($n = 5$) vs. wild-type 0.21 ± 0.017 ng/islet/30 min ($n = 4$), $P < 0.001$; 30 mM K^+ in the presence of 2.8 mM glucose: rSey^2/+ 0.58 ± 0.035 ng/islet/30 min ($n = 5$) vs. wild-type 0.39 ± 0.040 ng/islet/30 min ($n = 4$), $P < 0.01$; 100 μM tolubutamide in the presence of 2.8 mM glucose: rSey^2/+ 0.45 ± 0.034 ng/islet/30 min ($n = 6$) vs. wild-type 0.31 ± 0.022 ng/islet/30 min ($n = 6$), $P < 0.01$) (Fig. 3C).

$[Ca^{2+}]_i$ elevation in pancreatic islets induced by 30 mM K^+-induced membrane depolarization in rSey^2/+ rats

To determine if the increase in K^+-induced insulin release in rSey^2/+ isolated islets is associated with increased intracellular Ca^{2+}, fura-PE3 was used to measure changes in $[Ca^{2+}]_i$. Five minutes before and 15 min after exposure to 30 mM K^+ in the presence of 2.8 mM glucose, $[Ca^{2+}]_i$ of rSey^2/+ islets was somewhat lower than that of wild-type islets (Fig. 4A and B). However, there was no difference between the depolarization-stimulated increment ratio in

Table 1
Blood glucose and plasma pancreatic hormone levels

	Blood glucose (mg/dl)		Plasma insulin (ng/ml)		Plasma glucagon (ng/ml)	
	Fed	Fasted	Fed	Fasted	Fed	Fasted
Wt	91 ± 2.1 ($n = 14$)	76 ± 1.0 ($n = 17$)	2.4 ± 0.36 ($n = 12$)	0.30 ± 0.09 ($n = 8$)	0.80 ± 0.03 ($n = 9$)	1.2 ± 0.44 ($n = 9$)
rSey^2/+	$80 \pm 1.8^{**}$ ($n = 11$)	72 ± 1.3 ($n = 15$)	1.9 ± 0.23 ($n = 11$)	$0.91 \pm 0.15^{*}$ ($n = 6$)	0.73 ± 0.05 ($n = 9$)	1.1 ± 0.22 ($n = 9$)

Values indicated as means ± SE.
* $P < 0.01$.
** $P < 0.001$ for rSey^2/+ vs. Wt.

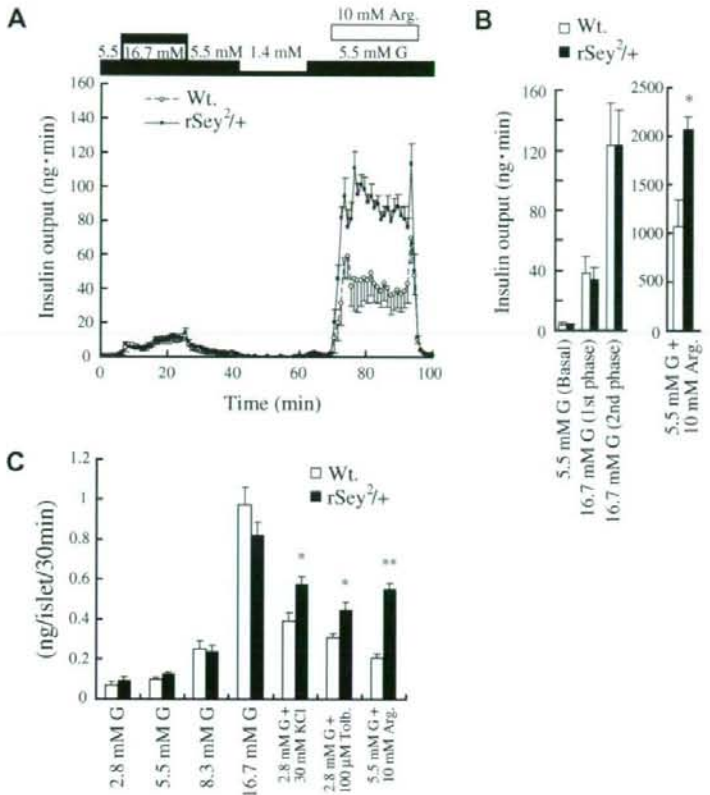

Fig. 3. (A,B) Insulin secretory responses from isolated perfused pancreas. Insulin release in response to glucose and arginine from the pancreas of rSey^2/+ (■) and wild type littermates (○). Values are expressed as mean ± SE. (B) AUC of insulin release at 5.5 mM glucose (basal level, 1–6 min), first (6–13 min) and second (13–26 min) phase insulin release at 16.7 mM glucose, and insulin release in response to 5.5 mM glucose and 10 mM arginine (70–96 min) from the perfused pancreas of wild type (open bars, $n = 5$) and rSey^2/+ (filled bars, $n = 5$). Values are expressed as mean ± SE. * $P < 0.05$, for rSey^2/+ vs. wild type. G: glucose, Arg.: arginine. (C) Depolarization-induced insulin release from isolated islets. Insulin release from batch-incubated islets of wild type (open bars) and rSey^2/+ (filled bars) was examined in response to the indicated concentrations of glucose with or without membrane depolarizing insulin secretagogues. Values are expressed as mean ± SE of 4–7 determinations from several experiments. * $P < 0.01$, ** $P < 0.001$ for rSey^2/+ vs. wild type. G: glucose, Tolb.: tolbutamide, Arg.: arginine.

rSey^2/+ islets and wild-type islets (rSey^2/+ 1.076 ± 0.0033 vs. wild-type 1.073 ± 0.0044, $P = 0.54$) (Fig. 4B).

Ca^{2+} efficacy in insulin release under low ATP condition in rSey^2/+ pancreatic islets

To determine if intracellular Ca^{2+} efficacy is altered in rSey^2/+ islets, we measured insulin release from pancreatic β-cell at $[Ca^{2+}]_i$ clamped by extracellular medium. As shown in Fig. 4C and D, raising the Ca^{2+} concentration from 30 nM to 10 μM elicited an increase in insulin release from electrically permeabilized islets. In the presence of 5 mM ATP, insulin release in rSey^2/+ was similar to wild-type at all Ca^{2+} concentrations (Fig. 4C). This result is commensurate with the findings that rSey^2/+ has a similar insulin secretory response to glucose as wild-type. However, in the presence of 1 mM ATP, insulin release in rSey^2/+ islets at Ca^{2+} concentrations from 30 to 1000 nM was greater than in wild-type islets (at 30 nM Ca^{2+}: rSey^2/+ 0.44 ± 0.031 vs. wild-type 0.30 ± 0.032 ($n = 8$), $P < 0.01$; at 100 nM Ca^{2+}: rSey^2/+ 0.44 ± 0.039 vs. wild-type 0.32 ± 0.035 ($n = 8$), $P < 0.05$; at 300 nM Ca^{2+}: rSey^2/+ 0.66 ± 0.040 vs. wild-type 0.45 ± 0.052 ($n = 7$), $P < 0.01$; at 1000 nM Ca^{2+}: rSey^2/+ 0.85 ± 0.033 vs. wild-type 0.54 ± 0.074 ng/islet/30 min ($n = 8$), $P < 0.01$) (Fig. 4D). This might well underlie the increased insulin secretion seen in rSey^2/+ β-cells in response to membrane depolarizing stimuli at the basal glucose level.

Thus, our findings show that pax6 gene mutation modifies the insulin secretory profile of adult pancreatic islets and that the disturbance in the insulin secretory mechanism in rSey^2/+ pancreatic islets is in the triggering of insulin granule exocytosis by the rise in $[Ca^{2+}]_i$, although the molecular mechanism remains to be determined.

In this study, rSey^2/+ islets showed increased insulin response to membrane-depolarizing stimuli such as

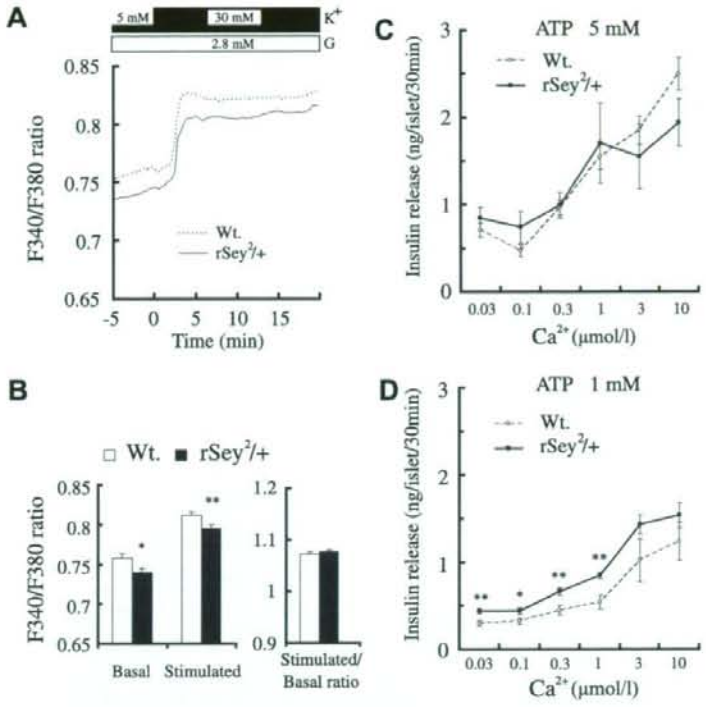

Fig. 4. (A,B) Fluorescence measurement of $[Ca^{2+}]_i$ elevation induced by 30 mM K^+-induced depolarization in the presence of 2.8 mM glucose. (A) Time course of $[Ca^{2+}]_i$ in perfused islets. Values represent mean of 83 (wild-type) and 74 (rSey^2/+) determinations from the several experiments. (B) Left, average values calculated from the data from (A). Basal, average values from −5 to 0 min in the presence of 5 mM K^+ with 2.8 mM glucose. Stimulated, average value from 0 to 15 min in the presence of 30 mM K^+ with 2.8 mM glucose. Right, values represent means ± SE of the ratio of stimulated value to basal value. $^*P < 0.05$, $^{**}P < 0.01$ for rSey^2/+ vs. wild-type. G, glucose. (C,D) Ca^{2+} dose–response of insulin release from electrically permeabilized islets. After preincubation with 2.8 mM glucose for 50 min, islets were electrically permeabilized and incubated with medium containing Ca^{2+} and ATP at the concentration indicated in the figure. Ca^{2+} dose-dependent insulin release from electrically permeabilized islets of wild-type (○) and rSey^2/+ (■) in the presence of 5 mM (C) or 1 mM (D) ATP. Values represent means ± SE of 7–8 determinations in the same experiment for each. $^*P < 0.05$, $^{**}P < 0.01$ for rSey^2/+ vs. wild-type.

arginine. This findings may underlie the relative hyperinsulinemia of rSey^2/+ rats in vivo. In contrast to rSey^2/+, it recently has been reported that rSey/+, another small eye rat strain (Fig. 1), in which the pax6 mutation is located in the transactivation domain [20], has impaired insulin response to glucose but show normal insulin secretory response to arginine [21]. These differences in pancreatic islet function suggest that pax6 plays a key role in regulating the insulin secretory response to various nutrients in pancreatic islets.

