Table 1 – Clinical characteristics of the subjects in six subgroups of three WHO categories at 10.0 mmol/l

	NGT		Isolated IFG		Isolated IGT	
	NGT-LG	NGT-HG	IFG-LG	IFG-HG	IGT-LG	IGT-HG
Total N	179		44		103	
N	121	58	14	30	32	71
Age (years)	47.2 ± 1.1	53.1 ± 1.3**	54.4 ± 2.1	51.5 ± 1.9	52.0 ± 1.6	52.2 ± 1.1
BMI (kg/m2)	23.1 ± 1	24.2 ± 0.4*	22.6 ± 0.8	24.2 ± 0.6*	22.8 ± 0.5	24.0 ± 0.3*
FPG (mmol/l)	5.2 ± 0.0	5.6 ± 0.0***	6.3 ± 0.1	6.3 ± 0.0	5.2 ± 0.1	5.6 ± 0.0***
1-h PG(mmol/l)	7.7 ± 0.1	11.5 ± 0.2***	8.2 ± 0.4	12.5 ± 0.4***	7.9 ± 0.3	12.2 ± 0.2***
2-h PG(mmol/l)	5.7 ± 0.1	6.2 ± 0.1*	6.0 ± 0.4	6.5 ± 0.1	8.6 ± 0.1	9.2 ± 0.1**
Fasting insulin(pmol/l)	31 ± 1	30 ± 2	26 ± 2	33 ± 3	34 ± 4	34 ± 2
1-h insulin(pmol/l)	250 ± 18	287 ± 23	287 ± 68	238 ± 29	146 ± 16	221 ± 18*
2-h insulin(pmol/l)	191 ± 16	196 ± 16	133 ± 17	192 ± 27	211 ± 22	254 ± 20
HbA1c(%)	5.1 ± 0.1	5.4 ± 0.1***	5.6 ± 0.1	5.6 ± 0.1	5.2 ± 0.1	5.6 ± 0.1*
Triglycerides(mmol/l)	1.28 ± 0.08	1.47 ± 0.16	1.14 ± 0.17	1.31 ± 0.15	1.74 ± 0.29	1.85 ± 0.25
Total cholesterol(mmol/l)	5.33 ± 0.1	5.31 ± 0.11	5.01 ± 0.3	5.41 ± 0.14	5.3 ± 0.16	5.41 ± 0.1
HDL-cholesterol(mmol/l)	1.45 ± 0.05	1.47 ± 0.07	1.46 ± 0.11	1.51 ± 0.11	1.37 ± 0.10	1.39 ± 0.06

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LG. Data are mean ± S.E.

Fig. 1A and B. The insulinogenic index in the HG groups was remarkably lower than in the LG groups. The insulinogenic index values were 25.6 ± 0.3 vs. 75.9 ± 1.6 (NGT-HG vs. NGT-LG: $p < 0.01$), 23.1 ± 0.5 vs. 67.0 ± 3.5 (IFG-HG vs. IFG-LG: $p < 0.05$) and 22.6 ± 0.3 vs. 56.4 ± 1.9 (IGT-HG vs. IGT-LG: $p < 0.01$). The HOMA-β cell index of the HG group was significantly lower than that of the LG group in NGT and isolated IGT. There was no difference between IFG-HG and IFG-LG in HOMA β-cell index. We also estimated the insulin sensitivity indices using ISI composite and HOMA-IR in the three categories. The ISI composite index represents insulin sensitivity during OGTT, while HOMA-IR represents insulin resistance at fasting state. The ISI composite and the HOMA-IR values were similar in the HG group and the LG group in all three WHO categories. ISI composite values were 7.5 ± 0.6 vs. 9.2 ± 0.4 (NGT-HG vs. NGT-LG: n.s.), 6.9 ± 0.6 vs. 8.0 ± 0.8 (IFG-HG vs. IFG-LG: n.s.) and 7.0 ± 0.4 vs. 7.8 ± 0.7 (IGT-HG vs. IGT-LG: n.s.). HOMA-IR values were 1.2 ± 0.1 vs. 1.2 ± 0.1 (NGT-HG vs. NGT-LG: n.s.), 1.4 ± 0.1 vs. 1.2 ± 0.1 (IFG-HG vs. IFG-LG: n.s.) and 1.4 ± 0.1 vs. 1.4 ± 0.1 (IGT-HG vs. IGT-LG: n.s.).

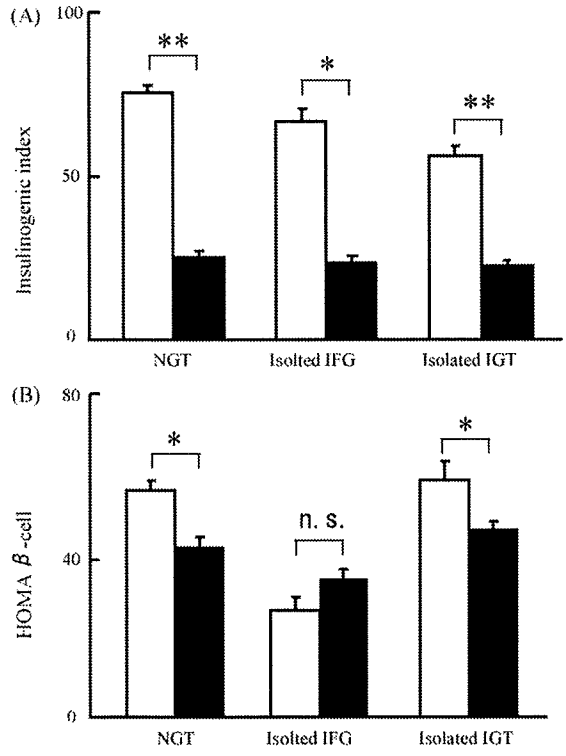

Fig. 1 – Indices of insulin secretion in six subgroups of three WHO categories: (A) insulinogenic index, (B) HOMA β-cell; light bars indicate subjects without elevated 1-h PG, dark bars indicate subjects with elevated 1-h PG. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LG, data are mean ± S.E.

In addition, we then analyzed the relationship between 1-h PG and the indices of insulin secretion and insulin sensitivity. Scattered plots of simple regression analysis between 1-hPG and the four indices are shown in Fig. 2. 1-h PG had a significant correlation with the insulinogenic index, HOMA β-cell, and ISI composite. Multiple regression analysis revealed that HOMA β-cell, ISI composite, and insulinogenic index were the independent factors in the variation of 30.0% in 1-h PG ($p < 0.001$). The correlation coefficients of these indices with 1-h PG in simple regression analysis, and the β values and p-values of multiple regression analysis are shown in Table 2. The insulinogenic index was the strongest factor to explain the 1-h PG levels.

4. Discussion

In the present study, we found that elevated 1-h PG is strongly associated with decreased insulinogenic index, indicating reduced capacity of early-phase insulin secretion [24,25]. The insulinogenic index in NGT-HG became remarkably lower than in NGT-LG at about 20, declining to the absolute levels of IFG-HG and IGT-HG. Multiple regression analysis showed that the insulinogenic index was the strongest factor among the four indices in elevated 1-h PG. These results indicate that decreased insulinogenic index is the major factor in elevated 1-h PG during oral glucose tolerance test. Since NGT-HG showed significantly higher area under the curve of glucose

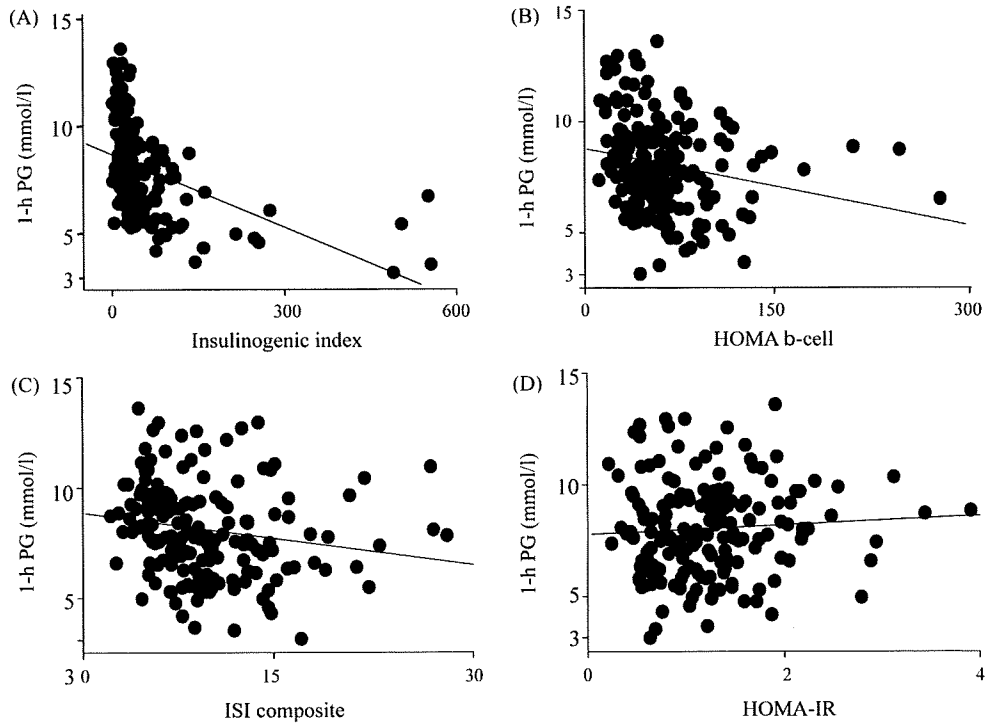

Fig. 2 – Relationship between 1-h PG and indices of insulin secretion and insulin sensitivity; (A) insulinogenic index, (B) HOMA β-cell, (C) ISI composite, (D) HOMA-IR; in insulin secretion, insulinogenic index and HOMA β-cell had significant relationships with 1-h PG ($r = -0.46$, $p < 0.001$, and $r = -0.2$, $p < 0.01$, respectively) In insulin sensitivity, there was a only mild significant relationship between 1-h PG and ISI composite ($r = -0.18$, $p < 0.05$). HOMA-IR had no significant relationship with 1-h PG ($r = 0.06$).

