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ORIGINAL ARTICLE

Long-term engraftment and function of transplanted pancreatic islets in vascularized segments of small intestine

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Summary

This study evaluated the potential of vascularized small intestinal segments for pancreatic islet transplantation. Islets isolated from Lewis rats were transplanted into diabetic syngeneic recipients. Segments of small intestine were prepared by denudation of the mucosal layer prior to implantation of pancreatic islets into the segments. Animal groups were established to determine engraftment, survival and function of islets transplanted into either intestinal segments or portal vein over up to 60 days. We found transplantation of functionally intact pancreatic islets into small intestinal segments was well tolerated. Transplanted islets were rapidly engrafted in intestinal segments as demonstrated vascularization and expression of insulin and glucagon throughout the 60-day duration of the studies. Transplantation of islets restored euglycemia in diabetic rats, which was similar to animals receiving islets intraportally. Moreover, animals treated with islet transplants showed normal responses to glucose challenges. Removal of graft-bearing intestinal segments led to recurrence of hyperglycemia indicating that transplanted islets were responsible for improved outcomes. Therefore, we concluded that vascularized intestinal segments supported reorganization, survival and function of transplanted islets with therapeutic efficacy in streptozotocin-treated diabetic rats. The approach described here will be appropriate for studying islet biogenesis, reorganization and function, including for cell therapy applications.

Introduction

Type-1 diabetes mellitus is caused by progressive destruction of insulin-producing pancreatic ß-cells and affects all age groups. Maintenance of normoglycemia by intense insulin treatment reduces or prevents diabetes complications [1], although this is frequently associated with recurrent episodes of severe hypoglycemia. Moreover, people with brittle diabetes or diabetes complications are often difficult to treat. In contrast, cell therapy by grafting of insulin-producing tissue or cells offers a physiological alternative to insulin alone, as indicated by experiences with islet transplantation into the portal vein [2]. On the other hand, intraportal islet transplantation may be associated with complications, e.g., portal thrombosis or subcapsular hematomas [3], and is hampered by a significant early loss of transplanted islets.

Development of alternative implantation sites for pancreatic islets should minimize surgical risks and improve therapeutic successes of islet transplantation. Previously, many transplantation sites were examined for islet transplants, including subcapsular space of the kidney [4], peritoneal cavity for implantation of vascularized biohybrid devices [5], the intramural small bowel site [6,7],

gastric submucosa [8], muscle [9], and subcutaneous sites for vascularized devices [10,11].

We considered that transplanted islets will benefit from appropriate microenvironments supporting their engraftment and survival, as well as regulated expression of required genes for maintaining euglycemia. Specific extracellular matrix components (ECM), cell–cell interactions, and access to vascular supply for endocrine functions should be among the desirable features of such microenvironments. Recently, we demonstrated that an auxiliary liver could be developed in vascularized segments of the small intestine, with synthesis and secretion of hepatic proteins, i.e., albumin, into the blood. Also, radiotracer studies demonstrated that hepatobiliary excretion pathways were appropriately preserved in transplanted auxiliary liver tissue [12,13]. These studies showed that transplanted liver tissue underwent significant reorganization and angiogenesis, such that isolated small intestinal segments maintained hepatic functions over the long term [13]. Recapitulation of this small intestinal system in transplanted pancreatic islets may be of significant interest, as tissue revascularization shall provide extensive benefits for islet engraftment, reorganization, survival and functions.

Here, we report that pancreatic islets can be successfully transplanted into vascularized small intestinal segments with the potential to correct hyperglycemia in diabetic rats, which offers a new approach to cell therapy in diabetes mellitus.

Materials and methods

Animals

Inbred male Lewis rats were obtained from Charles River Deutschland (Sulzfeld, Germany). All animal studies were performed at the Department of Clinical Anatomy of Tbilisi State Medical University. Experimental protocols and use of animals were approved by the Institutional Animal Care Committee.