Morphological and functional analyses of rSey^2/+ pancreatic islets have important implications regarding gene dosage on pancreatic islet architecture and function. The maintenance of islet morphology in adult pancreas showed low sensitivity to quantity of pax6 gene. In contrast, islet function is necessarily more sensitive to alterations in pax6 gene. While the pax6 gene mutation in rSey^2/+ altered the insulin secretory profile, glucagon secretion was unaffected by the same mutation. These findings indicate that for displaying normal function, insulin-secreting β-cells among the pancreatic islet hormone-secreting cells require higher quantity of pax6 gene than glucagon-secreting α-cells. Outside of the pancreas, it is well known that pax6 is a key regulator of eye formation and that heterozygous pax6 mutations result in eye size reduction due to sensitivity to pax6 gene dosage for eye formation [22,23]. rSey^2/+ eye size also is reduced [14], indicating that the pancreas and the eye of same individual has differing sensitivity to the quantity of pax6 gene. Thus, there are differential dosage requirements for the pax6 gene between organs as well as between morphological and functional characteristics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.105.

References

[1] H. Edlund, Pancreatic organogenesis—developmental mechanisms and implications for therapy, Nat. Rev. Genet. 3 (2002) 524–532.
[2] M.E. Wilson, D. Scheel, M.S. German, Gene expression cascades in pancreatic development, Mech. Dev. 120 (2003) 65–80.
[3] A. Mansouri, M. Hallonet, P. Gruss, Pax genes and their roles in cell differentiation and development, Curr. Opin. Cell Biol. 8 (1996) 851–857.
[4] E. Dahl, H. Koseki, R. Balling, Pax genes and organogenesis, Bioessays 19 (1997) 755–765.
[5] B. Sosa-Pineda, K. Chowdhury, M. Torres, G. Oliver, P. Gruss, The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas, Nature 386 (1997) 399–402.
[6] L. St-Onge, B. Sosa-Pineda, K. Chowdhury, A. Mansouri, P. Gruss, Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas, Nature 387 (1997) 406–409.
[7] T.I. Simpson, D.J. Price, Pax6; a pleiotropic player in development, Bioessays 24 (2002) 1041–1051.
[8] C. Dohrmann, P. Gruss, L. Lemaire, Pax genes and the differentiation of hormone-producing endocrine cells in the pancreas, Mech. Dev. 92 (2000) 47–54.
[9] M. Sander, A. Neubuser, J. Kalamaras, H.C. Ee, G.R. Martin, M.S. German, Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development, Genes Dev. 11 (1997) 1662–1673.
[10] F.G. Andersen, J. Jensen, R.S. Heller, H.V. Petersen, L.I. Larsson, O.D. Madsen, P. Serup, Pax6 and Pdx1 form a functional complex on the rat somatostatin gene upstream enhancer, FEBS Lett. 445 (1999) 315–320.
[11] C.C. Martin, J.K. Oeser, R.M. O'Brien, Differential regulation of islet-specific glucose-6-phosphatase catalytic subunit-related protein gene transcription by Pax-6 and Pdx-1, J. Biol. Chem. 279 (2004) 34277–34289.
[12] S.E. Samaras, M.A. Cissell, K. Gerrish, C.V. Wright, M. Gannon, R. Stein, Conserved sequences in a tissue-specific regulatory region of the pdx-1 gene mediate transcription in Pancreatic beta cells: role for hepatocyte nuclear factor 3β and Pax6, Mol. Cell. Biol. 22 (2002) 4702–4713.
[13] R. Ashery-Padan, X. Zhou, T. Marquardt, P. Herrera, L. Toube, A. Berry, P. Gruss, Conditional inactivation of Pax6 in the pancreas causes early onset of diabetes, Dev. Biol. 269 (2004) 479–488.
[14] N. Osumi, A. Hirota, H. Ohuchi, M. Nakafuku, T. Iimura, S. Kuratani, M. Fujiwara, S. Noji, K. Eto, Pax-6 is involved in the specification of hindbrain motor neuron subtype, Development 124 (1997) 2961–2972.
[15] K. Tsuji, T. Taminato, M. Usami, H. Ishida, N. Kitano, H. Fukumoto, G. Koh, T. Kurose, Y. Yamada, H. Yano, Y. Seino, H. Imura, Characteristic features of insulin secretion in the streptozotocin-induced NIDDM rat model, Metabolism 37 (1988) 1040–1044.
[16] Y. Ihara, S. Toyokuni, K. Uchida, H. Odaka, T. Tanaka, H. Ikeda, H. Hiai, Y. Seino, Y. Yamada, Hyperglycemia causes oxidative stress in pancreatic β-cells of GK rats, a model of type 2 diabetes, Diabetes 48 (1999) 927–932.
[17] T. Kurose, Y. Seino, S. Nishi, K. Tsuji, T. Taminato, K. Tsuda, H. Imura, Mechanism of sympathetic neural regulation of insulin, somatostatin, and glucagon secretion, Am. J. Physiol. 258 (1990) E220–E227.
[18] S. Fujimoto, E. Mukai, Y. Hamamoto, T. Takeda, M. Takehiro, Y. Yamada, Y. Seino, Prior exposure to high glucose augments depolarization-induced insulin release by mitigating the decline of ATP level in rat islets, Endocrinology 143 (2002) 213–221.
[19] R.S. Heller, D.A. Stoffers, A. Liu, A. Schedl, E.B. Crenshaw 3rd, O.D. Madsen, P. Serup, The role of Brn4/Pou3f4 and Pax6 in forming the pancreatic glucagon cell identity, Dev. Biol. 268 (2004) 123–134.
[20] T. Matsuo, N. Osumi-Yamashita, S. Noji, H. Ohuchi, E. Koyama, F. Myokai, N. Matsuo, S. Taniguchi, H. Doi, S. Iseki, et al., A mutation in the Pax-6 gene in rat small eye is associated with impaired migration of midbrain crest cells, Nat. Genet. 3 (1993) 299–304.
[21] A. Kuroda, H. Kaneto, Y. Fujitani, H. Watada, Y. Nakatani, Y. Kajimoto, M. Matsuhisa, Y. Yamasakai, M. Fujiwara, Mutation of the Pax6 gene causes impaired glucose-stimulated insulin secretion, Diabetologia 47 (2004) 2039–2041.
[22] A. Schedl, A. Ross, M. Lee, D. Engelkamp, P. Rashbass, V. van Heyningen, N.D. Hastie, Influence of PAX6 gene dosage on development: overexpression causes severe eye abnormalities, Cell 86 (1996) 71–82.
[23] C.D. van Raamsdonk, S.M. Tilghman, Dosage requirement and allelic expression of PAX6 during lens placode formation, Development 127 (2000) 5439–5448.

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Curcumin inhibits glucose production in isolated mice hepatocytes

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ABSTRACT

Curcumin is a compound derived from the spice turmeric, and is a potent anti-oxidant, anti-carcinogenic, and anti-hepatotoxic agent. We have investigated the acute effects of curcumin on hepatic glucose production. Gluconeogenesis and glycogenolysis in isolated hepatocytes, and gluconeogenetic enzyme activity after 120 min exposure to curcumin were measured. Hepatic gluconeogenesis from 1 mM pyruvate was inhibited in a concentration-dependent manner, with a maximal decrease of 45% at the concentration of 25 μM. After 120 min exposure to 25 μM curcumin, hepatic gluconeogenesis from 2 mM dihydroxyacetone phosphate and hepatic glycogenolysis were inhibited by 35% and 20%, respectively. Insulin also inhibited hepatic gluconeogenesis from 1 mM pyruvate and inhibited hepatic glycogenolysis in a concentration-dependent manner. Curcumin (25 μM) showed an additive inhibitory effect with insulin on both hepatic gluconeogenesis and glycogenolysis, indicating that curcumin inhibits hepatic glucose production in an insulin-independent manner. After 120 min exposure to 25 μM curcumin, hepatic glucose-6-phosphatase (G6Pase) activity and phosphoenolpyruvate carboxykinase (PEPCK) activity both were inhibited by 30%, but fructose-1,6-bisphosphatase (FBPase) was not reduced. After 120 min exposure to 25 μM curcumin, phosphorylation of AMP kinase α-Thr^{172} was increased. Thus, the anti-diabetic effects of curcumin are partly due to a reduction in hepatic glucose production caused by activation of AMP kinase and inhibition of G6Pase activity and PEPCK activity.

1. Introduction

Curcumin is the major yellow pigment extracted from turmeric, the powdered rhizome of the herb *curcuma longa*. Turmeric is a spice used extensively in curries and mustards as a coloring and flavoring agent. Curcumin is reported to have a wide range of effects: it is anti-inflammatory [1], anti-oxidant [2,3] anti-hepatotoxic [4], and hypocholesterolemic [5,6]. Curcumin also is reported to have a beneficial effect on blood glucose in diabetic rats [7,8]. However, while elevated hepatic glucose production is

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Abbreviations: DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphophatase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; AMP kinase, adenosine monophosphate activated protein kinase.

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found frequently in type 2 diabetes, it is not known whether curcumin affects glucose metabolism in the liver. In the present study, we demonstrate that curcumin suppresses hepatic glucose production in an insulin-independent manner in isolated hepatocytes. We also investigated the inhibitory effect of curcumin on the activity of gluconeogenetic enzymes in isolated hepatocytes. Our results show that curcumin activates AMP kinase and suppresses both hepatic glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), thus inhibiting hepatic glucose output.

2. Materials and methods

2.1. Animals

C57/BL6J mice were purchased from Shimizu (Kyoto, Japan). The mice were allowed access to food, standard rat chow (Oriental Yeast, Osaka, Japan), and water *ad lib*. The mice were housed in an air-controlled (temperature 25 ± 2 °C and 50% humidity) room with a 12 h light/dark-cycle. For gluconeogenesis measurements, the mice were fasted 24 h with free access to water before the experiment. For glycogenolysis measurements, the mice were allowed access to food and water *ad lib* before the experiment.

2.2. Hepatocyte preparation

Liver of 10-week-old mice was perfused through the inferior *vena cava* with a buffer consisting of 140 mM NaCl, 2.6 mM KCl, 0.28 mM Na_2HPO_4, 5 mM glucose, and 10 mM Hepes (pH 7.4) after pentobarbital sodium anesthesia as described previously in Refs. [9,10]. The perfusion was first for 5 min with the buffer supplemented with 0.1 mM EGTA and then for 15 min with the buffer containing 5 mM $CaCl_2$ and 0.2 mg/ml collagenase type 2 (Worthington, Lakewood, NJ). All of the solutions were prewarmed at 37 °C and gassed with a mixture of 95% O_2/5%CO_2, resulting in pH 7.4. The isolated hepatocytes were filtered with nylon mesh (0.75 mm in diameter) and washed twice with the buffer above without collagenase, and suspended in a small volume of DMEM (GIBCO, Rockville, MD) without glucose or pyruvate, and counted. The viability of hepatocytes was evaluated by trypan blue staining. Samples with viability of less than 90% were discarded.