(G-AUC) compared to NGT-LG (19930 ± 256 vs. 15131 ± 181: $p < 0.05$), mildly impaired glucose tolerance due to reduced early-phase insulin secretion may already be present in NGT-HG. In addition, NGT-HG showed normal 2-h PG despite the elevated 1-h PG in the present study. Regarding the serum insulin level during OGTT, the 90 min insulin level in NGT-HG was significantly higher than in NGT-LG subjects (56.4 ± 7.3 vs. 40.3 ± 4.1: $p < 0.05$) in the cases we could analyze. Since late-phase insulin secretion in NGT-HG was sufficient to normalize 2-h PG, the regulatory mechanisms of elevated 1-h PG and 2-h PG are at least partly distinct.

HOMA β-cell measures insulin secretion capacity in the fasting state. HOMA β-cell values in NGT-HG and IGT-HG were significantly lower than those in NGT-LG and IGT-LG, but were similar to those in IFG-LG and IFG-HG. The values of HOMA β-cell is influenced with fasting PG per se. Isolated IFG subjects whose fasting PG levels are higher than those of NGT and isolated IGT had already low HOMA β-cell. It may explain for no significant difference between IFG-HG and IFG-LG in HOMA β-cell. Further studies are necessary to elucidate the other factors to influence HOMA β-cell in isolated IFG subjects.

Table 2 – Relationship of indices of insulin secretion and insulin sensitivity with 1-h PG

	Correlation coefficients	Standardized β	p-Value
Insulinogenic index	−0.47	0.42	<0.001
HOMA β-cell	−0.2	−0.31	<0.05
ISI composite	−0.18	−0.23	<0.05
HOMA-IR	0.06	0.13	n.s.

ISI composite and HOMA-IR are used to estimate insulin sensitivity [23]. We found both indices in LG and HG subjects to be similar in all three WHO categories. However, the insulin sensitivity of these subjects was higher than in Mexican Americans and Caucasians, as previously reported [26–28]. Since Japanese diabetes subjects are less obese than Caucasians, and insulin secretion rather than insulin sensitivity is the more important factor in the progression from NGT to diabetes in Japanese, it is likely that elevated 1-h PG in these subjects is mainly due to decreased early-phase insulin secretion rather than to impaired insulin sensitivity [19,29–31].

The ratio of NGT-HG subjects to total NGT subjects was 33% (58/121), while it was 69% (14/30) and 68% (32/71) for isolated IFG and isolated IGT subjects, respectively. The fact that the ratios increased similarly and progressively from NGT to isolated IFG and isolated IGT also suggests the use of 1-h PG as a marker to detect early stages of impaired glucose tolerance.

In conclusion, we have elucidated that impaired early-phase insulin secretion is strongly associated with an elevated 1-h PG level in Japanese subjects, suggesting that elevated 1-h PG may be a convenient marker to screen for decreased early-phase insulin secretion in early stage glucose intolerance.

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Conflict of interest

There are no conflict of interest.

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Comparison of M-Kyoto Solution and Histidine–Tryptophan–Ketoglutarate Solution With a Trypsin Inhibitor for Pancreas Preservation in Islet Transplantation

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The use of University of Wisconsin (UW) preservation solution in islet transplantation has some disadvantages, including inhibition of collagenase activity for pancreatic digestion. Histidine–tryptophan–ketoglutarate (HTK) solution has demonstrated an efficacy similar to UW solution for organ preservation in clinical pancreas transplantation. Recently, we reported that islet yield from porcine pancreata was significantly gtreater when they were preserved using M-Kyoto solution compared with UW solution. Here, we compared HTK solution with ulinastatin (M-HTK) and M-Kyoto solution for islet yield. In porcine islet isolation, islet yield after purification was significantly greater in the M-Kyoto/perfluorochemical (PFC) group compared with the M-HTK/PFC group. The M-Kyoto/PFC group had a significantly lower ADP/ATP ratio compared with the M-HTK/PFC group, suggesting that different islet yields might be due to the differences as energy sources of the solutions used. In conclusion, M-Kyoto/PFC solution is better for pancreas preservation before islet isolation than M-HTK/PFC solution.

Keywords: Islet transplantation, Islet isolation, M-Kyoto solution, Histidine–tryptophan–ketoglutarate solution, Trypsin inhibitor.

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Pancreatic islet transplantation represents a viable option for the treatment of patients with unstable type 1 diabetes mellitus who have frequent severe hypoglycemia and hypoglycemia unawareness (*1–6*). Since the Edmonton protocol was announced, more than 500 type 1 diabetes patients in more than 50 institutions have undergone islet transplantation to cure their disease. However, treatment of diabetic patients by pancreatic islet transplantation often requires the use of islets from two to four donors to produce insulin independence in a single recipient (*1, 2, 5, 6*). After isolation and transplantation, islets are susceptible to apoptosis, which limits their function and probably long-term islet graft survival.

Donor pancreata usually are preserved with University of Wisconsin (UW) solution. Recent reports have shown that the two-layer method (TLM), which uses oxygenated perfluorochemical (PFC) and UW solution, is superior to simple cold storage in UW not only for preserving the whole pancreas but also for improved viable islet yield in subsequent islet transplantation (*7, 8*). However, use of UW solution in islet transplantation has some disadvantages. The high potassium concentration in UW solution causes insulin release from pancreatic β cells (*9*), and the high viscosity of UW solution may prevent sufficient flushing. Moreover, UW solution inhibits the activity of Liberase, an enzyme blend used for pancreatic digestion (*10, 11*). Our previous study showed that ET-Kyoto (Kyoto solution®, Otsuka Pharmaceutical, Tokyo, Japan) with ulinastatin (Miraclid®, Mochida Pharmaceutical, Tokyo, Japan) (M-Kyoto) in combination with PFC significantly improved viable islet yields compared with UW/PFC preservation (*12*). The effectiveness of ET-Kyoto solution has also been demonstrated in clinical lung transplantation (*13, 14*) and skin flap storage (*15*). ET-Kyoto solution contains trehalose and gluconate. Trehalose has a cytoprotective effect against stress, and gluconate acts as an extracellular anti-oncotic agent, which prevents cells from swelling (*16*). Histidine–tryptophan–ketoglutarate (HTK) solution (Custodiol®, Alsbach, Hähnlein, Germany), originally developed for cardioplegia, is being used with increasing frequency in cardiac, renal, and hepatic transplantation (*17, 18*). The protective effect of HTK solution is based on the strong buffering capacity of histidine. This solution has a low viscosity, easy handling properties and a relatively low cost. Some studies have demonstrated comparable results between UW and HTK solution for pancreas preservation not only in experimental animal models (*19–21*) but also clinical pancreas transplantation (*22–24*).

In this study, we compared M-Kyoto solution with HTK solution containing ulinastatin (M-HTK) for islet isolation. Animal studies were approved by the Institu-

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TABLE 1. Pig islet isolation characteristics

	M-Kyoto (n=6)	M-HTK (n=4)
Pancreas size (g)	105.2±17.8	108.9±29.0
Operation time (min)	7.8±2.1	7.0±2.2
Warm ischemic time (min)	26.7±3.6	26.0±2.9
Cold ischemic time (min)	123.0±3.5	123.5±3.1
Phase I period (min)	10.2±3.6	9.5±2.1
Phase II period (min)	35.3±6.1	32.8±8.6

Data are expressed as mean±SD.

TABLE 2. Pig islet characteristics

	M-Kyoto (n=6)	M-HTK (n=4)
Islet yield before purification (IE/g)	10,121±1,674	7,904±4,970
Islet yield after purification (IE/g)[a]	6,599±1,854	3,147±1,979
Viability (%)	96.5±2.7	96.4±4.4
Score	9.3±0.6	9.5±1.0
Purity (%)	70.0±16.7	82.2±21.9
Recovery rate (%)	65.6±17.9	43.4±13.3
Stimulation iIndex	2.29±0.67	1.58±0.23

Data are expressed as mean±SD.
[a] Islet yield after purification was significantly greater in the M-Kyoto/PFC group than in the M-HTK/PFC group ($P<0.05$).

tional Animal Research Committees of Kyoto University, Nagoya University, and Fujita Health University. Porcine pancreata were obtained at a local slaughterhouse. About 10 minutes after the cessation of heart beating, the surgery was started. After removing the pancreas, we immediately inserted a cannula into the main pancreatic duct, infused either M-Kyoto or M-HTK preservation solution for ductal protection, and placed the pancreas into the respective two-layer preservation container (M-Kyoto/PFC or M-HTK/PFC). Islet isolation was conducted in accordance with the Kyoto Islet Isolation Method modified in the Edmonton protocol (*1, 4–6, 8, 12*). The characteristics of the porcine islet isolation protocols are shown in Table 1. There were no significant differences in pancreas size, operation time, warm ischemic time, cold ischemic time, Phase I period, or Phase II period between the two groups. Islet yield before purification was higher, but not significantly so, in the M-Kyoto/PFC group (n=6) compared with the M-HTK/PFC group (n=4). Islet yield after purification was significantly higher in the M-Kyoto/PFC group compared with the M-HTK/PFC group (Table 2). Other porcine islet characteristics are shown in Table 2. There were no other significantly different characteristics between the two groups.