Islet isolation and culture

Islets were isolated from Lewis rats weighing 340–400 g as previously described [14]. Briefly, the pancreatic duct was occluded near the duodenum and then distended by intraductal injection of 10 ml Hank's Balanced Salt Solution (HBSS) containing 0.4 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride as trypsin inhibitor, 20 PZ-U of NB1 collagenase and 0.4 DMC-U of neutral protease (Serva Electrophoresis GmbH, Germany). The pancreas was then excised and incubated for 19–21 min at 37 °C in a shaking water bath. Pancreatic tissue was vortexed on a desktop vortexer at full speed thrice for 10 s each. During digestion, tissue samples were evaluated microscopically to monitor dissociation, and undigested material was carefully removed. After three washes with HBSS supplemented with 10% newborn calf serum, the tissue pellet was resuspended in ice-cold University of Wisconsin solution and kept on ice for 30 min. Islets were then sedimented in Ficoll (Biochrom, Berlin, Germany). Discontinuous density gradients of Ficoll were formed with HBSS. Dissociated tissue was resuspended in 1.090 g/ml Ficoll and placed at the bottom followed by a layer of 1.077 mg/ml and 1.040 g/ml of Ficoll. After centrifugation at 800 *g* for 5 min, islets were collected from the interface of 1.077 and 1.040 g/ml Ficoll. After two washes, islets were picked by hand.

Isolated islets were counted manually. Islet viability was determined by trypan blue dye exclusion**.** Purity of islets was verified by dithiocarbazone staining.

Assessment of islets function *in vitro*

Islets were cultured in CMRL 1066 medium containing L-glutamine, 1 mol/l HEPES and10% fetal calf serum in humidified atmosphere of 95% air, 5% CO2 for 24 h at 37 °C. After overnight culture, insulin secretion was measured by an immunoassay specific for rat insulin (ELISA kit; Mercodia, Uppsala, Sweden) and expressed as ng/ml. Glucose-stimulated insulin secretion was determined during static glucose incubation of 20 islets for 120 min and this was expressed as stimulation index represented by the ratio of insulin released in the presence of 2.8 vs. 20 mM glucose.

Preparation of intestinal segments and islet transplantation

The intestinal segments were created as previously described [12]. Briefly, 1-cm-long segment of ileum with intact arteriovenous supply was isolated and inverted inside-out by grasping with anatomic forceps. The mucosa of the inverted intestinal segment was scarified by a surgical blade until mucosal denudation was complete. The denuded segment was irrigated with normal saline and restored to its normal inside-in position. One end of the separated intestinal segment was then closed with 7-0 Prolene. Fibrin Glue (Tissel; Baxter AG, Vienna, Austria) was applied for hemostasis and fixating/immobilizing islets. Islets were gathered by a Hamilton Syringe (Reno, NV, USA), and transferred to a polyethylene catheter, for implantation of 500 islets per recipient ($n = 6$) into the small intestinal segment, followed by application of Fibrin Glue. The other end of the intestinal segment was then closed with 7-0 Prolene. The integrity of the gastrointesti-

nal tract was restored by entero-entero anastomosis with 8-0 surgical silk. The segment containing pancreatic islets was anchored to anterior abdominal wall with Fibrin Glue. After closing the abdominal wall, animals were kept warm until recovery from anesthesia. To verify that islets were functionally intact and viable *in vivo*, 500 islets were injected into the portal vein in control rats ($n = 4$).

Animals were given amoxicillin 250 mg/l in drinking water for up to 2 weeks after surgical procedures for protection against bacterial infection.

Histopathologic evaluation

Graft-bearing intestinal segments were collected, fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections of 5 μm thickness were stained with hematoxylin and eosin and Masson's trichrome for morphological evaluation.

Immunohistochemistry

Expression of insulin and glucagon was analyzed by staining with mouse monoclonal anti-insulin (1:1000) and mouse monoclonal anti-glucagon antibodies (1:2000) (ab6995 and ab10988 respectively; Abcam plc, Cambridge, UK). To demonstrate proliferation in transplanted islet cells, tissue sections were stained with rabbit monoclonal Ki-67 antibody (1:150) (ab16667; Abcam plc). Tissue revascularization was verified by immunostaining with α-smooth muscle actin (SMA) antibody (1:100) (ab18147; Abcam plc). Secondary detection was performed with rabbit-specific goat IgG for Ki-67 (ab6721; Abcam plc) and mouse-specific rabbit IgG for insulin, glucagon and α-SMA (ab6728; Abcam plc) with diaminobenzidine (DAB) substrate (Abcam plc).