2.3. Hepatic glucose production

For gluconeogenesis measurements, hepatocytes (7.5×10^5) were incubated at 37 °C in a humidified atmosphere (5% CO_2) in 0.5 ml of DMEM without glucose but containing 1 mM pyruvate or 2 mM dihydroxyacetone phosphate (DHAP), 0.24 mM 3-isobutyl-1-methylxanthine in the presence or absence of curcumin or insulin. For glycogenolysis measurements, hepatocytes (7.5×10^5) were incubated at 37 °C in a humidified atmosphere (5% CO_2) in 0.5 ml of DMEM without glucose or pyruvate but containing 0.24 mM 3-isobutyl-1-methylxanthine in the presence or absence of curcumin or insulin. Curcumin was dissolved in DMSO to a concentration in the medium that did not interfere with cell viability (maximally 0.1%, v/v). Incubation was stopped by placing the cells on ice, followed by centrifugation at 4 °C for 60 s at 600 × *g*. The sampling was done at 0, 30, 60, and 120 min. The supernatant was removed, the cells were lysed in 0.1% of SDS in phosphate buffered saline, and the protein content was determined (BCA kit, Pierce). The glucose content of the supernatant was measured by glucose oxidation method (100 Trinder kit, Sigma). The dose–response of curcumin in gluconeogenesis and glycogenolysis were obtained at the incubation time of 120 min.

2.4. DNA synthesis measurement

DNA synthesis of hepatocytes was determined as the uptake of 5-bromo-2′-deoxyuridine (BrdU) according to the instruction manual (Cell Proliferation ELISA, BrdU (colorimetric), Roche Diagnostics, Manheim, Germany). After a 24-h preincubation of isolated hepatocytes with curcumin (25 μM) or vehicle in DMEM without glucose but with 10% fetal calf serum, hepatocytes were incubated for an additional 2 h with BrdU. The hepatocytes were fixed, and BrdU incorporation into DNA in hepatocytes was detected by ELISA. The results of incorporation of BrdU were expressed as photo-absorbance (wavelength 370–492 nm).

2.5. Enzyme activities

Hepatocytes were incubated at 37 °C in a humidified atmosphere (5% CO_2) in DMEM without glucose but containing 1 mM pyruvate and 0.24 mM 3-isobutyl-1-methylxanthine in the presence of 25 μM curcumin or vehicle (DMSO) for 120 min. Incubation was stopped by placing the cells on ice followed by centrifugation at 4 °C for 60 s at 600 × *g*. The supernatant was removed, and the cells were homogenized using a glass/Teflon homogenizer. In the microsomal preparation for the G6Pase assay, 50 mM Tris–HCl, pH 7.5, containing 250 mM sucrose, and 0.2 mM EDTA, was used as the homogenizing buffer [11]. For assay of G6Pase, liver microsomal fraction was prepared as follows: homogenate obtained as above was centrifuged at 20,000 × *g* for 20 min at 4 °C, and was then ultracentrifuged at 105,000 × *g* for 1 h at 4 °C. The resulting sediments were used for G6Pase assay [11]. The G6Pase activity was measured with intact microsomal preparation. Activity of G6Pase was determined as described by Passonneau and Lowry [12].

For liver PEPCK and fructose-1,6-bisphophatase (FBPase) assays, the homogenizing buffer contained 0.1 M Tris–HCl, pH 7.5, 0.15 M KCl, 5 mM EDTA, 5 mM dithiothreitol, and 5 mM $MgSO_4$ [11]. The homogenate was centrifuged at 105,000 × *g* for 1 h at 4 °C, and the supernatant was collected. Activity of FBPase was determined as described by Passonneau and Lowry [12].

Activity of PEPCK was determined as described by Nakagawa and Nagai [13]. All enzyme activity was measured photometrically using BIO-RAD Benchmark Plus.

Enzyme activities are expressed as the number of substrate molecules converted by 1 mg cytosolic or microsomal protein per minute. The liver microsomal fraction was solubilized by addition of 0.1% SDS before protein determination.

2.6. Immunoblotting analysis

Hepatocytes were incubated at 37 °C in a humidified atmosphere (5% CO_2) in 10 ml of DMEM without glucose but

containing 1 mM pyruvate and 0.24 mM 3-isobutyl-1-methylxanthine in the presence of 25 μM curcumin or vehicle (DMSO) for 120 min. Incubation was stopped by placing the cells on ice followed by centrifugation at 4 °C for 60 s at 600 × *g*. The supernatant was removed, and the cells were homogenized in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonylfluoride, 0.2 mM sodium vanadate, 250 mM mannitol, 1% Triton X-100, and 5 μg/ml soybean trypsin inhibitor). The cell lysates were sonicated twice for 10 s and centrifuged at 13,000 × *g* for 5 min. The pellets were discarded, and supernatants were assayed for protein concentration. Equal amounts of proteins (50 μg) were subjected to SDS-polyacrylamide (8%) gel electrophoresis and transferred onto nitrocellulose membranes (PROTRAN, Schleicher & Schuell) by electroblotting. After pre-incubation with blocking buffer (PBS containing 0.1% Tween 20 and 5% nonfat dry milk) for 2 h at room temperature, blotted membranes were incubated with each primary antibody (phospho-AMP kinase α-Thr^{172} antibody or AMP kinase α antibody, Cell Signaling Technology, Danvers, MA) overnight at 4 °C, followed by washing twice with blocking buffer. Membranes were then incubated with a horseradish peroxidase-linked anti-rabbit IgG (Amersham) for 1 h at room temperature, washed twice in PBS containing 0.04% Tween 20, and visualized by ECL Western blotting detection reagents (Amersham). Densitometry was carried out to measure band intensities and phosphorylated AMP kinase α-Thr^{172} was normalized by the levels of AMP kinase α protein.

2.7. *Materials*

Curcumin was purchased from Wako Chemicals (Osaka, Japan). Standard rat chow was from Oriental Yeast (Osaka, Japan). Human insulin was from Novo-Nordisk (Copenhagen, Denmark). All other chemicals were of reagent grade.

2.8. *Statistical analysis*

Results are mean ± S.E.M. (*n* = number of animals). Statistical significance was evaluated using two-tailed Student's *t*-tests. Differences among groups were also statistically examined by one-way ANOVA (Fisher's PLSD test). $P < 0.05$ was considered significant.

2.9. *Ethical considerations*

All studies were performed in the laboratories of the Department of Diabetes and Clinical Nutrition, Kyoto University, in accordance with the Declaration of Helsinki.

3. Results

3.1. *Effect of curcumin on hepatic gluconeogenesis in freshly isolated hepatocytes*

Fig. 1A shows the time course of inhibition by curcumin of hepatic gluconeogenesis from pyruvate. After 30, 60, and 120 min exposure to 25 μM curcumin, hepatic gluconeogenesis was significantly inhibited by approximately 45%, 40%, and 45%, respectively. The viability of the hepatocytes was not affected by 120 min exposure to 25 μM curcumin (control: 78 ± 1% vs. curcumin: 79 ± 2%). Fig. 1B shows the time course of inhibition by curcumin of hepatic gluconeogenesis from dihydroxyacetone phosphate. After 120 min exposure to 25 μM curcumin, hepatic gluconeogenesis was significantly inhibited by approximately 35%.

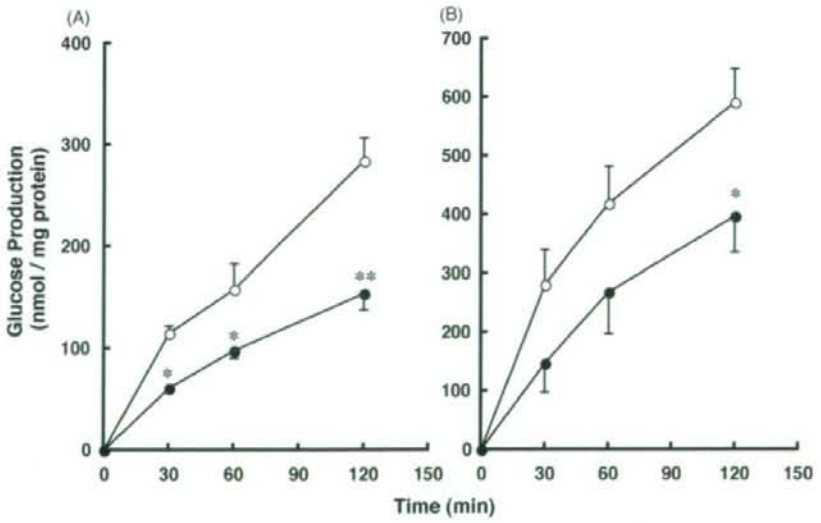

Fig. 1 – Time course of inhibition in hepatic gluconeogenesis from 1 mM pyruvate (A) and 2 mM DHAP (B). Isolated hepatocytes from fasted mice were incubated in the presence of 25 μM curcumin or vehicle for 2 h. Glucose content in supernatant was measured by glucose oxidation method. Each point shows mean ± S.E.M. (*n* = 6). *$P < 0.05$, **$P < 0.01$ compared with control by unpaired Student's *t*-test. Control (○), curcumin (●).

As shown in Fig. 2, curcumin inhibited hepatic gluconeogenesis from pyruvate at the incubation time of 120 min in a concentration-dependent manner.

As shown in Fig. 3, after 120 min exposure to insulin, hepatic gluconeogenesis from pyruvate was inhibited in a concentration-dependent manner. After 120 min exposure to various concentrations of insulin (0.1, 1, and 10 nM) in the presence of 25 μM curcumin, hepatic gluconeogenesis from pyruvate was further inhibited by approximately 45% when compared to that in the absence of 25 μM curcumin.

3.2. *Effect of curcumin on DNA synthesis in isolated hepatocytes*

To determine whether curcumin is toxic to hepatocytes, we examined the effect of curcumin on DNA synthesis in isolated hepatocytes. After 24 h exposure to 25 μM curcumin, BrdU incorporation into DNA in isolated hepatocytes was not decreased compared to control, indicating no suppressive effects of curcumin on DNA synthesis (Fig. 4).