Islet function was assessed by the adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio, which shows the energy status of islets and correlates with transplantation outcome, according to a procedure described by Goto and colleagues (*25*). The ADP/ATP ratio was measured using the ApoGlowTM kit (Cambrex Bio Science Nottingham Ltd., Nottingham, UK). The ADP/ATP ratio in the M-Kyoto/PFC group was significantly lower than in the M-HTK/PFC group (Fig. 1A). These data suggest that different islet isolation effects between the two preservation solutions might be due to their differences as energy sources. To assess the islet graft function of each group in vivo, mice with severe combined immunodeficiency disease (SCID; CLEA Japan, Inc., Meguro, Tokyo) were used for the experiments. The recipients were rendered diabetic by a single injection of streptozotocin (STZ) at a dose of 220 mg/kg. The 1,500 or 2,000 IE pig islets obtained from each group were transplanted into the renal subcapsular space of the left kidney of diabetic SCID mice as previously described (*26–28*). When 1500 IEs from each group were transplanted below the kidney capsule of STZ-induced diabetic SCID mice, the normoglycemic rate was

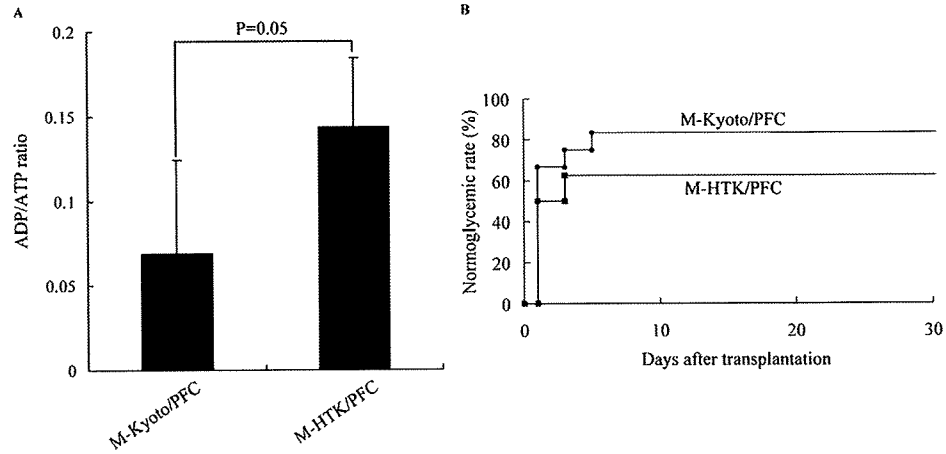

FIGURE 1. ADP/ATP ratio and transplant experiment. (A) The ADP/ATP ratio was measured to evaluate the energy status of cultured islets using the ApoGlowTM kit. The ADP/ATP ratio in the M-Kyoto/PFC group was barely lower than in the M-HTK/PFC group. Data are expressed as the mean±standard deviation. (B) Normoglycemic rate of STZ-induced diabetic SCID mice after islet transplantation. Immediately after isolation, 1,500 IEs were transplanted below the kidney capsule of diabetic SCID mice. Normoglycemia was defined as two consecutive posttransplant blood glucose levels showing less than 200 mg/dl (M-Kyoto group n=12; M-HTK group n=8).

greater, but not significantly so, in the M-Kyoto/PFC group (n=12) compared with the M-HTK/PFC group (n=8) (Fig. 1B). When 2000 IEs from each group were transplanted below the kidney capsule of STZ-induced diabetic SCID mice, the normoglycemic rate was more than 80% in both groups.

Inhibitory effects of collagenase on preservation solutions, such as UW solution, result in poor islet yield and islets of poor viability (*10, 11*). It has been reported that the components in UW solution found to be most inhibitory were magnesium, low Na+/high K+, hydroxyethyl starch (HES), and adenosine. Allopurinol, in combination with either lactobionate or glutathione, was markedly inhibitory, and the most inhibitory solution tested was a combination of three components, raffinose, glutathione, and lactobionate (*11*). M-Kyoto solution has high Na+/low K+ and, of the UW components, it contains only HES at a lower concentration. Moreover, trehalose and ulinastatin in M-Kyoto solution inhibit collagenase digestion less than UW solution (*12*). The M-HTK solution includes magnesium, but does not include HES, adenosine, allopurinol, lactobionate, glutathione, or raffinose. It has also been shown that the adenosine, allopurinol, and glutathione are not essential for the cold storage of pancreatic digests prior to islet purification (*29*). To assess the inhibitory effects on collagenase by M-Kyoto and M-HTK solutions, the rate of inhibition on collagenase digestion was measured in accordance with the modified method as previously described (*11*). The median digestion time was 79.0±2.9 min for M-Kyoto solution and 77.0±1.3 min for M-HTK solution. There are no significant differences between the two solutions on collagenase activity. Therefore, the different islet yields after purification are not due to differences in collagenase inhibition between these two solutions.

The TLM is important for preserving pancreata before islet isolation because it helps to preserve the organ, whereas UW preservation results in the deterioration of both islet isolation efficacy and posttransplant islet function (*7, 8*). The pancreas is directly oxygenated by PFC during pancreas preservation and maintains a high level of ATP in tissues. ATP drives a sodium pump, maintains cell integrity and repairs warm ischemic injury (*30, 31*). After significant warm ischemic injury, adenosine in UW solution is used as a substrate of ATP synthesis, however, without significant warm ischemia, ATP is generated from ADP or AMP located in the cells. We showed previously that ATP levels after preservation in M-Kyoto/PFC were similar to those in UW/PFC preservation and that adenosine is not importance for ATP generation when the warm ischemic time was less than thirty minutes (*12*). In this study, neither the M-Kyoto solution nor the M-HTK solution included adenosine. The ADP/ATP ratio in the M-Kyoto/PFC group was around half of that measured in the M-HTK/PFC group (Fig. 1), suggesting that different islet isolation effects between the two preservation solutions might be because of their differences as energy sources. Because energy status is an excellent predictor of successful pancreas transplantation (*32*), a low ADP/ATP ratio also might reflect effective two-layer preservation. Gluconate as an energy source or trehalose as a cytoprotectant might contribute to the effective M-Kyoto/PFC preservation of warm ischemically damaged pancreata.

In conclusion, M-Kyoto solution is superior to M-HTK solution for islet isolation. The improved pancreas preservation and islet isolation by M-Kyoto solution was not due to differences in collagenase digestion between the two solutions. However, a significantly lower ADP/ATP ratio was observed in the M-Kyoto/PFC group, compared to the M-HTK/PFC group, suggesting that the energy sources might be a factor in the islet yield differences observed between the two solutions. On the basis of these data, we now use M-Kyoto solution for clinical islet transplantation from non-heart-beating donor (NHBD) pancreata. M-Kyoto/PFC preservation makes it feasible to use NHBDs for efficient islet transplantation into type 1 diabetes.

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Original Article

Cell-Permeable Pentapeptide V5 Inhibits Apoptosis and Enhances Insulin Secretion, Allowing Experimental Single-Donor Islet Transplantation in Mice

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OBJECTIVE—Treatment of diabetic patients by pancreatic islet transplantation often requires the use of islets from two to four donors to produce insulin independence in a single recipient. Following isolation and transplantation, islets are susceptible to apoptosis, which limits their function and probably long-term islet graft survival.

RESEARCH DESIGN AND METHODS—To address this issue, we examined the effect of the cell-permeable apoptosis inhibitor pentapeptide Val-Pro-Met-Leu-Lys, V5, on pancreatic islets in a mouse model.

RESULTS—V5 treatment upregulated expression of anti-apoptotic proteins Bcl-2 and XIAP (X-linked inhibitor of apoptosis protein) by more than 3- and 11-fold and downregulated expression of apoptosis-inducing proteins Bax, Bad, and nuclear factor-κB–p65 by 10, 30, and nearly 50%, respectively. Treatment improved the recovered islet mass following collagenase digestion and isolation by 44% and in vitro glucose-responsive insulin secretion nearly fourfold. Following transplantation in streptozotocin-induced diabetic mice, 150 V5-treated islet equivalents functioned as well as 450 control untreated islet equivalents in normalizing blood glucose.

CONCLUSIONS—These studies indicate that inhibition of apoptosis by V5 significantly improves islet function following isolation and improves islet graft function following transplantation. Use of this reagent in clinical islet transplantation could have a dramatic impact on the number of patients that might benefit from this therapy and could affect long-term graft survival. ***Diabetes* 56:1259–1267, 2007**

Pancreatic islet transplantation holds great promise as a treatment for type 1 diabetes. Insulin independence has been accomplished using a glucocorticoid-free immunosuppression regimen but often requires transplantation of islets from two to four donors (1–3). Since there is a considerable shortage of pancreas donors suitable for islet isolation, relatively few diabetic patients have benefited from this form of therapy. More effective recovery of islets from donor pancreata would dramatically increase the number of patients that could be treated by islet transplantation and could improve long-term graft survival (4–6).

Following transplantation, islets undergo apoptosis and necrosis from transient local hypoxia, a lack of nutrient support (7,8), and hyperglycemia-induced toxicity (9,10). While use of fibroblast growth factor-2 (FGF-2) at the time of transplantation improves revascularization of islet grafts and facilitates their engraftment (11), this approach addresses only part of the problem. Collagenase digestion of the pancreas has been shown to induce apoptosis of isolated islets from anoikis and loss of cell-matrix interactions (12,13). In addition, islets express proinflammatory nuclear factor-κB (NF-κB)–dependent genes after isolation, amplifying apoptosis signaling and potentially inducing immunological rejection (14–16). Optimization of the isolation process to reduce islet stress has failed to improve recovery, leaving uncontrolled apoptosis as the main cause of poor islet yield (3,13,17).

Investigators have attempted to prevent β-cell apoptosis by transferring antiapoptosis molecules (A20, Bcl-2, the Iκβ [inhibitor of κβ] repressor, and X-linked inhibitor of apoptosis protein [XIAP]) and growth factors (hepatocyte and vascular endothelial) into islet grafts (18–27). In most cases, these genes have been delivered by recombinant adenovirus. This approach, however, is not without potential risk (28). Use of IGF-II (29), leptin (30), and 17 β-estradiol (31) has improved islet mass recovery and viability but has not affected the number of islets that are needed following transplantation to correct diabetes in

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FGF-2, fibroblast growth factor-2; HBSS, Hank's balanced salt solution; IL, interleukin; KRBB, Krebs-Ringer balanced buffer; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; TUNEL, transferase-mediated dUTP nick-end labeling; XIAP, X-linked inhibitor of apoptosis protein.

mice. The caspase-3 inhibitor Z-DEVD-fmk (32) improves islet recovery and viability but inhibits only caspase-3. Whereas these methods have helped reduce apoptosis during islet isolation, few methods have been developed to reduce apoptosis after islet transplantation.

We examined the efficacy of the cell-permeable pentapeptide apoptosis inhibitor V5, which inhibits a wide range of caspases, on improving pancreatic islet recovery. This molecule binds Bax and prevents mitochondrial cytochrome c translocation (33), resulting in global inhibition of caspases through activation of NF-κB–dependent and BH1–4 genes (14,15,22). In this study, we demonstrate that culture of isolated islets with V5 improved islet recovery and their capacity for glucose-responsive insulin secretion. Use of FGF-2 and V5 decreased the number of islets needed to correct diabetes following transplantation threefold and allowed routine correction of glucose homeostasis in mice using islets from a single donor.