Studies of islet function

Diabetes was induced in inbred male Lewis rats of 6–9 weeks of age, weighing 160–220 g, by one intraperitoneal dose of 65 mg/kg streptozotocin (STZ; Sigma, Steinhein, Germany). After islet transplantation, nonfasting blood glucose levels were measured daily during the first week, twice per week for the first month, and once per week until the end of the study. Animals were considered hyperglycemic when blood glucose levels were ≥350 mg/dl for two consecutive days.

Islets were transplanted in diabetic animals 5–7 days after STZ treatment. Graft function was defined as nonfasting glycemic levels ≤200 mg/dl. Graft failure was defined as blood glucose levels ≥350 mg/dl for at least two consecutive days. To exclude residual function of the native pancreas, graft-bearing intestinal segments were removed 7, 12, 30 and 60 days after initial islet transplantation surgery followed by further monitoring of blood glucose levels.

Glucose tolerance test was performed 58 days after islet transplantation to assess the metabolic activity of transplanted islets in intestinal segments. Fasting animals were given 2 g/kg glucose in normal saline intravenously followed by blood glucose measurements after 15, 30, 60 and 120 min.

Statistical analysis

Data were expressed as means ± SE as appropriate. The differences were considered significant when P-value was <0.05 utilizing the Mann–Whitney test.

Results

Islet quality assessment

The number of islets isolated was 1600 ± 76 per donor pancreas. Isolated islets appeared to be morphologically intact, had a viability of 94 ± 2%, and were dithizone positive immediately after isolation, as well as after culture (not shown). The glucose stimulation index was 3.1 ± 0.5.

Engraftment of islets in small intestinal segment

The surgical procedure to create a vascularized intestinal segment required approximately 40 min. Figure 1 shows key steps of the surgical procedure. Rats recovered promptly and all animals survived after pancreatic islet transplantation into the intestinal segment. At later times (see below), the reanastomosed small intestine appeared normal without any evidence of gastrointestinal obstruction and intestinal segments containing transplanted islets showed normal morphological appearance.

Histological examination of intestinal segments demonstrated that the intestinal mucosa was completely denuded, while the submucosa was well preserved in intestinal segments containing transplanted islets (Fig. 2a). This preservation of intestinal submucosa with capillaries and other structures should have been important for maintaining the viability of the intestinal segments, along with transplanted islets. We found abundant vascular structures throughout the intestinal segments at various time points after islet transplantation. Remarkably, we did not observe inflammatory infiltrates in intestinal segments containing transplanted islets (Fig. 2a–f). The overall morphological structure of transplanted islets, which were embedded within connective tissue in the intestinal segments, appeared to be well preserved over up to 60 days (Fig. 2c–f).