3.3. *Effect of curcumin on hepatic glycogenolysis in freshly isolated hepatocytes*

Fig. 5A shows the time course of inhibition by curcumin of hepatic glucose production from glycogenolysis. After 60 and 120 min exposure to 25 μM curcumin, hepatic glycogenolysis was significantly inhibited by approximately 10% and 20%, respectively. As shown in Fig. 5B, curcumin inhibited hepatic glycogenolysis at the incubation time of 120 min in a concentration-dependent manner. As shown in Fig. 6, after

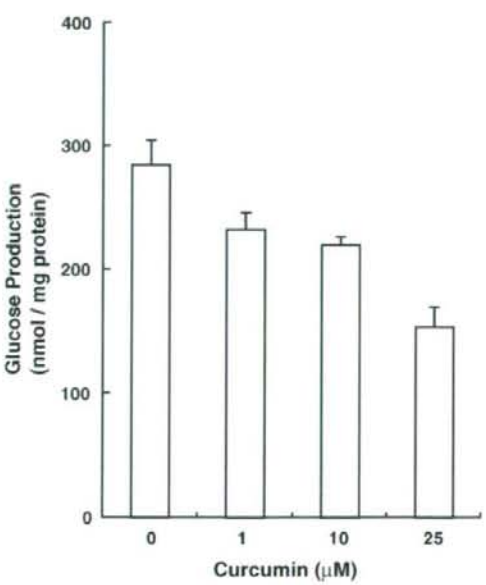

Fig. 2 – Concentration-dependence of inhibition in gluconeogenesis from 1 mM pyruvate by curcumin at the incubation time of 120 min in isolated mice hepatocytes. Each point shows mean ± S.E.M. ($n = 6$). $P < 0.001$ by one-way ANOVA.

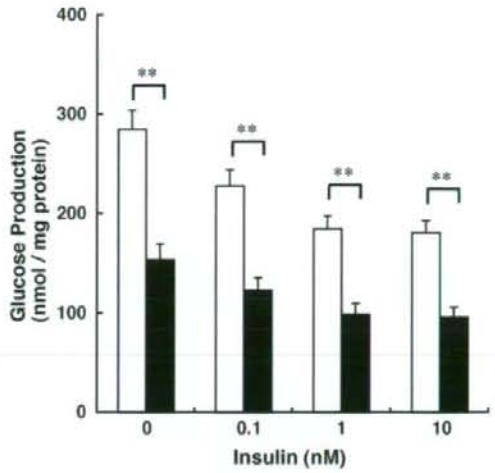

Fig. 3 – Concentration-dependence of inhibition in gluconeogenesis from 1 mM pyruvate by insulin in the presence and absence of 25 μM curcumin in isolated mice hepatocytes. Each point shows mean ± S.E.M. ($n = 6$). $P < 0.001$ by one-way ANOVA. "$P < 0.01$ compared with insulin alone by unpaired Student's t-test. Insulin alone (open bar), insulin plus curcumin (closed bar).

120 min exposure to insulin, hepatic glycogenolysis was inhibited in a concentration-dependent manner. After 120 min exposure to various concentrations of insulin (0.1, 1, and 10 nM) in the presence of 25 μM curcumin, hepatic glycogenolysis was further inhibited by approximately 20% compared to that in the absence of 25 μM curcumin.

3.4. *Effect of curcumin on activities of hepatic gluconeogenetic enzymes*

To further investigate inhibition of hepatic glucose production by curcumin, we measured the activities of key gluconeogenetic enzymes, G6Pase, FBPase, and PEPCK. After 120 min exposure to 25 μM curcumin, hepatic G6Pase activity and PEPCK activity were significantly inhibited by approximately 30%, but FBPase was not inhibited (Fig. 7).

3.5. *Effect of curcumin on phosphorylation of AMP kinase*

AMP kinase activation was monitored in Western blots by staining with a specific antibody against phosphorylated Thr^{172} of AMP kinase α, which is essential for AMP kinase activity. After 120 min exposure to 25 μM curcumin, phosphorylation of AMP kinase α-Thr^{172} was significantly increased by 70% when normalized by total content of AMP kinase α, clearly indicating curcumin activation of AMP kinase (Fig. 8).

4. Discussion

This is the first study to show that curcumin reduces hepatic glucose production. Our results demonstrate that curcumin

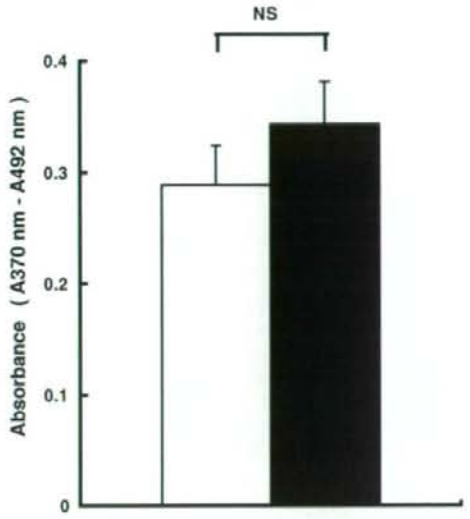

Fig. 4 – The effect of curcumin on DNA synthesis in isolated hepatocytes. After 24-h pre-incubation of isolated mice hepatocytes with 25 μM curcumin or vehicle in DMEM without glucose but with 10% fetal calf serum, hepatocytes were incubated for an additional 2 h with BrdU. The results of incorporation of BrdU were expressed as photo-absorbance (wavelength 370–492 nm). Control (open bar), curcumin (closed bar).

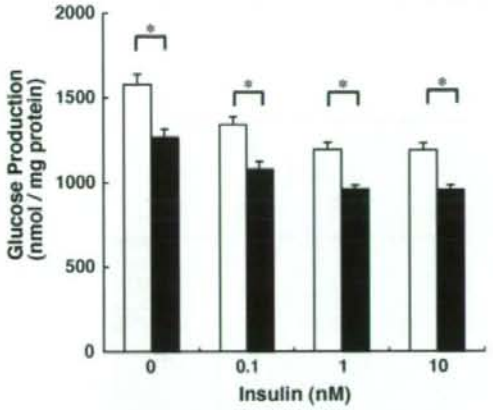

Fig. 6 – Concentration-dependence of inhibition in glycogenolysis by insulin in the presence and absence of 25 μM curcumin at the incubation time of 120 min in isolated mice hepatocytes. Each point shows mean ± S.E.M. ($n = 6$). $P < 0.001$ by one-way ANOVA. *$P < 0.05$ compared with insulin alone by unpaired Student's t-test. Insulin alone (open bar), insulin plus curcumin (closed bar).

inhibits both hepatic gluconeogenesis and glycogenolysis by suppressing both G6Pase activity and PEPCK activity. As curcumin had no suppressive effect on DNA synthesis in isolated hepatocytes, the inhibition of hepatic glucose production should not be a toxic effect. Indeed, Shen et al.

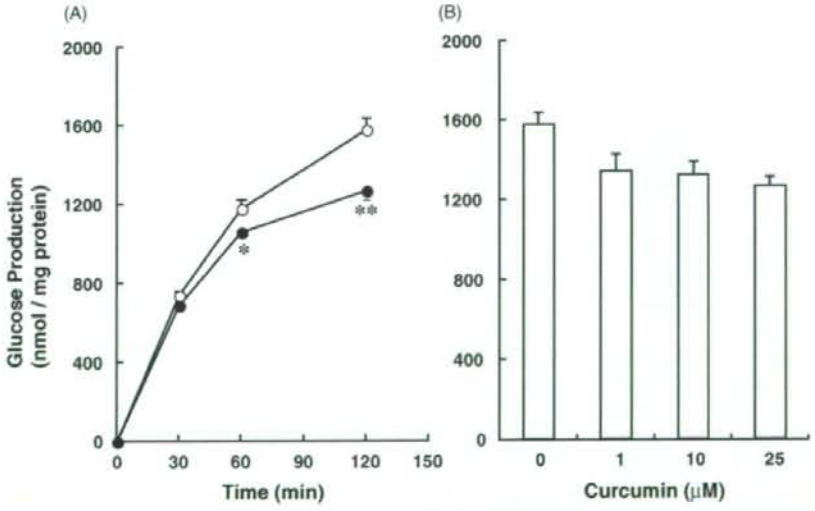

Fig. 5 – (A) Time course of inhibition of hepatic glucose production from glycogenolysis by curcumin. Isolated hepatocytes from fed mice were incubated in the presence of 25 μM curcumin or vehicle for 2 h. Glucose content in supernatant was measured by glucose oxidation method. Each point shows mean ± S.E.M. ($n = 5$). *$P < 0.05$, **$P < 0.01$ compared with control by unpaired Student's t-test. Control (○), curcumin (●). (B) Concentration-dependence of inhibition of glycogenolysis by curcumin at the incubation time of 120 min in isolated mice hepatocytes. Each point shows mean ± S.E.M. ($n = 6$). $P < 0.05$ by one-way ANOVA.

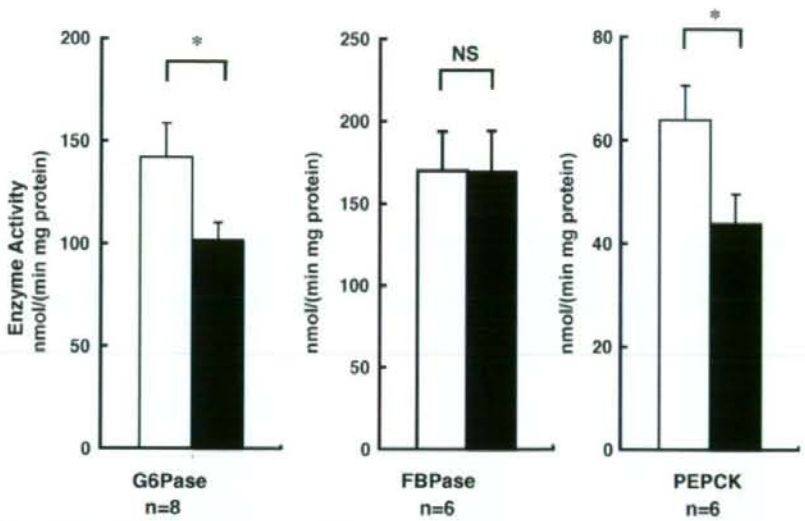

Fig. 7 – Effects of curcumin on hepatic gluconeogenetic activities of G6Pase, FBPase, and PEPCK in isolated mice hepatocyte. Isolated hepatocytes from fasted mice were incubated in the presence of 25 μM curcumin or vehicle for 2 h. All enzyme activities were measured photometrically. Enzyme activities are expressed as the number of substrate molecules converted by 1 mg cytosolic or microsomal protein per minute. Each point shows mean ± S.E.M. *P < 0.05 compared with control by unpaired Student's *t*-test. Control (open bar), curcumin (closed bar).