RESEARCH DESIGN AND METHODS

Peptide synthesis and preparation. Synthesis of a V5 was carried out at Sigma Genosis (Ishikari, Japan). The purity of the material was 98.8%, and the total amount of the product was 48.9 mg. Dried peptide powders were stored at −80°C and dissolved in fresh pure water for the experiments.

Islet isolation and culture. Male inbred Balb/C mice, 20 g and 10–12 weeks old, were used as pancreas donors. All the experiments performed were approved by the institutional ethical committee and were conducted according to its guidelines. Mouse islet isolation was performed with Hank's balanced salt solution (HBSS) (GibcoBRL, Grand island, NY), containing 2 mg/ml type-V collagenase (Sigma-Aldrich, St. Louis, MO), 2 mg/ml soybean trypsin inhibitor (Sigma-Aldrich), and 0.2% BSA (Sigma-Aldrich), using a modified Gotoh's method with Histopaque 1077-RPMI 1640 medium gradient (Sigma-Aldrich).

Islets were handpicked up under a microscope and cultured with RPMI-1640 (GibcoBRL) at 37°C and 5% CO_2 for in vitro analyses. Freshly isolated islets were used immediately for transplantation experiments. Islet viability was evaluated using a Live & Dead detection kit (Molecular Probes, Eugene, OR) in accordance with the manufacturer's instructions. Purity of islets was assessed by dithizone staining, and islet equivalents' yield was determined using a phase-contrast microscopy with a squared calibrated grid. One islet equivalent was equal to a spherical islet of 150 μm in diameter. ATP content of islets, directly after isolation and following 24 h of culture, was measured at SRL (Tokyo, Japan) using 500 islet-equivalent aliquots per experiment in three separate studies.

Measurement of mitochondrial activity of islets. Islets of 50 islet equivalents were cultured for 24 h with or without 100 μmol/l V5 in each well of six-well plates (BD Biosciences, San Jose, CA), and mitochondrial dehydrogenases activity of islets were comparatively measured using 0.5 mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT reagent; Sigma). Freshly isolated islets were used as a positive control (34). Three independent experiments were performed.

Detection of apoptosis of islets by annexin-V expression. Expression of annexin-V was measured in islets treated with or without 100 μmol/l V5 at 24 h of culture. Islets were washed twice with HBSS and dispersed to a single cell by gentle pipeting in trypsin-EDTA (Sigma-Aldrich). Single islet cells were washed twice with HBSS containing 10% newborn calf serum (Sigma-Aldrich) and labeled with an annexin V–enhanced green fluorescent protein apoptosis detection kit (MBL, Nagoya, Japan), according to the manufacturer's instructions, and analyzed by a MoFlo cell sorter (Dako-Cytomation, Tokyo, Japan). Apoptotic cells were identified by the fluorescence of enhanced green fluorescent protein. Freshly isolated islets were used as a control (3). Three independent experiments were performed.

Power blot analysis for apoptosis-associated molecules. Islets (1,000 islet equivalents) were cultured with RPMI-1640-S with or without V5 (100 μmol/l) for 24 h. Then, islets were washed with HBSS twice, rinsed with ice-cold lysis buffer of PBS containing 1.0% Triton X-100 (Sigma-Aldrich), sonicated for 30 s, and placed on ice for 10 min. Lysates were centrifuged at 15,000 rpm for 10 min at 4°C to exclude cellular debris. Protein concentrates were collected and analyzed for 50 apoptosis-associated molecules according to the manufacture's protocol (Clonotech, Tokyo, Japan). Three independent experiments were performed.

Measurement of insulin secretion, insulin content, and stimulation index of islets. Islets (10 islet equivalents/well of six-well plates) were cultured in RPMI-1640-S supplemented with or without V5 (100 μmol/l) in both standard and ultralow attachment plates (BD Bioscience, Tokyo, Japan). Islets were treated for 20 min in 2 ml of Krebs-Ringer balanced buffer (KRBB) (containing 143.0 mmol/l Na, 5.8 mmol/l K, 2.5 mmol/l Ca, 1.2 mmol/l Mg_2, 124.1 mmol/l Cl, 1.2 mmol/l PO-4, 1.2 mmol/l SO-4, 25 mmol/l HCO-3, 10 mmol/l HEPES, 0.2% BSA, and 3.3 or 25 mmol/l glucose) at pH 7.4 for RPMI-1640-S equilibration. Insulin secretion of the islets was measured under static incubation using a Mercodia mouse insulin enzyme-linked immunosorbent assay kit (Uppsala, Sweden) at 0, 24, 72, 120, and 168 h, as previously reported (35,36). Briefly, islets were first incubated at 37°C and 5% CO_2 for 2 h in KRBB with 3.3 mmol/l glucose, then in KRBB with 25 mmol/l glucose for 2 h, and finally in KRBB with 3.3 mmol/l glucose. Amount of insulin content of islets was measured at the end of static incubation. Three independent experiments were performed.

Transplantation experiments. Female inbred Balb/C mice, 20 g and 10 weeks old, received a single intraperitoneal injection of 220 mg streptozotocin per kg body wt. Mice with blood glucose levels >360 mg/dl on a minimum of two consecutive measurements were selected as recipients (34). Gelatinized microspheres for islet transplantation (30–50 μm in size) containing both FGF-2 (100 ng) and V5 (100 μmol/l) or FGF-2 (100 ng) only were prepared through glutaraldehyde cross-linking of an aqueous gelatin solution, as previously reported (37). For transplantation, freshly isolated islets were suspended in 10 μl RMPI-1640-S medium, embedded with the gelatinized microspheres, and then transplanted under the kidney capsule of diabetic mice.

Diabetic mice were divided into the following four groups: *1*) $n = 21$, transplantation with 150 islet equivalents obtained from one mouse prepared with FGF-2 only; *2*) $n = 21$, transplantation with 150 islet equivalents obtained from one mouse prepared with both FGF-2 and V5; *3*) $n = 21$, transplantation with 450 islet equivalents obtained from three mice prepared with FGF-2 only; and *4*) $n = 5$, animals received no islet transplants. Normal healthy mice were used as a positive control (group 5; $n = 5$).

In vivo evaluation after islet transplantation in diabetic mice. Blood glucose levels were monitored at regular intervals for 27 weeks. Normoglycemia was defined to be <126 mg/dl in at least two consecutive measurements. An intraperitoneal glucose tolerance test was performed at 24 weeks. Mice were fasted overnight and then glucose (1g/kg body wt) was injected intraperitoneally, as previously described (34). Nephrectomy was performed at 26 weeks in the mice of groups 1, 2, and 3, and total insulin content of the samples was measured per microgram graft (34).

Histological studies of kidneys bearing islet grafts. Kidneys bearing islet grafts were removed at 3 days and at 26 weeks after transplantation ($n = 3$ from groups 1, 2, and 3), fixed in 10% formalin for 24 h, and embedded in paraffin for hematoxylin-eosin staining, insulin staining, and transferase-mediated dUTP nick-end labeling (TUNEL) staining. Serial-matched paraffin sections were used for these stainings. Polyclonal anti-insulin guinea pig primary antibodies (Dakocytomation) were applied. Then, secondary antibody phycoethrin-labeled anti–guinea pig (Amersham Biosciences) was added. Green fluorescent nuclear counterstaining was used for all the samples. Immunofluorescent stained slides were observed under a confocal laser-scanning microscope (LSM510; Carl Zeiss) (34). An in situ cell death detection tetramethylrhodamine red kit (Roche, Mannheim, Germany) was used for TUNEL staining.

PCR analysis. For detection of inflammatory molecules, total RNA was extracted from kidneys bearing islet grafts 2 days after transplantation using RNA Trizol (Invitrogen), as previously reported (38). RT-PCR was performed at 22°C for 10 min and then at 42°C for 20 min using 1.0 μg RNA per reaction to ensure that the amount of cDNA amplified was proportional to the mRNA present in the original samples. The following specific primers were used: interleukin (IL)-1β (NM_008361), 5′-caggcaggcagtatcactca-3′ forward and 5′-agctcatatgggtccgacag-3 reverse; tumor necrosis factor (TNF)α (NM_01369), 5′-agtccgggcaggtctacttt-3 forward and 5′-ggtcactgtcccagcatctt-3 reverse; Bcl-2 (NM_009741), 5′-aggagcaggtgcctacaaga-3 forward and 5′-gcattttcccaccactgtct-3 reverse; and GAPDH (NM_008084) 5-acccagaagactgtggatgg-3 forward and 5′-cacattgggggtaggaacac-3 reverse.

Immunoelectron microscopic examination of kidneys bearing islet grafts. At 26 weeks, kidneys bearing islet grafts were harvested ($n = 3$ each from groups 1, 2, and 3) and fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/l phosphate buffer at pH 7.4. Samples were embedded in LR White (London Resin, London, U.K.). Ultrathin sections on nickel grids were incubated with 6 mol/l urea in 0.1 mol/l glycine-HCl buffer (pH 3.5) for 5 min to etch the surface of sections. The grids were incubated with polyclonal anti-insulin–guinea pig primary antibodies (DakoCytomation) at 4°C overnight. After washing, the grids were incubated for 1.5 h with 10 nm colloidal gold-conjugated goat anti–guinea pig IgG (British Biocell Interna-

TABLE 1
Effect of V5 on mouse islets isolated in association with 12 h of cold ischemia and 30 min of warm ischemia

Experiments	V5	Islet yield	Islet viability (%)			ATP (pg/islet equivalent)	
			0 h	12 h	24 h	0 h	24 h
Cold ischemia	+	110.8 ± 9.4	93.3 ± 2.1	92.1 ± 2.3*	88.9 ± 3.8*	39.4 ± 8.2	129.3 ± 11.2*
	−	108.3 ± 10.8	92.4 ± 3.7	71.4 ± 6.4*	57.3 ± 5.6*	40.2 ± 7.9	68.4 ± 8.4*
Warm ischemia	+	83.5 ± 7.3	58.9 ± 10.6	46.0 ± 8.2§	39.2 ± 7.9*	19.8 ± 3.5	45.9 ± 8.5*
	−	82.3 ± 8.6	60.3 ± 9.2	37.8 ± 4.5†	14.8 ± 4.7*	21.6 ± 2.8	16.1 ± 5.8*

Data are means ± SE. *$P < 0.01$ for V5(+) vs. V5(−); †$P < 0.05$ for V5(+) vs. V5(−).

tional). The grids were washed, postfixed with 2% glutaraldehyde, rinsed with ddH2O, and dried. The sections were stained with 2% uranyl acetate for 15 min and 3% lead citrate for 1 min and observed with a Hitachi H-7100 transmission electron microscope (35).