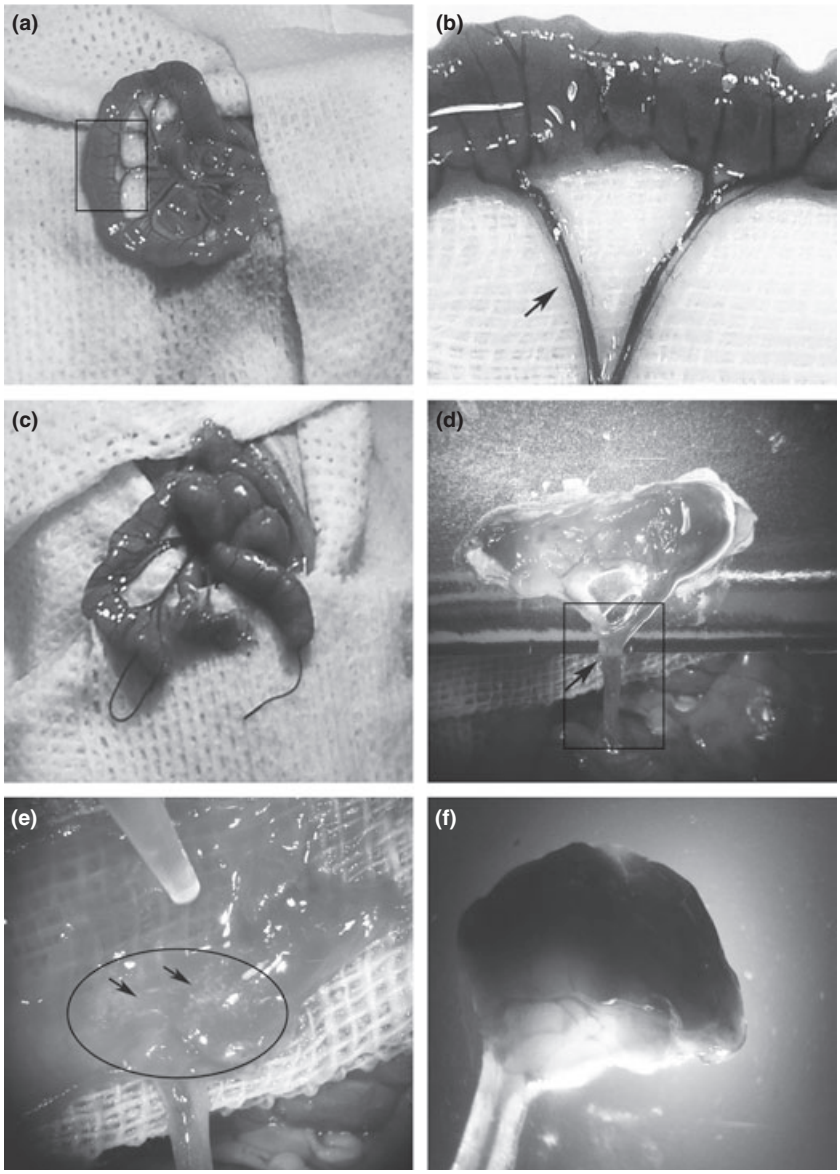

Figure 1 Surgical procedure. (a, b) Identification of an appropriate small intestinal loop (box in a) with intact arteriovenous pedicle (arrow in b). (c) Separation of the small intestinal segment and (d) closure of one end by a ligature and the final appearance of the segment with intact vascular pedicle (arrow). (e) Showing placement of pancreatic islets on the submucosa of the intestinal segment (arrows). (f) Completion of the small intestinal segment filled with pancreatic islets.

To demonstrate the functional integrity of transplanted islets, we analyzed expression of insulin and glucagon *in situ*. Insulin and glucagon were expressed in transplanted islets throughout the observation period of 60 days (Fig. 3a–d), at intensity levels similar to donor pancreas (data not shown). Similarly, immunostaining for α-SMA verified presence of mature blood vessels in peri-islet areas, as well as within the intestinal submucosa (Fig. 3e and f), which indicated vascularization of transplanted islets. We did not observe any evidence of apoptosis in transplanted islets (data not shown). Moreover, we did not detect proliferation in transplanted islets, as indicated by Ki-67 immunostaining in explanted tissue 60 days after islet transplantation (not shown).

Post-transplant function

In all diabetic animals subjected to islet transplantation, irrespective of whether islets were transplanted into intestinal segments or portal vein, blood glucose returned to a