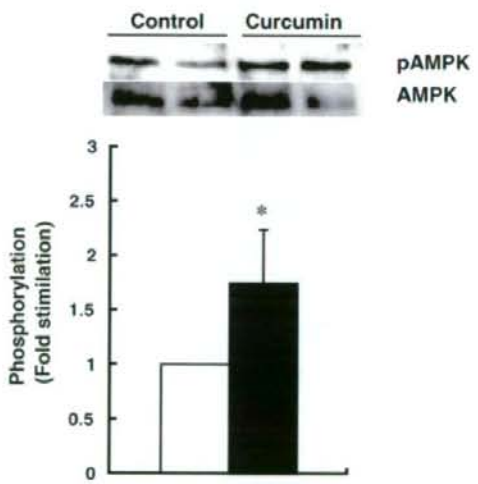

Fig. 8 – Effect of curcumin on activation of AMP kinase in isolated mice hepatocytes. AMP kinase activation was monitored in Western blots by staining with a specific antibody against phosphorylated Thr^{172} of AMP kinase α. After 120 min exposure to 25 μM curcumin, the level of phosphorylation of AMP kinase α-Thr^{172} (pAMPK) was significantly increased by 70% when normalized by total content of AMP kinase α (AMPK), and is expressed as fold stimulation over the control (mean ± S.E.M., *n* = 5) (lower panel). Upper panel shows a representative immunoblot of pAMPK and AMPK in hepatocytes from two mice in each group. *P < 0.05 compared with control by unpaired Student's *t*-test. Control (open bar), curcumin (closed bar).

recently reported a protective effect of curcumin against warm ischemia/reperfusion injury in rat liver [14].

Arun and Nalini reported that curcumin reduced blood glucose in alloxan-induced diabetic rats [7], but the mechanism of the anti-diabetic action was left unclear in that study. In the present study, insulin was found to dose-dependently inhibit hepatic gluconeogenesis, reaching a plateau at a concentration of 10 nM. In the presence of 10 nM insulin, the addition of 25 μM curcumin enchanced the inhibitory effect of insulin on hepatic gluconeogenesis, demonstrating that curcumin inhibits hepatic gluconeogenesis by a pathway independent of insulin signaling. Thus, curcumin is an insulin-sentitizing agent.

Recently, the major effect of metformin, a biguanide, was reported to be inhibition of hepatic G6Pase activity and hepatic glucose production in rats fed a high-fat diet [15]. Zhou et al. reported that metformin activated AMP kinase in hepatocytes [16]. The activation of AMP kinase is known to suppress gene expression of G6Pase and PEPCK and to inhibit hepatic glucose production in an insulin-independent manner [17,18]. In the present study, curcumin was found to inhibit both G6Pase and PEPCK activity; we therefore measured the effect of curcumin on AMP kinase activity to clarify the underlying mechanism. Zang et al. reported that resveratrol, a polyphenol and an anti-oxidant, which is a key component in red wine, stimulates AMP kinase in hepatoma HepG2 cells [19]. Kim et al. reported that cryptotanshinone, another anti-oxidant and a diterpene, which was originally isolated from dried roots of *Salvia mlitorrhiza* Bunge, showed anti-diabetic effects through activation of AMP kinase [20]. Considering these findings together, the potent anti-oxidant effect of curcumin may

well be involved in the activation of AMP kinase. Further investigation is required to clarify the anti-diabetic action of curcumin in hepatocytes.

Biguanide sometimes shows the lethal adverse effect of lactic acidosis in diabetic patients when prescribed inappropriately. On the other hand, since curcumin is derived from an extensively used dietary spice, the compound may well be safely administered to humans. Indeed, Sharma et al. administered oral daily curcumin to advanced colorectal cancer patients without major adverse effects [21]. Cheng et al. also administered oral daily curcumin to patients with high risk or pre-malignant lesions [22].

Considered together with our results, these data suggest that curcumin might provide a valuable new therapy in the treatment of type 2 diabetes.

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Conflict of interest

The authors state that they have no conflict of interest.

REFERENCES

[1] R.C. Srimal, B.N. Dhawan, Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent, J. Pharm. Pharmacol. 25 (1973) 447–452.

[2] B. Joe, B.R. Lokesh, Role of capsaicin, curcumin and dietary n-3 fatty acids in lowering the generation of reactive oxygen species in rat peritoneal macrophages, Biochim. Biophys. Acta 1224 (1994) 255–263.

[3] A.C. Reddy, B.R. Lokesh, Studies on the inhibitory effects of curcumin and eugenol on the formation of reactive oxygen species and the oxidation of ferrous iron, Mol. Cell. Biochem. 137 (1994) 1–8.

[4] Y. Kiso, Y. Suzuki, N. Watanabe, Y. Oshima, H. Hikino, Antihepatotoxic principles of *Curcuma longa* rhizomes, Planta Med. 49 (1983) 185–187.

[5] D.S. Rao, N.C. Sekhara, M.N. Satyanarayana, M. Srinivasan, Effect of curcumin on serum and liver cholesterol levels in the rat, J. Nutr. 100 (1970) 1307–1315.

[6] T.N. Patil, M. Srinivasan, Hypocholesteremic effect of curcumin in induced hypercholesteremic rats, Indian J. Exp. Biol. 9 (1971) 167–169.

[7] N. Arun, N. Nalini, Efficacy of turmeric on blood sugar and polyol pathway in diabetic albino rats, Plant Foods Hum. Nutr. 57 (2002) 41–52.

[8] T. Nishiyama, T. Mae, H. Kishida, M. Tsukagawa, Y. Mimaki, M. Kuroda, et al., Curcuminoids and sesquiterpenoids in turmeric (*Curcuma longa* L.) suppress an increase in blood glucose level in type 2 diabetic KK-Ay mice, J. Agric. Food Chem. 53 (2005) 959–963.

[9] M. Hosokawa, B. Thorens, Glucose release from GLUT2-null hepatocytes: characterization of a major and a minor pathway, Am. J. Physiol. Endocrinol. Metab. 282 (2002) E794–E801.

[10] M. Uldry, M. Ibberson, M. Hosokawa, B. Thorens, GLUT2 is a high affinity glucosamine transporter, FEBS Lett. 524 (2002) 199–203.

[11] K. Aoki, T. Saito, S. Satoh, K. Mukasa, M. Kaneshiro, S. Kawasaki, et al., Dehydroepiandrosterone suppresses the elevated hepatic glucose-6-phosphatase and fructose-1,6-bisphosphatase activities in C57BL/Ksj-db/db mice: comparison with troglitazone, Diabetes 48 (1999) 1579–1585.

[12] J.V. Passoneau, O.H. Lowry, Enzymic Analysis: A Practical Guide by Passoneau JV and Lowry OH., Humana Press, Totowa, NJ, 1993, pp. 249–253.

[13] H. Nakagawa, K. Nagai, Cold adaptation. I. Effect of cold-exposure on gluconeogenesis, J. Biochem. (Tokyo) 69 (1971) 923–934.

[14] S.Q. Shen, Y. Zhang, J.J. Xiang, C.L. Xiong, Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes, World J. Gastroenterol. 13 (2007) 1953–1961.

[15] G. Mithieux, L. Guignot, J.C. Bordet, N. Wiernsperger, Intrahepatic mechanisms underlying the effect of metformin in decreasing basal glucose production in rats fed a high-fat diet, Diabetes 51 (2002) 139–143.

[16] G. Zhou, R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, et al., Role of AMP-activated protein kinase in mechanism of metformin action, J. Clin. Invest. 108 (2001) 1167–1174.

[17] P.A. Lochhead, I.P. Salt, K.S. Walker, D.G. Hardie, C. Sutherland, 5-Aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase, Diabetes 49 (2000) 896–903.

[18] T. Yamauchi, J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, et al., Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase, Nat. Med. 8 (2002) 1288–1295.

[19] M. Zang, S. Xu, K.A. Maitland-Toolan, A. Zuccollo, X. Hou, B. Jiang, et al., Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice, Diabetes 55 (2006) 2180–2191.

[20] E.J. Kim, S.N. Jung, K.H. Son, S.R. Kim, T.Y. Ha, M.G. Park, et al., Antidiabetes and antiobesity effect of cryptotanshinone via activation of AMP-activated protein kinase, Mol. Pharmacol. 72 (2007) 62–72.

[21] R.A. Sharma, S.A. Euden, S.L. Platton, D.N. Cooke, A. Shafayat, H.R. Hewitt, et al., Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance, Clin. Cancer Res. 10 (2004) 6847–6854.

[22] A.L. Cheng, C.H. Hsu, J.K. Lin, M.M. Hsu, Y.F. Ho, T.S. Shen, et al., Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions, Anticancer Res. 21 (2001) 2895–2900.

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Glycemic instability in type 1 diabetic patients: Possible role of ketosis or ketoacidosis at onset of diabetes

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ABSTRACT

Aims: In type 1 diabetic patients, some have glycemic instability while others glycemic stability. We have developed criteria for evaluating glycemic instability and investigated the factors responsible.

Methods: Glycemic instability in 52 type 1 diabetic patients was assessed by the mean amplitude of glycemic excursions (MAGE) and M-value, and clinical characteristics of good, fair and poor control groups were compared.

Results: The median MAGE and M-value was 6.6 mmol/L and 18.7, respectively. Then MAGE ≥6.6 mmol/L and M-value ≥18.7 was defined as poor control. In the 32 patients without detectable C-peptide levels, 18 patients (56%) showed poor control. The frequency of ketosis or ketoacidosis at onset of diabetes was dramatically higher in the poor control group not only in the patients as a whole but also in those without detectable C-peptide levels.

Conclusions: A decreased level of C-peptide is a significant factor in glycemic instability. However, some patients have glycemic stability though β-cell function is completely depleted. The presence of ketosis or ketoacidosis at onset of diabetes may be a factor in later glycemic instability, suggesting the importance of examining patients in detail at onset of diabetes for careful follow-up to prevent progression of acute and chronic complications of diabetes.

1. Introduction

Type 1 diabetes mellitus is characterized by various forms of β-cell destruction, exhibiting various modes of onset [1]: acute-onset ('classical'), slow-onset [2] and fulminant [3]. While the incidence rate and mode of onset may differ among various populations, being 20-fold greater in Finland than in Japan, for example [4], β-cell destruction usually results in their complete loss and insulin-dependent diabetes mellitus (insulin-dependency) [5]. In patients with insulin-dependency, not only does the HbA_{1c} level increase, which represents the mean blood glucose level [6], but, as we previously reported [7], the blood glucose level remains unstable despite all efforts to optimize the use of exogenous insulin. Patients with glycemic

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instability are at increased risk of chronic macroangiopathic complications [8,9] as well as acute complications such as recurrent ketoacidosis [10], life-threatening hypoglycemia unawareness, and even "dead-in-bed syndrome" [11].

To quantify glycemic instability, the mean amplitude of glycemic excursions (MAGE) [12] and M-value [13] are commonly used [14,15]. However, glycemic instability has not been assessed by these indexes in a large population recently though the development of various new drugs has considerably facilitated glycemic control, and there are no established criteria for glycemic instability suitable for clinical use.

The blood glucose levels of some type 1 diabetic patients are as stable as those of type 2 diabetic patients [7]. To examine the distribution of glycemic instability and clarify the factors responsible for glycemic instability is of clinical importance for careful follow-up to prevent onset and progression of both acute and chronic secondary complications of diabetes.