Statistical analysis. Results were expressed as means ± SE. For comparisons between two groups, the paired or unpaired Student's *t* test (two tailed) was used. For multiple comparisons, the one-way ANOVA was used. Kaplan-Meier method was used to calculate the survival data. A *P* value <0.05 was considered significant when determined by the Mann-Whitney *U* test.

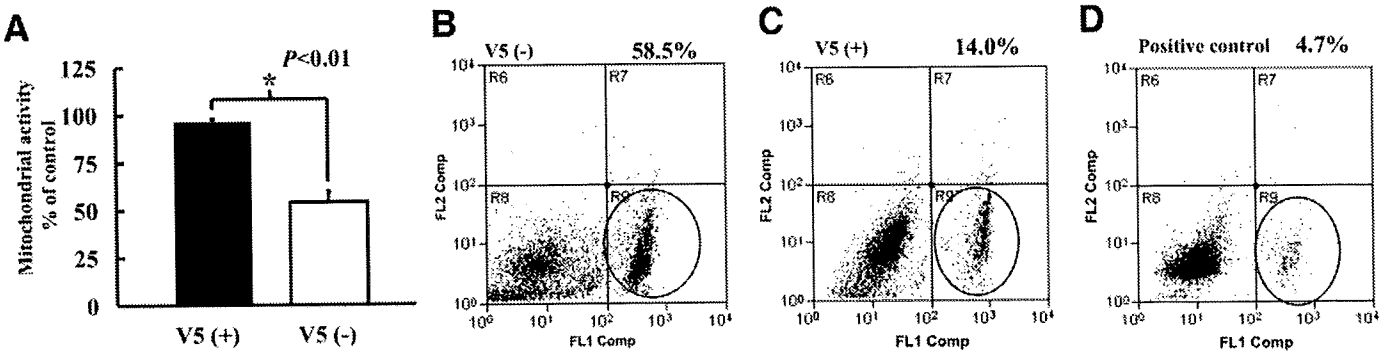
RESULTS

Effect of treatment with V5 on islet viability and function, as well as parameters associated with apoptosis in vitro. Pancreatic islet yield following isolation was 152.5 ± 3.46 islet equivalents per mouse. Islet viability immediately after isolation, and 12 and 24 h later in control culture, was 99.1 ± 0.7%, 81.0 ± 1.2%, and 72.0 ± 0.9%, respectively, whereas viability at the same time points after culture in the presence of 100 μmol/l V5 was 98.9 ± 0.6%, 95.4 ± 0.8%, and 93.5 ± 0.6%. To assess the effect of V5 on islets recovered under clinically relevant donor recovery conditions, we also evaluated the effect of V5 on mouse islets isolated in association with 12 h of cold ischemia and 30 min of warm ischemia. We found that V5 was effective in protecting mouse islets under these conditions (Table 1). Since apoptosis is often reflected in mitochondrial function, we assessed mitochondrial dehydrogenase activity 24 h after isolation. As shown in Fig. 1*A*, mitochondrial dehydrogenase activity was significantly greater in V5-treated islets (96.4 ± 1.5%) than that in control untreated islets (54.0 ± 5.7%). We also measured the expression of annexin-V, an early marker of apoptosis, 24 h after isolation. V5-treated islets expressed significantly less annexin-V (14.0%) than control untreated islets (58.5%) (Fig. 1*B–D*). A power blot was then performed to identify proteins significantly (more than twofold) altered in isolated islets after treatment for 24 h in V5. Power blot analysis screened over 50 proteins associated with apoptosis (Fig. 1*E*). Western blot analysis showed a 0.11-fold reduction in the expression of the pro-apoptotic protein Bax, a 0.34-fold reduction in Bad, and a 0.46-fold reduction in NF-κB–p65. Treatment of islets with V5 also generated an 11.76-fold upregulation in XIAP and a 3.31-fold increase in Bcl-2 expression (Fig. 1*E* and F).

To further investigate the effect of V5 on recovered islets, we analyzed glucose-responsive insulin secretion, or insulin secretion index, immediately after isolation and 24, 72, 120, and 168 h later. Since islets adherent to culture plates lose function quickly, analysis was performed on islets under both adherent and nonadherent conditions. V5-treated islets had a 2.7- to 3.7-fold higher insulin secretion index than control untreated islets at all time points under both adherent and nonadherent conditions (Fig. 2*A* and *B*), and V5-treated islets maintained their insulin content (128 ± 1 at 24 h and 76 ± 6 at 168 h under adherent culture conditions; 128 ± 3 at 24 h and 101 ± 5 at 168 h under nonadherent culture conditions) significantly better than untreated control islets (116 ± 5 at 24 h and 9 ± 2 at 168 h under adherent culture conditions; 117 ± 4 at 24 h and 80 ± 4 at 168 h under nonadherent culture conditions) (Fig. 2*C* and *D*), respectively.

Effect of V5 on recovered islets after transplantation. To investigate the effect of V5 treatment on recovered islets after transplantation, we transplanted V5-treated and control untreated islets into streptozotocin-induced diabetic mice. Transplantation procedures induce a meaningful grade of apoptosis, dramatically reducing the islet engraftment capacities and its survival by induction of the inflammatory reactions. We examined the potential prevention of apoptosis in vivo by microspheres containing V5 and FGF-2 within the islets grafts, 3 days after transplantation, by insulin staining and TUNEL assay. We found that the number of insulin-positive cells was significantly maintained by V5 treatment (150 control untreated islet equivalents: 51 ± 9 cells/high power field vs. 150 V5-treated islet equivalents: 202 ± 23 cells/high power field; Fig. 3*A–F* and *J*). Notably, a significantly larger number of TUNEL-positive cells were observed in control untreated islet grafts than found in V5-treated islets (Fig. 3*G–I* and *K*). V5 treatment significantly reduced inflammatory molecule IL-1β and TNF-α gene expression and enhanced Bcl-2 gene expression in islet grafts (Fig. 3).

In consistency with this, following transplantation of 150 V5-treated islet equivalents (recovered from one donor), normoglycemia was achieved in all diabetic recipients within 12 days and 100% 6-month survival obtained (Fig. 4*A* and *B*). In contrast, transplantation of 150 control untreated islet equivalents (recovered from one donor) failed to tightly control blood glucose levels, and 60% of diabetic recipients died 6 months after transplantation. Transplantation of 450 control untreated islet equivalents (recovered from three donors) corrected hyperglycemia to the same degree as transplantation of 150 V5-treated islet equivalents and produced a similar degree of blood glucose control to that after transplantation of 150 V5-treated islet equivalents following glucose challenge (Fig. 4*C*). Removal of kidneys bearing islet grafts 26 weeks after transplantation produced hyperglycemia in all transplanted mice, indicating that the islet grafts were responsible for correction of diabetes. Histological analysis of V5-treated islet grafts showed a comparatively equal amount of both total insulin content (16.1 ± 0.4 μg/graft), compared with those (16.7 ± 1.4 μg/graft) of V5-untreated 450 islet equivalents (Fig. 4*D*), and size of the functional islet grafts (Fig. 5*B*, *C*, *E*, and *F*). Insulin immunoelectron microscopy 26 weeks after transplantation showed β- cells with numerous secretory granules and well-preserved ultrastructure organelles following V5-treatment (Fig. 5*H* and *J*), whereas control untreated islet grafts showed



E

Lane1	Lane4	Lane7
ApaF-1	Bif	DAP Kinase
Ku70	GOK/Stim1	CAS
MST3	RIP2/RICK	DFF45
EB3	Selenocysteine Lyase	Peroxiredoxin V
Bcl-x	PI31	Lane8
		Mitosin
Bax	Bcl-2	Dlg
Lane2	Lane5	BMX
SLK	DNA Polymerase e catalytic	
SLK	IKAP	APP-BP1
Kua0	IKKb	DAP3
NF-KB p65	hILP/XIAP	TRADD
IKKg/NEMO	IkBa/MAD-3	Lane9
		Integrin a5
Bad	Bad	LRP
Lane3	Lane6	FIP-2
DNA Polymerase d	TopBP1	
RIP	DNA Ligase III	PKR
MST1	Nup88	Smac/DIABLO
Fas/CD95/APO -1	hILP/XIAP	
BH1	IkBe	
	FADD	

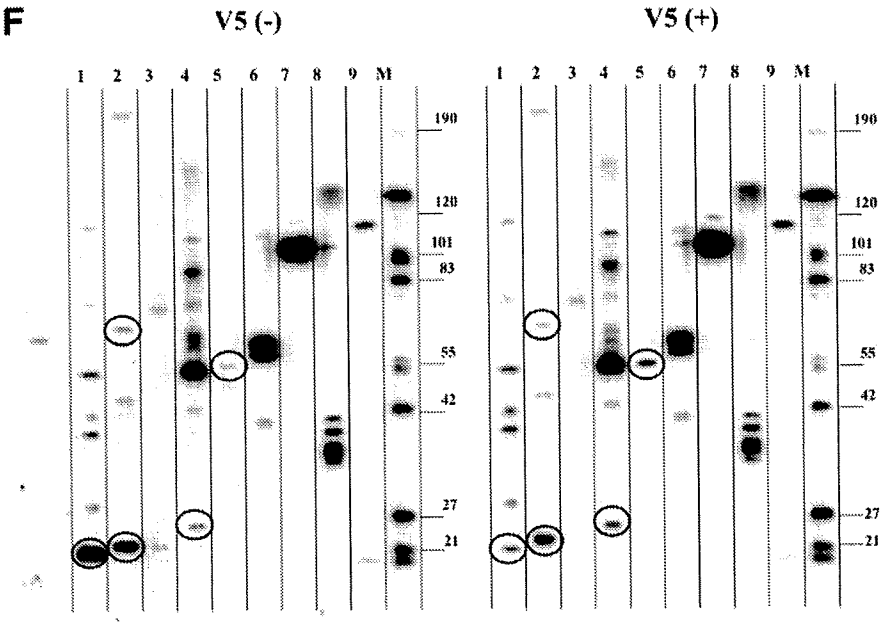


FIG. 1. Mitochondrial function, annexin-V expression, and power blot assay of the islets treated with V5. *A*: Comparative measurements of mitochondrial function of the islets at 24 h by an MTT assay. V5-treated islets showed significantly better function than untreated islets [$P < 0.01$ for V5 (+): 96.4 ± 1.5%] and V5 (−): 54.0 ± 5.7%). The value for islets immediately after isolation was 100%. *B–D*: Detection of annexin-V expression. V5-untreated islets revealed the high expression of annexin-V (58.5%) 24 h after isolation. The significantly lower expression of annexin-V (14.0%) was observed in the V5-treated islets at 24 h, which was relatively comparable with that of normal islets immediately after isolation (8.7%). *E*: List of the molecules assessed by a power blot study. *F*: The differential expression of the molecules in V5-treated or -untreated islets at 24 h. The marked reduction of the expression of pro-apoptotic proteins of Bax, Bad, and NF-κB p65 and the upregulated expression of XIAP and Bcl-2 were observed in V5-treated islets. The data are representative of three independent experiments.

fewer β-cells, each containing fewer insulin secretory granules and organelles (Fig. 5*G* and *J*).