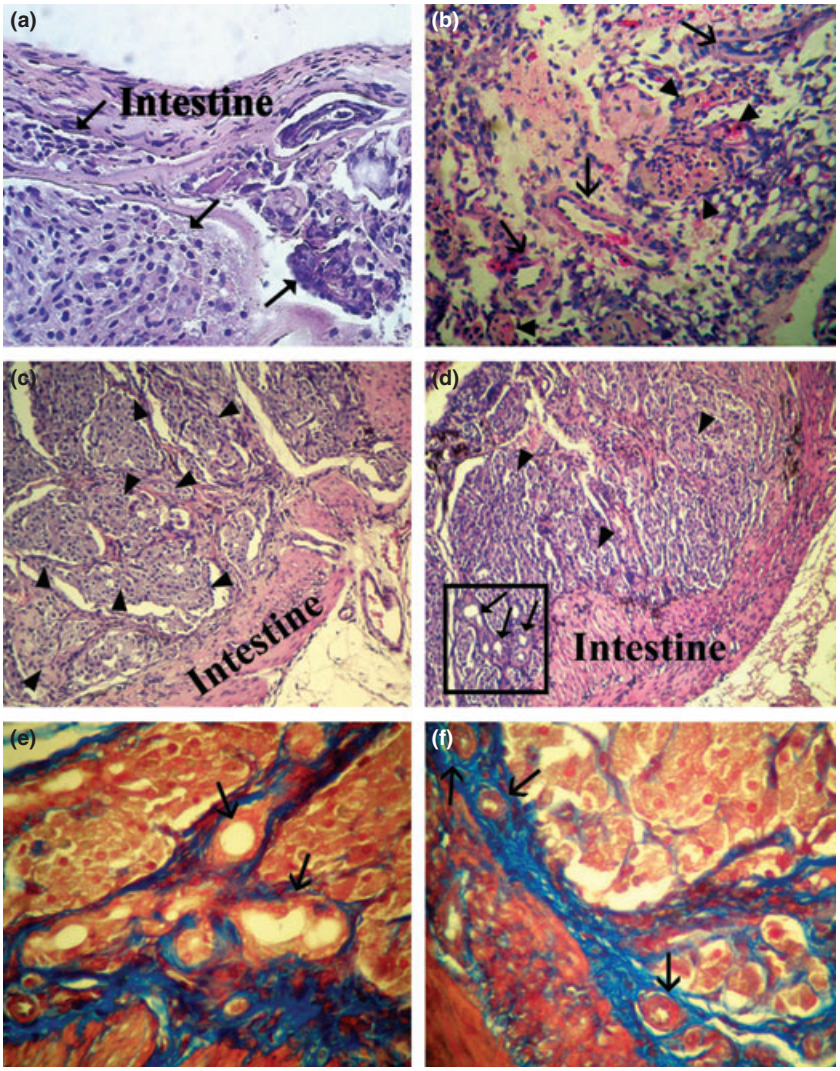

Figure 2 Histopathological assessment of explanted islet-containing intestinal segments. (a) Shows the overall organization of islets (arrowheads) bounded by the intestinal wall (intestine) 7 days after their transplantation. Note that the intestinal mucosa is completely denuded and is without obvious inflammatory infiltrates. (b) Shows numerous vascular structures (arrows) throughout the section, 12 days after transplantation of islets in the intestinal segment. (c, d) Islets (arrowheads) 60 days after transplantation into the intestinal segment. Abundant vascular structures are indicated in the box. (e, f) Collagen formation stained by blue Masson's trichrome surrounding islet structures. (a–d) Hematoxilin and eosin staining; e and f, Masson's trichrome. Original magnification: a and b, ×200; c and d, ×100; e and f, ×400.

normoglycemic level within 20 days (Fig. 4a). Consequently, transplanted animals showed improvements in clinical condition including a steady gain in body weight (Fig. 4b). On the other hand, when intestinal segments bearing transplanted islets were removed, hyperglycemia invariably returned in all rats within 24 h after graft removal, confirming that normoglycemia was restored by transplanted islets in the intestinal segments.

Glucose tolerance tests revealed excellent functional capacity of transplanted islets. In diabetic rats treated with islets transplanted into the intestinal segment, glucose challenge on day 58 showed rapid return to normoglycemia (0 min: 85 ± 12 mg/dl; 15 min: 446 ± 54 mg/dl; 30 min: 397 ± 35 mg/dl; 60 min: 260 ± 28 mg/dl; and 120 min: 87 ± 11 mg/dl). This was comparable to normal controls (0 min: 68 ± 6 mg/dl; 15 min: 418 ± 22 mg/dl; 30 min: 261 ± 28 mg/dl; 60 min: 122 ± 11 mg/dl; 120 min: 79 ± 11 mg/dl), However, diabetic rats without islet transplantation showed pathologic glucose tolerance tests (0 min: 420 ± 15 mg/dl; 15 min: 590 ± 24 mg/dl; 30 min: 520 ± 25 mg/dl; 60 min: 486 ± 13 mg/dl; 120 min: 435 ± 26 mg/dl) (Fig. 4c).