In the present study, we assessed glycemic instability just before discharge after hospital treatment of at least a week period to exclude the influence of poor adherence to diet and exercise therapy, inappropriate use of exogenous insulin, and stressful circumstances of home or work place. We then compared the clinical characteristics and laboratory data of the patients with poor control and those with good or fair control to clarify the factors responsible.

2. Subjects and methods

2.1. Subjects

We examined all of the type 1 and type 2 diabetic patients admitted to Kyoto University Hospital from January, 2003 to July, 2007, except those meeting the exclusion criteria. A total of 52 type 1 diabetic patients (27 women and 25 men) (median age, 49 years; range, 16–80: median duration of diabetes, 6 years; range, 0–37) and 160 type 2 diabetic patients (64 women and 96 men) (median age, 65 years; range, 16–83: median duration of diabetes, 11 years; range, 0–53) were involved. American Diabetes Association Criteria was used as the criteria of type 1 diabetes [5]. Patients with renal insufficiency (Cre $\geq$ 1.5 mg/dl) were excluded, as were those with liver failure, acute illness such as infection, psychological comorbidities such as eating disorders, depression, needle phobia, those taking steroid medication, or inadequately monitoring blood glucose, and those on short-term admission (<7 days). No patients have learning disorders and apparent manipulative behaviour.

2.2. Assessment of glycemic instability

Seven capillary glucose measurements (before meals, 120 min after meals, and at bedtime) for two successive days just before discharge were analyzed to calculate the mean amplitude of glycemic excursions (MAGE) [12] and M-value, the indexes of glycemic instability. For calculating M-value, the modification [16] of the method of Schlichtkrull et al. [13,17] is commonly used: the average of M_{BS}^{BS} values; $M_{BS}^{BS} = |10 \times \log \frac{BS}{120}|^3$, which is the logarithmic transformation of the deviation of glycemia from the arbitrarily selected standard (120 mg/dl). Recently, 90 mg/dl is often used as the selected standard [18], and we used 100 mg/dl to give greater emphasis to hypoglycemia. Thus, in the present study, M-value is defined as the mean of M_{BS}^{BS} of each successive day: $M_{BS}^{BS} = |10 \times \log \frac{BS}{100}|^3$. The MAGE and M-value of the type 1 diabetic patients suggested a criterion for glycemic instability: MAGE $\geq$ the median and M-value $\geq$ the median representing poor control, MAGE < the first quartile and M-value < the first quartile indicating good control, and fair control comprising the middle ranges.

2.3. Factors responsible for glycemic instability

To clarify the factors responsible for glycemic instability, we compared the clinical characteristics and laboratory data of the good or fair groups and the poor control group of the 51 type 1 diabetic patients receiving intensive insulin therapy based on the guidelines by Japan Diabetes Society [19]. Ketosis was established by ketonuria, elevated serum ketones, or both. Serum and urinary C-peptide levels were measured using a commercially available EIA kit (ST AIA-PACK C-peptide, TOSOH corporation, Tokyo, Japan) with a detection limit of 0.1 ng/ml (intra-assay coefficient of variation [CV] 1.3–2.2%, interassay CV 1.7–2.0%) for serum C-peptide and 0.1 ng/dl (intra-assay CV 3.1–3.8%, inter-assay CV 1.9–2.1%) for urinary C-peptide. This kit has good reproductivity even when the level of C-peptide is lower than 1.0 ng/ml [20]. Diabetic sensorimotor distal symmetric polyneuropathy was assessed by pinprick, vibration perception threshold, and ankle reflexes. Autonomic function was evaluated by the coefficient of variation of the R-R interval (CVR-R) during deep breathing monitored on an electrocardiogram, and values below the reference values of healthy subjects [21] were counted abnormal. The presence of diabetic neuropathy was established by at least one abnormal result in the tests described above.

2.4. Statistical analysis

Qualitative variables were compared using Fisher's exact test. The Mann–Whitney *U* test was used to compare quantitative variables, which were expressed as medians and ranges. Two-sided *P* values <0.05 were considered statistically significant. Data were analyzed using StatView 5.0 (SAS Institute, Cary, NC).

3. Results

3.1. Criteria for glycemic instability

The median MAGE of type 1 diabetic patients was 6.6 mmol/L, significantly higher than that of type 2 diabetic patients ($P < 0.001$) (Fig. 1A). The first quartile MAGE of type 1 diabetic patients was 4.3 mmol/L and the third quartile was 7.5 mmol/L. The median M-value of type 1 diabetic patients was 18.7, significantly higher than that of type 2 diabetic patients ($P < 0.05$) (Fig. 1B). The first quartile M-value of type 1 diabetic patients was 9.2 and the third quartile was 30.2. MAGE was

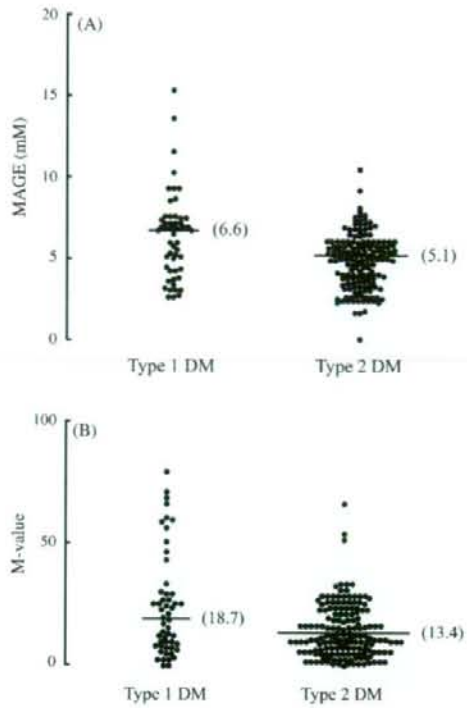

Fig. 1 – (A) MAGE of 52 type 1 diabetic patients and 160 type 2 diabetic patients at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians. (B) M-value of 52 type 1 diabetic patients and 160 type 2 diabetic patients at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians.

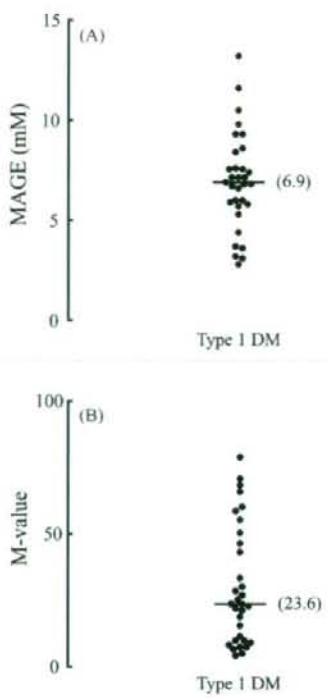

Fig. 2 – (A) MAGE of 32 type 1 diabetic patients without detectable C-peptide levels at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians. (B) M-value of 32 type 1 diabetic patients without detectable C-peptide levels at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians.

significantly correlated with M-value ($r = 0.71$, $P < 0.0001$). Then MAGE≥6.6 mmol/L and M-value ≥18.7 was defined as poor control, MAGE <4.3 mmol/L and M-value <9.2 good control, and the middle ranges fair control. By these criteria, 57.7% of the type 1 diabetic patients exhibited good or fair control (good: 15.4%, fair: 42.3%, respectively), while 90.0% of the type 2 diabetic patients exhibited good or fair control (good: 20.6%, fair: 69.4%, respectively). Thus, type 1 diabetic patients exhibited significantly greater glycemic instability than type 2 diabetic patients ($P < 0.001$).

3.2. *Clinical characteristics and laboratory data in 51 type 1 diabetic patients*

Clinical characteristics and laboratory data of the good or fair groups vs. the poor control group in 51 type 1 diabetic patients are shown in Table 1. There were no significant differences between the good or fair groups and the poor control group regarding age, sex, BMI, duration of diabetes, IA-2 antibodies, insulin antibodies, HbA_{1c} level, the state of diabetic complications and thyroid function at present admission, age at onset, and GAD antibodies at onset. Serum C-peptide at prestimulation, post glucagon stimulation and urinary C-peptide at present admission were significantly lower in the poor control group than in the good or fair groups ($P < 0.05$). The frequency of ketosis or ketoacidosis at onset of diabetes was significantly higher in the poor control group compared to the good or fair groups ($P < 0.01$; risk ratio [RR] 3.5 [95% CI 1.4–9.0]). In other words, in those with ketosis or ketoacidosis at onset of diabetes, glycemic instability was markedly higher than in those without ketosis or ketoacidosis at onset of diabetes (14 of 23 vs. 4 of 23; $P < 0.01$; [RR] 3.5 [95% CI 1.4–9.0]). There were no significant differences regarding the frequency of ketosis or ketoacidosis at onset of diabetes between those with detectable C-peptide levels and those without (11 of 18 vs. 12 of 28; $P = 0.37$). The frequency of positive GAD antibodies (≥1.5 U/ml) at present admission was significantly lower in the poor control group than in the good or fair groups ($P < 0.05$; [RR] 2.3 [95% CI 1.1–4.8]).

3.3. *Glycemic instability in type 1 diabetic patients without detectable C-peptide levels*

Thirty-two type 1 diabetic patients without detectable serum C-peptide levels at post glucagon stimulation and without detectable urinary C-peptide levels in 24-h urine collections

Table 1 – Clinical characteristics of type 1 diabetic patients

Glycemic instability	Good or fair control	Poor control	P-value or RR (95% CI) good or fair vs. poor
	MAGE <6.6 or M-value <18.7	MAGE ≥6.6 and M-value ≥18.7	
Clinical characteristics at present admission			
Age (years)	49 (16–80)	43 (18–79)	0.76
Sex (female/male)	16/13	11/11	0.78
BMI (kg/m^2)	21.0 (17.8–26.9)	20.8 (16.1–25.3)	0.95
Duration of diabetes (years)	6 (0–37)	7.5 (0–31)	0.95
GAD antibodies (<1.5/≥1.5 U/ml)	11/17	16/6	0.024 2.3 (1.1–4.8)
IA-2 antibodies (<0.4/≥0.4 U/ml)	7/3	6/2	0.99
Insulin antibodies (%)	10.2 (3.7–77.6)	9.8 (6.6–89.6)	0.67
HbA_{1c} (%)	8.1 (5.6–16.3)	8.3 (5.0–11.6)	0.73
Serum C-peptide at prestimulation (ng/ml)	0 (0–0.90)	0 (0–0.43)	0.023
Serum C-peptide at post glucagon stimulation (ng/ml)	0.15 (0–2.0)	0 (0–0.92)	0.010
Serum ΔC-peptide (ng/ml)	0.05 (0–1.63)	0 (0–0.49)	0.010
Urinary C-peptide (μg/day)	0 (0–57.7)	0 (0–21.0)	0.019
Diabetic retinopathy (NDR/NPDR/PDR)	22/3/4	14/8/0	0.78
Diabetic nephropathy (normoalbuminuria/ microalbuminuria/proteinuria)	25/3/1	17/5/0	0.59
Diabetic neuropathy (negative/positive)	14/15	8/14	0.27
CVR-R (%) (normal/abnormal)	11/10	8/6	0.99
Ankle brachial pressure index (ABI)	1.10 (0.88–1.24)	1.11 (0.72–1.23)	0.86
Carotid intima-media thickness (mm)	0.9 (0.6–1.5)	0.8 (0.7–1.6)	0.69
TSH (μU/ml)	1.80 (0.59–8.02)	1.31 (0–54.8)	0.62
Free T_4 (ng/dl)	1.22 (0.89–1.90)	1.14 (0.57–2.69)	0.42
Clinical characteristics at onset			
Age at onset (years)	40 (7–78)	34 (10–74)	0.52
Ketosis or ketoacidosis (negative/positive)	19/9	4/14	0.0058 3.5 (1.4–9.0)
GAD antibodies (<1.5/≥1.5 U/ml)	3/18	6/8	0.11 2.2 (1.0–4.5)

Data are median (range) or number of patients. Serum C-peptide, urinary C-peptide, GAD antibodies and IA-2 antibodies below detection limits are expressed as 0.

were then selected. Of these patients, 18 patients (56%) showed poor control, and the other 14 patients (44%) showed good or fair control (MAGE <6.6 mmol/L in 12 patients, M-value <18.7 in 12 patients) (Fig. 2A and B). In contrast, of the 19 patients with detectable C-peptide levels, 15 (79%) showed good or fair control, and the other 4 (21%) showed poor control.