DISCUSSION

Type 1 diabetes results from the loss of insulin-producing pancreatic β-cells by β-cell–specific autoimmune responses. Pancreatic islet transplantation is one possible method for the cure of diabetes; however, the shortage of human donor pancreata limits the widespread application of this procedure (1,5,6). Because a relatively large β-cell mass from two to four donor pancreata is needed to achieve normoglycemia in the recipient, it is crucial to develop treatments that would reduce loss of transplanted islets due to apoptosis and maximize use of the limited amount of donor tissue. Isolation of human islets is very stressful on the cells as it disrupts cell-cell and cell-matrix interactions and results in islet apoptosis (9,12,13,17, 32,38,39). Alterations in islet fine structure can be seen shortly after isolation and culture in vitro. Prevention of apoptosis has been a target to maintain islet mass for transplantation; for example, overexpression of XIAP (26) or A20 (23) in mouse islets by adenoviral vector–mediated delivery prevented early posttransplant apoptosis and reduced the islet cell mass needed to achieve normoglycemia. Although ex vivo gene transfer procedures using viral vectors are attractive, adenovirally transduced islets should be cultured to eliminate the risk of viral gene transfer to recipients. Thus, it would be beneficial to develop a simple, efficient method to protect islets from apoptosis and reduce the number of islets required for transplantation.

Bax is a member of the Bcl-2 family of proteins and plays a key role in the induction of apoptosis. In response to apoptotic stimuli, Bax translocates from the cytosol to mitochondria and causes release of apoptogenic factors (40). Inhibition of Bax would be extremely useful in islet culture immediately after isolation procedure. Ku70 plays an important role in DNA double-strand break repair in the nucleus (41). Ku70 binds Bax in the cytosol and inhibits its translocation into mitochondria (33). The Bax-binding

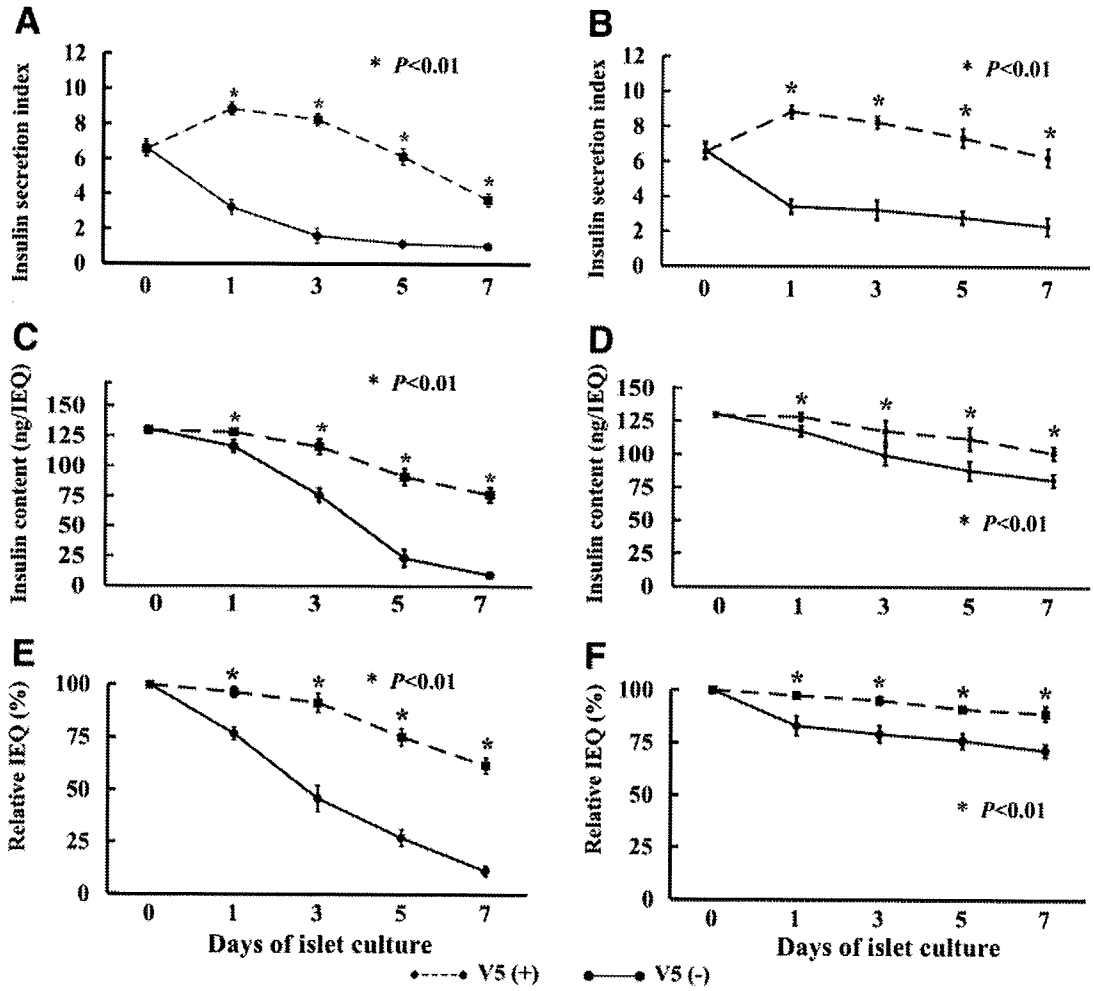


FIG. 2. Insulin secretion index and insulin content of islets. *A*: V5-treated islets showed significantly better insulin secretion index than V5-untreated islets at days 1, 3, 5, and 7. *C*: The insulin content was significantly higher in V5-treated islets at days 1, 3, 5, and 7 compared with V5-untreated islets. These data are representative of three independent experiments. *E*: Treatment of V5 allowed significantly favorable maintenance of islet mass at days 1, 3, 5, and 7. *B–F*: These findings, observed in adherent culture, were also confirmed in islets in floating culture using ultralow attachment dishes. IEQ, islet equivalent.

domain of human Ku70 consists of residues 578–583, and a pentapeptide (i.e.,V5) contained within these residues is cell permeable and suppressed Bax-mediated apoptotic cell death in several types of human cells, including hepatoma Hep3B cells and myeloid 32D (EpoR wt) cells (14). We previously found that V5 treatment of monkey hepatocyte cultures improved differentiated function and prolonged cell survival (42). In this study, we investigated the effect of V5 on islet viability and functionality during islet isolation and transplantation into diabetic mice.

First, we examined the effect of V5 on apoptosis of islets in vitro. We found that fluorescein isothyocyanate–labeled V5 (100 μmol/l) was uniformly taken up by islet cells within 3 h when added into the culture, and no cytotoxic effects were observed with a dose ≤500 μmol/l (data not shown). Treatment of islets isolated from Balb/c mice with V5 peptide significantly increased viability and inhibited apoptosis.

The mitochondrial metabolite, succinate, is a key metabolic mediator of glucose-stimulated preproinsulin gene transcription and translation (43). Therefore, we examined mitochondrial function in V5-treated islets and found that mitochondrial function was increased by ~42% compared with untreated islets. Preservation of mitochondrial function in β-cells is critical for preserving their capacity to produce, store, and secrete insulin. V5 treatment significantly enhanced ATP levels in both 12-h cold preserved and 30-min warm preserved islets (Table 1) and markedly reduced apoptosis. Consistent with these findings, glucose-responsive insulin secretion was also increased by 2.7- to 3.7-fold in V5-treated islets. Islets treated with V5 maintained the insulin content of freshly isolated islets, even after 1 week of culture. Since islet culture seems to be an important step in the islet transplantation, the use of V5 might constitute an important tool for maintaining more viable islets with enhanced insulin secretion over the conventional cultures, floating better than adherent culture with matrices. Second, we examined the expression of proteins involved in the regulation of apoptosis in V5-treated islets. We found that the expression of pro-apoptotic molecules Bax, Bad, and NF-κB was markedly reduced, and the expression of anti-apoptotic molecules XIAP and Bcl-2 was upregulated. XIAP has previously been shown to improve β-cell growth, survival, and metabolic function during stress (35,44,45) and affects Akt/protein kinase B phosphorylation (36,45), modulating Bad, caspase-9, Bcl-2, cyclic AMP–response element-binding