The good or fair and poor control groups in type 1 diabetic patients without detectable C-peptide levels were then compared. The frequency of ketosis or ketoacidosis at onset was dramatically higher in the poor control group than in the good or fair groups (11 [73%] of 15 vs. 1 [8%] of 13; $P < 0.001$; [RR] 3.7 [95% CI 1.5–8.7]). In other words, of the 13 patients without detectable C-peptide levels but with glycemic stability, 12 patients did not have ketosis or ketoacidosis at onset. The frequency of positive GAD antibodies at present admission was significantly lower in the poor control group than in the good or fair groups (3 [17%] of 18 vs. 8 [62%] of 13; $P < 0.05$; [RR] 2.8 [95% CI 1.0–7.5]).

3.4. *Glycemic instability in acute-onset type 1 diabetic patients*

In the 51 type 1 diabetic patients, 38 patients had acute-onset ('classical') type 1 diabetes, 6 patients fulminant type 1 diabetes [3], and 2 patients latent autoimmune diabetes of adults [2], and 5 patients unknown. Thirty-eight acute-onset type 1 diabetic patients were then examined to exclude the influence of fulminant type 1 diabetes, because the presence of ketosis or ketoacidosis at onset of diabetes, which has been found to be involved in glycemic instability in the present study, is a characteristic of fulminant type 1 diabetes [3], and glycemic instability in fulminant type 1 diabetes was markedly greater than in acute-onset type 1 diabetes (good: 0 of 6, fair: 1 of 6, poor: 5 of 6 vs. good: 6 of 38, fair: 18 of 38, poor: 14 of 38, $P < 0.05$). In acute-onset type 1 diabetic patients, the frequency of ketosis or ketoacidosis at onset was significantly higher in the poor control group than in the good or fair groups (8 [73%] of 11 vs. 7 [29%] of 24; $P < 0.05$; [RR] 3.6 [95% CI 1.1–11.2]).

4. Discussion

It has been reported that factors that influence glycemic instability are deficiency of endogenous insulin secretion [22,23], abnormal response of insulin-counteracting hormones [22,24,25], poor compliance with diet and exercise therapy, stressful life circumstances [26], and inappropriate use of exogenous insulin [27,28].

In the present study, we assessed glycemic instability by MAGE and M-value in 52 type 1 and 160 type 2 diabetic

patients, and proposed criteria for glycemic instability suitable for clinical use. MAGE and M-value of most of the type 2 diabetic patients showed good or fair glycemic stability.

Analysis of the 51 type 1 diabetic patients showed that serum C-peptide at post glucagon stimulation and urinary C-peptide at present admission were significantly lower in the poor control group than in the good or fair groups. In addition, of the 32 patients without detectable C-peptide levels, 18 (56%) showed poor control. These results indicate that decreased endogenous insulin secretion is a significant factor in glycemic instability. However, of the 32 patients without detectable C-peptide levels, 14 (44%) exhibited good or fair control. In addition, of these patients, MAGE of 6 (19%) and the M-value of 10 (31%) were lower than the median values of the type 2 diabetic patients. These results demonstrate that some patients with type 1 diabetic patients have glycemic stability though β-cell function is completely depleted.

The present study suggests that the presence of ketosis or ketoacidosis at onset of diabetes is a factor in later glycemic instability. This is not simply due to the including of fulminant type 1 diabetic patients, because analysis of acute-onset type 1 diabetic patients also showed that the frequency of ketosis or ketoacidosis at onset was significantly higher in the poor control group than in the good or fair groups. Moreover, analysis of the type 1 diabetic patients without detectable C-peptide levels also showed that the frequency of ketosis or ketoacidosis at onset was dramatically higher in the poor control group than in the good or fair groups. The same tendency of the higher frequency of ketosis or ketoacidosis at onset in the poor control group was shown in the patients with detectable C-peptide levels. These results suggest that the presence of ketosis or ketoacidosis at onset is a factor in later glycemic instability, and other factors than C-peptide also exist as the underlying mechanism besides decreased levels of C-peptide.

Then, what is the mechanism whereby ketosis or ketoacidosis at onset of diabetes leads to later glycemic instability? One possibility is decreased β-cell functional reserve as mentioned above. Other factors than C-peptide underlying the mechanism may be the increased levels of counter-regulatory hormones inducing insulin resistance, including glucagon, epinephrine, cortisol and growth hormone [29,30]. Glucagon levels are markedly elevated in certain patients with ketoacidosis including those having preserved endogenous insulin secretion [30]. In addition, not only insulin-dependent diabetic patients but also noninsulin-dependent diabetic patients with hyperglycemia display paradoxical hyperglucagonemia, which contributes to postprandial hyperglycemia and glycemic instability [24,31].

In addition, there have been no studies of type 1 diabetic patients to clarify the association of GAD antibodies with glycemic instability. The present study indicates that negative GAD antibodies at onset may contribute to later glycemic instability. This may be because there is an inverse relation between the level of GAD antibodies and β-cell-destructive T-cell responses [32] or partly because patients with fulminant type 1 diabetes were included.

The present study has limitations in that this is a cross-sectional study and we have little C-peptide data at onset of diabetes. However, the frequency of ketosis or ketoacidosis at onset was clearly higher in the poor control group than in the good or fair groups not only in the patients as a whole but also in those without detectable C-peptide levels at present admission.

In summary, we confirmed that a decreased level of C-peptide is a significant factor in glycemic instability. However, the present study showed that some patients with type 1 diabetic patients have glycemic stability though β-cell function is completely depleted. The present study suggested that ketosis or ketoacidosis at onset of diabetes is a factor in later glycemic instability. In addition, GAD antibodies at onset may also influence later glycemic instability. These results suggest the importance of examining patients in detail at the onset of diabetes. Patients with glycemic instability are at increased risk of both acute and chronic complications of diabetes, so patients exhibiting ketosis or ketoacidosis at onset of type 1 diabetes should be carefully monitored.

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Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

[1] E. Kawasaki, N. Matsuura, K. Eguchi, Type 1 diabetes in Japan, Diabetologia 49 (2006) 828–836.
[2] G. Stenström, A. Gottsäter, E. Bakhtadze, B. Berger, G. Sundkvist, Latent autoimmune diabetes in adults: definition, prevalence, beta-cell function, and treatment, Diabetes 54 (Suppl. 2) (2005) S68–S72.
[3] A. Imagawa, T. Hanafusa, J. Miyagawa, Y. Matsuzawa, A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. Osaka IDDM Study Group, N. Engl. J. Med. 342 (2000) 301–307.
[4] M. Karvonen, M. Viik-Kajander, E. Moltchanova, I. Libman, R. LaPorte, J. Tuomilehto, Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group, Diabetes Care 23 (2000) 1516–1526.
[5] The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, Report of the expert committee on the diagnosis and classification of diabetes mellitus, Diabetes Care 26 (Suppl. 1) (2003) S5–S20.
[6] C.L. Rohlfing, H.-M. Wiedmeyer, R.R. Little, J.D. England, A. Tennill, D.E. Goldstein, Defining the relationship between plasma glucose and HbA_{1c}: analysis of glucose profiles and HbA_{1c} in the Diabetes Control and Complications Trial, Diabetes Care 25 (2002) 275–278.