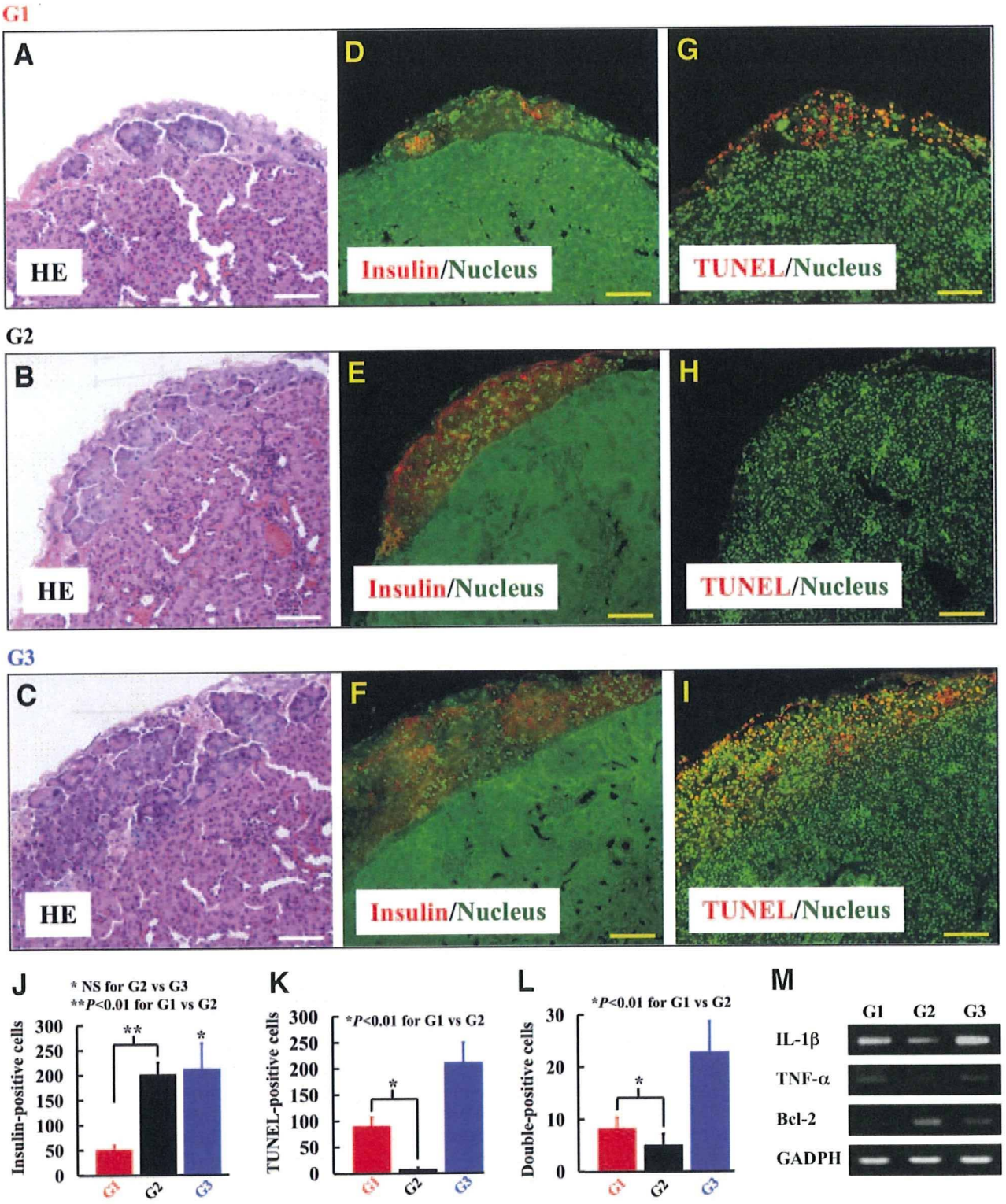


FIG. 3. Histological study of islets transplanted into the subrenal capsule in diabetic mice at 3 days. *A–C* (hematoxylin and eosin staining): A few clusters of islets were found in the graft of V5-untreated 150 islet equivalents (group 1: obtained from one donor), but much larger clusters were detected in that of V5-treated 150 islet equivalents (group 2: obtained from one donor). Amounts of islets were found in the graft of V5-untreated 450 islet equivalents (group 3: obtained from three donors), which served as a positive control. *D–F* (insulin staining): A few insulin-expressing cells, indicated by a red signal, were observed in the group 1 graft; in contrast, the group 2 graft showed significantly more insulin-positive cells, in which insulin intensity was stronger than in the cells in the group 3 graft. The observation was confirmed by counting the cell numbers of 10 different sections of each sample ($n = 3$) (*J*). The number of insulin-positive cells was 51.3 ± 8.7 for group 1, 202.1 ± 23.4 for group 2, and 212.5 ± 51.1 for group 3. *G–I* (TUNEL staining): Significantly higher ratios of TUNEL-positive cells, indicated by a red signal, were observed in the grafts of groups 1 and 3 compared with the group 2 graft. *K*: The number of TUNEL-positive cells was counted in 10 different sections of each sample ($n = 3$), and it was 91.2 ± 15.7 cells for group 1, 8.1 ± 3.1 for group 2, and 211.7 ± 37.6 for group 3. Scale bars = 200 μm. *L*: The number of double- (TUNEL- and insulin-) positive cells was counted in 10 different sections of each sample ($n = 3$), and it was 8.3 ± 2.0 cells for group 1, 5.1 ± 2.0 for group 2, and 22.9 ± 5.8 for group 3. Scale bars = 200 μm. *M*: V5 treatment significantly depressed inflammatory molecule IL-1β and TNF-α gene expression and enhanced Bcl-2 expression in islet grafts 2 days after transplantation.

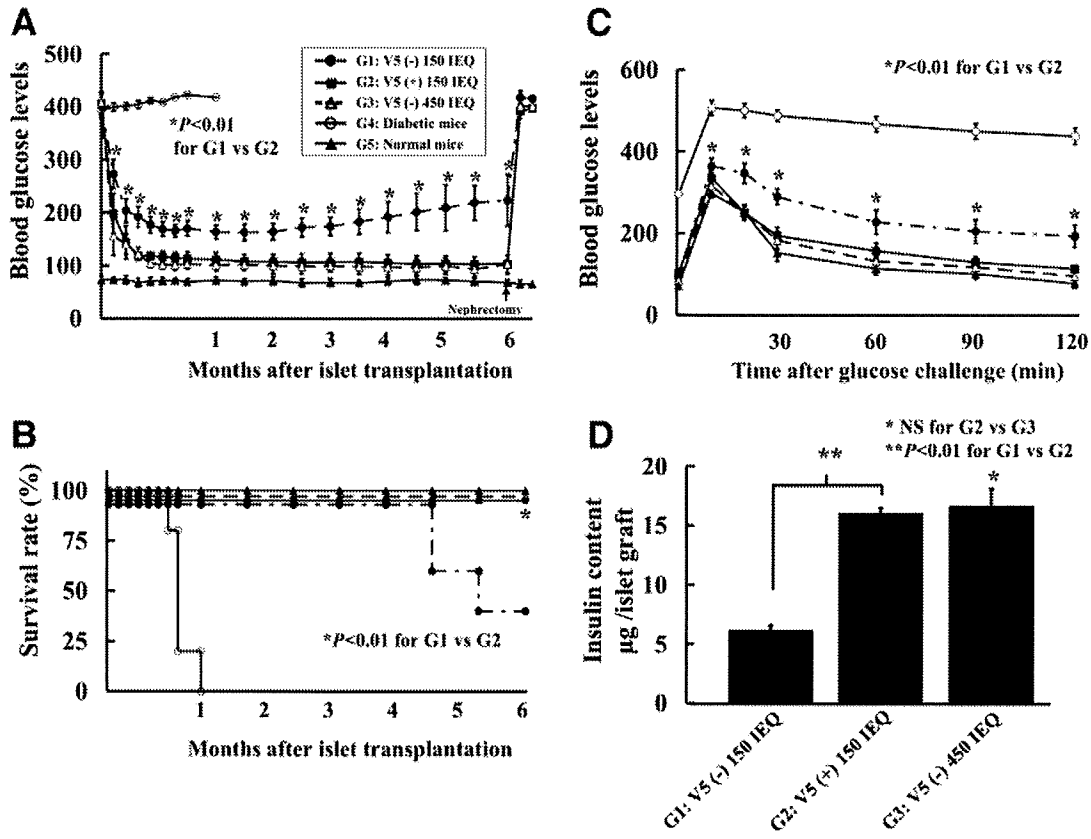


FIG. 4. Blood glucose levels, survival rate, and glucose challenge tests after islet transplantation and insulin content of islet grafts. *A*: Transplantation of V5-treated 150 islet equivalents (group 2) perfectly controlled the blood glucose levels of diabetic mice for 6 months; levels were comparable with those observed in group 3. *B*: All mice in group 2 survived for 6 months. *C*: Intraperitoneal glucose tolerance test performed at 24 weeks. The mice with group 2 showed a normal profile of blood glucose levels after glucose challenge. *D*: Insulin content of islet grafts. Significantly higher insulin content was observed in the group 2 graft (16.1 ± 0.4 mg/graft), which was comparable with that of the group 3 graft (16.7 ± 1.4), in comparison with the group 1 graft (6.2 ± 0.2). IEQ, islet equivalent.

protein, and insulin receptor substrate-1 downstream. These changes may help to protect V5-treated islets from apoptosis and increase graft survival. The beneficial effects of XIAP have been reported. Overexpression of XIAP markedly enhanced β-cell survival and functional recovery of islets in hypoxia- and cytokine-induced injury in vitro (27). Overexpression of XIAP in human islets reversed the negative effects of immunosuppressive drugs on insulin secretion and cell viability (46). Recently, it was reported that XIAP overexpression in human islets prevented posttransplant apoptosis and reduced the islet mass required to treat diabetes (26).

These promising in vitro results suggested that V5 treatment might preserve the islet mass in grafts and thus reduce the number of islets needed to obtain insulin independence. Local hypoxia and lack of nutrients can cause apoptosis in islet transplants (8). Early vascularization of islet grafts can overcome these problems and facilitate islet engraftment. We have previously found that use of gelatinized microspheres containing slow-release, cross-linked FGF-2 that persists for ~2 weeks produced rapid islet revascularization at the site of implantation (37). In those mouse islet transplantation studies, islet graft function was improved by improved vascularization. However, we also encountered considerable transplantation-associated apoptosis that resulted in loss of islet mass (11). Therefore, in this study, we transplanted islets embedded in microspheres containing FGF-2, to enhance vascularization, and V5, to reduce apoptosis, and found that apoptosis was decreased and the number of insulin-positive cells was increased in grafts containing islets treated with V5. When we embedded 150 islet equivalents within FGF-2–conjugated microspheres along with V5, diabetes was remitted within 12 days in streptozotocin-induced diabetic mice, similar to the results seen in mice transplanted with 450 islet equivalents with FGF-2 only, suggesting that normoglycemia could be achieved with islets from a single donor if V5 is provided. Interestingly, V5 treatment significantly depressed inflammatory molecule IL-1β and TNF-α gene expression in islet grafts. We found that V5 treatment similarly affected allogenic islets following transplantation in preliminary studies (supplementary Fig. 1 [available in an online appendix at http://dx.doi.org/10.2337/db06-1679]). We next plan to explore methods to protect transplanted islets from autoimmune attack and early recurrence of diabetes following allogeneic islet transplantation.