[7] M. Sassa, K. Fukuda, S. Fujimoto, K. Toyoda, Y. Fujita, S. Matsumoto, et al., A single transplantation of the islets can produce glycemic stability and reduction of basal insulin requirement, Diabetes Res. Clin. Pract. 73 (2006) 235–240.
[8] L. Monnier, E. Mas, C. Ginet, F. Michel, L. Villon, J.-P. Cristol, et al., Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes, JAMA 295 (2006) 1681–1687.
[9] A. Ceriello, Postprandial hyperglycemia and diabetes complications: is it time to treat? Diabetes 54 (2005) 1–7.
[10] G.V. Gill, The spectrum of brittle diabetes, J. R. Soc. Med. 85 (1992) 259–261.
[11] K.V. Allen, B.M. Frier, Nocturnal hypoglycemia: clinical manifestations and therapeutic strategies toward prevention, Endocr. Pract. 9 (2003) 530–543.
[12] F.J. Service, G.D. Molnar, J.W. Rosevear, E. Ackerman, L.C. Gatewood, W.F. Taylor, Mean amplitude of glycemic excursions, a measure of diabetic instability, Diabetes 19 (1970) 644–655.
[13] J. Schlichtkrull, O. Munck, M. Jersild, The M-value, an index of blood-sugar control in diabetics, Acta Med. Scand. 177 (1965) 95–102.
[14] C. Toso, R. Baertschiger, P. Morel, D. Bosco, M. Armanet, A. Wojtusciszyn, et al., Sequential kidney/islet transplantation: efficacy and safety assessment of a steroid-free immunosuppression protocol, Am. J. Transplant. 6 (2006) 1049–1058.
[15] M. Kishimoto, Y. Yamasaki, M. Kubota, K. Arai, T. Morishima, R. Kawamori, et al., 1,5-Anhydro-D-glucitol evaluates daily glycemic excursions in well-controlled NIDDM, Diabetes Care 18 (1995) 1156–1159.
[16] J. Mirouze, A. Satingher, C. Sany, C. Jaffiol, Insulin efficiency coefficient. M coefficient of Schlichtkrull corrected and simplified by the continuous blood glucose recording technic, Diabete 11 (1963) 267–273.
[17] J. Schlichtkrull, J. Funder, O. Munck, Clinical evaluation of a new insulin preparation, 4e Congrès de la Fédération Internationale du Diabète, Fédération Internationale du Diabète, Genève, 1961, pp. 303–305.
[18] F. Holleman, H. Schmitt, R. Rottiers, A. Rees, S. Symanowski, J.H. Anderson, Reduced frequency of severe hypoglycemia and coma in well-controlled IDDM patients treated with insulin lispro. The Benelux-UK Insulin Lispro Study Group, Diabetes Care 20 (1997) 1827–1832.
[19] Japan Diabetes Society, Treatment Guide for Diabetes, Bunkodo, 2007.
[20] H. Tsurumi, H. Kondoh, T. Horii, J. Igari, Fundamental evaluation of AIA-PACK C-peptide, J. Clin. Lab. Inst. Reag. 19 (1996) 885–892.
[21] J. Fujimoto, M. Hirota, M. Hata, M. Kondo, K. Shima, Normal reference values and prediction equations of autonomic nerve functions based on variations in the R-R interval in electrocardiographs, J. Japan Diab. Soc. 30 (1987) 167–173.
[22] C. Reynolds, G..D. Molnar, D.L. Horwitz, A.H. Rubenstein, W.F. Taylor, N.-S. Jiang, Abnormalities of endogenous glucagon and insulin in unstable diabetes, Diabetes 26 (1977) 36–45.
[23] The Diabetes Control and Complications Trial Research Group, Effect of intensive therapy on residual beta-cell function in patients with type 1 diabetes in the diabetes control and complications trial. A randomized, controlled trial, Ann. Intern. Med. 128 (1998) 517–523.
[24] S. Dinneen, A. Alzaid, D. Turk, R. Rizza, Failure of glucagon suppression contributes to postprandial hyperglycaemia in IDDM, Diabetologia 38 (1995) 337–343.
[25] N.C. Sjöbom, U. Adamson, P.-E. Lins, The prevalence of impaired glucose counter-regulation during an insulin-infusion test in insulin-treated diabetic patients prone to severe hypoglycaemia, Diabetologia 32 (1989) 818–825.
[26] R. Tattersall, R. Gregory, C. Selby, D. Kerr, S. Heller, Course of brittle diabetes: 12 year follow up, BMJ 302 (1991) 1240–1243.
[27] D.S. Schade, D.A. Drumm, W.C. Duckworth, R.P. Eaton, The etiology of incapacitating, brittle diabetes, Diabetes Care 8 (1985) 12–20.
[28] A.D. Morris, D.I. Boyle, A.D. McMahon, S.A. Greene, T.M. MacDonald, R.W. Newton, Adherence to insulin treatment, glycaemic control, and ketoacidosis in insulin-dependent diabetes mellitus. The DARTS/MEMO Collaboration. Diabetes Audit and Research in Tayside Scotland. Medicines Monitoring Unit, Lancet 350 (1997) 1505–1510.
[29] D.S. Schade, R.P. Eaton, Pathogenesis of diabetic ketoacidosis: a reappraisal, Diabetes Care 2 (1979) 296–306.
[30] W.A. Müller, G.R. Faloona, R.H. Unger, Hyperglucagonemia in diabetic ketoacidosis. Its prevalence and significance, Am. J. Med. 54 (1973) 52–57.
[31] A. Salehi, E. Vieira, E. Gylfe, Paradoxical stimulation of glucagon secretion by high glucose concentrations, Diabetes 55 (2006) 2318–2323.
[32] L.C. Harrison, M.C. Honeyman, H.J. DeAizpurua, R.S. Schmidli, P.G. Colman, B.D. Tait, et al., Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes, Lancet 341 (1993) 1365–1369.

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Secretory Unit of Islet in Transplantation (SUIT) and Engrafted Islet Rate (EIR) Indexes Are Useful for Evaluating Single Islet Transplantation

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The evaluation of engraftment is important to assess the success of islet transplantation, but it is complex because islet transplantation usually requires two or more donors to achieve euglycemia. Islet transplantation from NHBDs was evaluated using new assessment forms for the secretory unit of islet in transplantation (SUIT) and engrafted islet rate (EIR) indexes. Insulin independence was obtained when the SUIT index was more than 28, which might indicate that 28% of the β-cell mass of a normal subject is required for insulin independence. Because the average EIR for a single transplantation is about 30, the percentage of engrafted islets following one transplantation is about 30%, assuming that a normal subject has 1 million islet equivalents. Although few cultured islet transplants have been performed, the increase of the SUIT and EIR indexes in patients who received cultured islets was significantly lower than in patients who received fresh islets, suggesting that fresh islets may be more effective than cultured islets. The SUIT and EIR indexes are thus considered to be useful values for evaluating islet transplantation, especially for single islet transplantation.

Key words: Islet transplantation; Non-heart-beating donors; Secretory unit of islet in transplantation; Engrafted islet rate

INTRODUCTION

Islet transplantation is one of the options for treating type 1 diabetes. It has been shown to improve the quality of life in severe diabetic patients (32,39). The Edmonton protocol has been replicated by advanced islet transplantation centers (40), and islet transplantations have been obtained from brain-dead donors, as well as from non-heart-beating donors (NHBDs) (11,12,20) or living donors (13). These observations are proof that the protocol is viable and have intensified interest in treating diabetes or other diseases not only by cell transplantations but also by stem cells (19–22,24–28).

The evaluation of engraftment is important to assess the success of islet transplantation, but it is complex. Clinically, there is a spectrum of outcomes after islet transplantation, and some patients even achieve complete insulin independence with absolutely normal glucose profiles. Others have residual endogenous insulin secretion but may require supplementary insulin to maintain the appropriate glucose control, and some lose endogenous insulin secretion. The simple measure of success after islet transplantation is insulin independence. However, after islet transplantation, a patient may have a suboptimal outcome, where they no longer require insulin but still have glucose values that are elevated with a high HbA_{1C}. Equally, a patient may be taking insulin but, by virtue of endogenous insulin secretion, have perfectly stable glucose values and excellent glucose control. C-peptide levels are useful for the documentation of islet graft survival, but interpretation of the C-peptide values independent of glucose levels is of limited value to determine if the β-cell function is adequate. Simple measures of glucose control, such as fasting glucose and HbA_{1C}, are useful but difficult to interpret if insulin is used. The Edmonton group reported a β score

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that provides an integrated measurement of β-cell function after islet transplantation using these parameters (36). Although the β score can show the general evaluation of islet transplantation, it is difficult to evaluate the engrafted β-cell mass simply. Recently, the secretory unit of islet in transplantation (SUIT) (15,42) was developed for the simple evaluation of engraftment. The SUIT index is based on the correlation of fasting blood C-peptide and glucose levels in patients after islet transplantations (15,42). Moreover, we newly developed the engrafted islet rate (EIR), assessed by the SUIT index and transplanted islet equivalents for transplantation. EIR shows the percentage of engrafted islets for transplanted islets.

This study evaluated islet transplantation from NHBDs by SUIT and EIR indexes. These indexes are useful for evaluating single islet transplantation because two or three islet transplantations are needed to achieve insulin independence.

MATERIALS AND METHODS

Procurement of Human Pancreata

Nineteen human pancreata were obtained with informed consent from the relatives of NHBDs and procured through the Central Japan Region ($N = 17$, Aichi Prefecture, Japan) and the West Japan Region ($N = 2$, Osaka Prefecture, Japan) of the Japan Organ Transplantation Network between January 17, 2004 and November 18, 2005. After brain death had been confirmed, a cannula was inserted into the iliac vessels for rapid cooling of the pancreas (18). Cold lactate ringers solution was perfused via the cannula, after cessation of heart beating, until removal of the pancreata. M-Kyoto solution (29) was infused into the main pancreatic duct for ductal protection at the time of pancreas preservation (30,38). The pancreata were preserved using the modified two-layer (M-Kyoto/PFC) method and transported to the human islet isolation GMP facility (Center for Cell and Molecular Therapy). This study was approved by the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine.

Islet Isolation Protocols and Islet Evaluation

Upon arrival at the islet isolation GMP facility at Kyoto University, the pancreata were processed according to the Edmonton protocol with some modifications (12–14,39). Briefly, after the pancreas had been decontaminated, the ducts were perfused in a controlled fashion with a cold enzyme solution, Liberase HI (Roche Molecular Biochemicals, Indianapolis, IN). The distended pancreas was then cut into nine pieces and transferred to a Ricordi chamber. The pancreas was digested by repeated circulation of the enzyme solution through the Ricordi chamber at 37°C. The phase I period was defined as the time between the placement of the pancreas in the Ricordi chamber and the start of collection of the digested pancreas. The phase II period was defined as the time between the start and end of collection.

Islets were purified using a continuous density gradient with iodixanol-M-Kyoto solution in an apheresis system (COBE 2991 cell processor, Gambro Laboratories, Denver, CO). Because iodixanol has low viscosity, it requires less force during centrifugation in comparison with Ficoll. For the solution, low-density (density 1.070) and high-density (density 1.085–1.110) iodixanol-M-Kyoto solutions were produced by changing the volumetric ratio of iodixanol and M-Kyoto solution.

Islet preparations were evaluated for yield and purity by means of dithizone staining (2,14,33). Gross morphology, islet viability following purification, and islet function (stimulation index) were assessed according to a procedure described by Shapiro et al. (39).

Islet Transplantations Into Diabetic Patients

Sixteen of the 19 islet preparations were used to perform islet transplantations into seven type 1 diabetic patients between April 7 and November 18, 2005. The three remaining islet preparations were cryopreserved for future transplantation. All procedures were islet transplants alone. Three islet preparations were transplanted after an overnight culture. Five patients received multiple islet preparations and two patients received only one. Patient #3 received two islet preparations simultaneously because two donors with the same blood type were available. One patient was excluded from the analysis because her creatinine clearance was less than 80 ml/min.

Patients were sedated and a percutaneous transhepatic approach was used to gain access to the portal vein for all patients. Once access was confirmed, the Seldinger technique was used to place the Kumpe catheter within the main portal vein. Islets were infused by gravity using the bag technique (1).

Immunosuppression consisted of maintenance with tacrolimus (Prograf®, Fujisawa, Japan), at a target trough level of 4–6 ng/ml and sirolimus (Rapamune®, Wyeth Pharmaceuticals, Inc., Madison, NJ, USA), at a target trough level of 12–15 ng/ml for 3 months, and 40 mg of basiliximab (Simulect®, Novartis Pharma K.K., Tokyo, Japan) on POD 0 and 4. Three months after transplantation, 2 g/day of mycophenolate mofetil (MMF, CellCept®, Chugai Pharmaceutical Co., Ltd. Tokyo, Japan) was started and the trough level of sirolimus was maintained at 3–5 ng/ml (23).

Those subjects who were insulin independent with an HbA_{1C} within the normal range were considered to have achieved complete reversal of diabetes. Graft dysfunction is defined as more than three fasting blood glucose