In conclusion, we have shown that treatment of islets with V5 increases islet viability, enhances islet function, and prevents apoptosis. Transplantation of islets along with FGF-2 and V5 allowed a smaller islet mass (single-donor pancreas) to be used for transplantation; normoglycemia was achieved and insulin content and islet function were preserved posttransplantation. Timed release of V5, perhaps by gelatinization, may result in long-term prevention of apoptosis and improve outcomes in human islet transplantation.

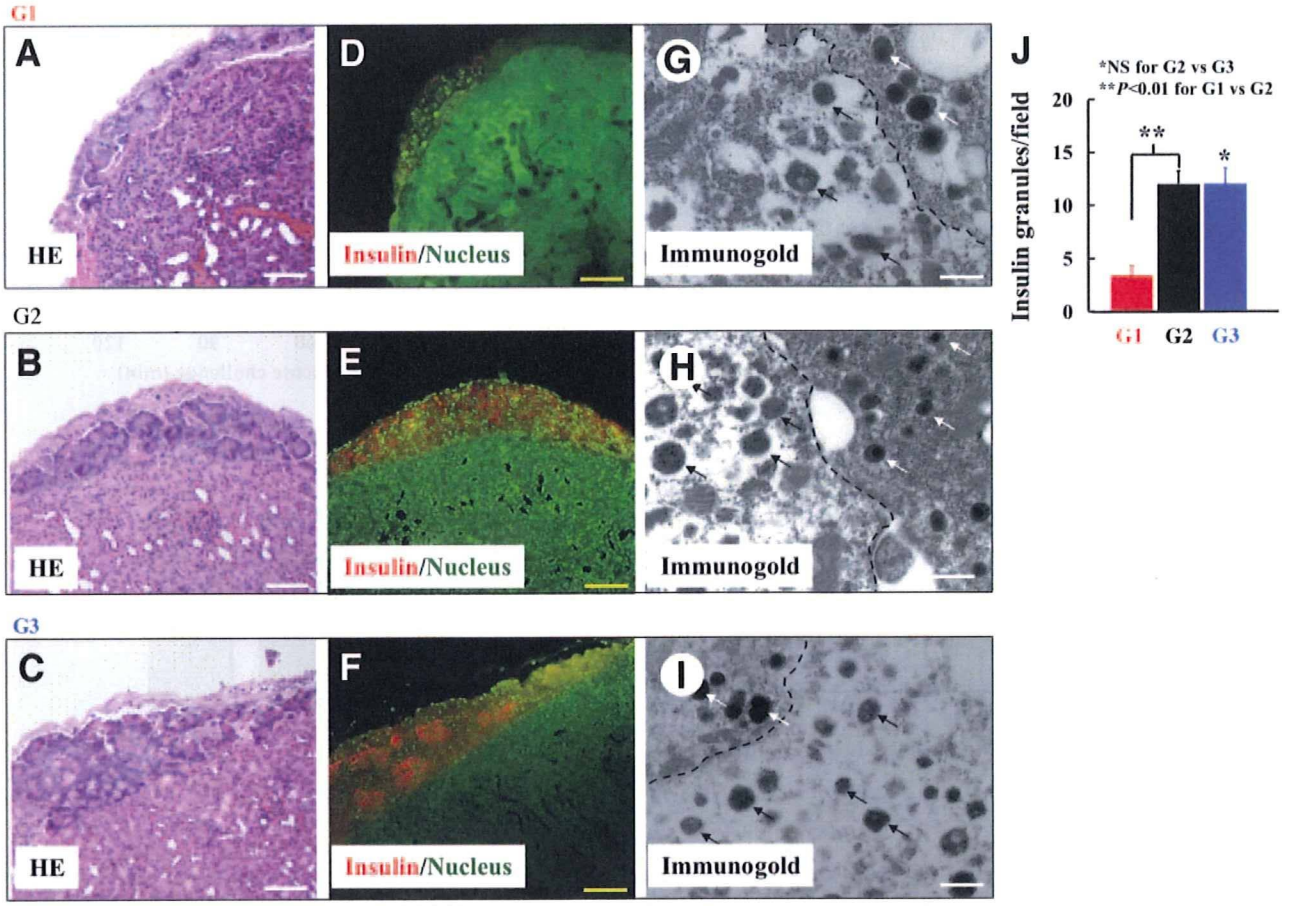


FIG. 5. Histological study of islets transplanted into the subrenal capsule in diabetic mice at 6 months. *A–C* (HE staining): Quite a few clusters of islets were found in the group 1 graft, but islet clusters were properly maintained in the group 2 graft, which was comparable with those in group 3 islets. Scale bars = 200 μm. *D–F* (insulin staining): Significantly more insulin-positive cells were observed in the group 2 graft than in the group 1 graft, which were comparable with the group 3 graft. Scale bars = 200 μm. *G–J* (transmission electron microscopy–immunogold staining): Numerous insulin secretory granules and well-preserved ultrastructural organelles were observed in the group 2 graft (12.0 ± 1.2 granules/field) when the grafts of group 1 (3.4 ± 0.09) and group 3 (12.0 ± 1.5) were considered. Such insulin granules were present in the apical zone of the β-cells in the group 2 graft. Black arrows indicate the insulin secretory granules labeled by immunogold, and white arrows show the glucagons granules (*J*). Scale bars = 300 nm. These data are representative of three different samples.

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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

levels of >140 mg/dl and/or 2-h postprandial blood glucose levels of >180 mg/dl in a single week, meriting the reintroduction of exogenous insulin (6).

Assessment of Transplanted Islet Function

Islet functioning was assessed in terms of daily serum glucose levels, the amount of insulin requirement, and HbA_{1C} before and after islet transplantation. The patients were also assessed using the SUIT index (15,42) for the simple evaluation of engraftment, and the EIR index, which shows the percentage of engrafted islets per transplanted islets. Assuming normoglycemic subjects aged <40 years have normal pancreatic β-cell mass, SUIT can be accessed from the fasting blood glucose (F-BG) and fasting C-peptide immunoreactivity (F-CPR) using the formula: 250 × F-CPR (nM) ÷ [F-BG (mM) − 3.43], where the SUIT index of normal subjects is 100.0 × 11.7 (42). For convenient daily usage the formula was simplified to: 1500 × F-CPR (ng/dl) ÷ [F-BG (mg/dl) − 63]. The SUIT index is based on the correlation of fasting blood C-peptide and glucose levels in patients after islet transplantations with that of normal individuals (42). A SUIT index of 30 means that the patient receiving islet transplantation has 30% of the functional islet mass of normal subjects. Undetectable C-peptide levels (<0.1 ng/ml) were taken as 0. The EIR was assessed using the SUIT index and transplanted islet mass (islet equivalents; IEQ) for transplantation by the formula: [SUIT after transplant − SUIT before transplant (ΔSUIT)] ÷ transplanted islet mass (IEQ) × 1,000,000. Because the SUIT index shows the percentage of functional (engrafted) islet mass compared with normal subjects, the ratio of ΔSUIT/transplanted islet mass might correlate with the rate of engrafted islets; therefore, the EIR shows the rate of functional (engrafted) islets from transplanted islets.

Assuming that a normal subject has 1 million IEQ, a SUIT index of 100 also means 1 million IEQ for that person and SUIT × 10,000 shows the number of engrafted islet equivalents. Therefore, EIR also shows the percentage of engrafted islets from transplanted islets, assuming that a normal subject has 1 million IEQ.

Statistics

The values for the collected data are represented by the mean ± SE. Two groups were compared using Student's *t*-test. A multiple regression analysis was performed with the data of the variables with a value of $p < 0.05$ in the univariate analysis. Values of $p < 0.05$ were considered to be significant.

RESULTS

Human Islet Characteristics

Islet characteristics based on the islet isolation protocol are shown in Table 1. The current criteria for the approval of clinical transplantation are an islet yield of more than 5,000 IEQ/kg body weight, purity more than 30%, viability more than 70%, tissue volume less than 10 ml, endotoxin level less than 5 EU/kg body weight, and a negative gram stain based on the Edmonton protocol (39). According to these criteria, all cases met the transplant criteria except for the islet yields. Sixteen of the 19 islet preparations were used to perform islet transplantations for seven type 1 diabetic patients. One patient was excluded from this study because her creatinine clearance was less than 80 ml/min, whereas the other six recipients were over 80 ml/min. The recipient characteristics and islet infusion characteristics are shown in Table 1.

Postoperative Course of all Cases

All transplanted islets started to secrete insulin-based on C-peptide measurements and all patients demonstrated improved blood glucose control without experiencing any hypoglycemic loss of consciousness. Three patients (#1, #4, #5) became insulin independent as shown previously (20), and the other three patients reduced their insulin amount. Patient #1 had a single positive autoantibody and patients #4 and #5 had negative autoantibodies, whereas patients #2 and #3 had double-positive autoantibodies and patient #6 had triple-positive autoantibodies (autoantibodies: anti-GAD antibody, anti-insulin antibody, anti-IA-2 antibody). The HbA_{1C} levels of all six patients gradually decreased and reach less than 6% within 3 months irrespective of their receiving single or multiple islet transplantation. C-peptide in patients #2 and #3 dramatically decreased from 3 months after the first transplantation to the next transplantation and became undetectable (C-peptide <0.1 ng/ml) before the next transplantation, as reported previously (20).

SUIT Index

To evaluate islet engraftment simply, the SUIT index was used (15,42). Table 2 shows the average SUIT index from day 7 to 3 months after transplantation or the next transplantation. The SUIT index of all patients before transplantation was 0, but increased in all patients after transplantation. The increase of the SUIT index in patients who received cultured islets was significantly lower than in patients who received fresh islets (fresh: 12.1 ± 1.7, $n = 11$; cultured: 3.8 ± 2.1, $n = 3$) (Table 4). The islet equivalents decreased by about 30% after overnight culture (before culture: 665,378 ± 101,075 IEQ; after culture: 481,469 ± 76,708 IEQ, $n = 3$). These data suggest that noncultured islets are more effective than cultured islets for islet transplantation from NHBDs. With the transplantation of fresh islets, the increase of the SUIT index in patients with double autoantibodies was significantly lower than in patients with a single or