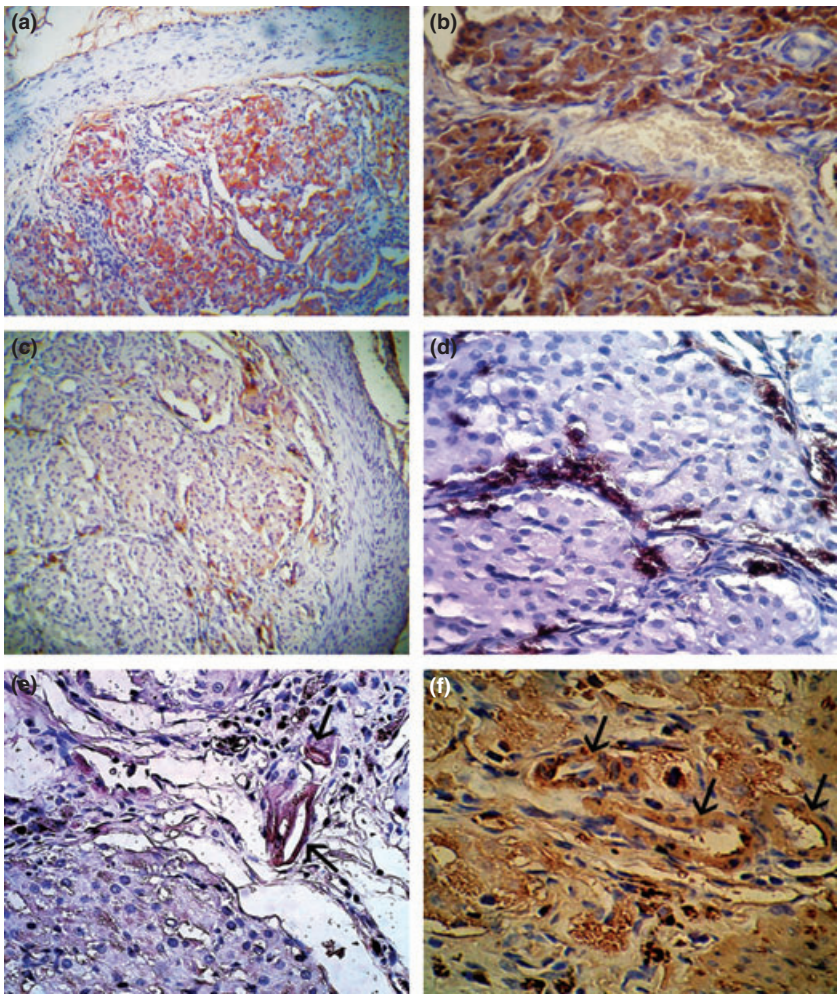

Figure 3 Endocrine and other functions in transplanted islets. Immunostaining for (a, b) insulin and (c, d) glucagon, as indicated by DAB staining in islet cells transplanted into intestinal segments. α-SMA immunostaining to identify mature blood vessels (arrows) in (e) islets transplanted into intestinal segments, as well as (f) intestinal submucosa. Original magnification: a and c, ×200; b, d, e and f, ×400.

Discussion

We demonstrated that pancreatic islets could be successfully transplanted into vascularized segments of the small intestine and this restored normoglycemia in STZ-treated diabetic rats. Histology studies revealed that islets transplanted in intestinal segments had normal morphology, and maintained expression of insulin as well as glucagon. Moreover, normoglycemia was restored in diabetic rats treated with islets transplanted in intestinal segments. Therefore, this tissue-engineering approach will be appropriate for addressing mechanisms in islet biology and for cell therapy in type-1 diabetes.

The impetus for developing novel approaches for islet transplants is driven by the limitations observed after intraportal infusion of islets, which is the commonly used procedure for islet transplantation at present. For instance, destruction of 50–80% of islets immediately after intraportal transplantation is a major problem that has not been solved yet [15,16]. Such early islet losses arise from multiple mechanisms, including onset of inflammation, blood clotting, shear forces generated by portal blood flow, as well as lack of suitable ECM to support islet engraftment and reorganization. Consequently, an excess of islets isolated from three or even more donor pancreata must be transplanted to achieve insulin independence. On the other hand, transplantation of increased number of islets may result in deleterious biological effects, e.g., release of tissue factor may activate platelets, granulocytes and monocytes with thrombotic events, tissue injury, inflammation, and loss of transplanted islets [17]. Among various deleterious mechanisms, the hyperglycemic liver environment in diabetes and exposure to immunosuppressive drugs or drug

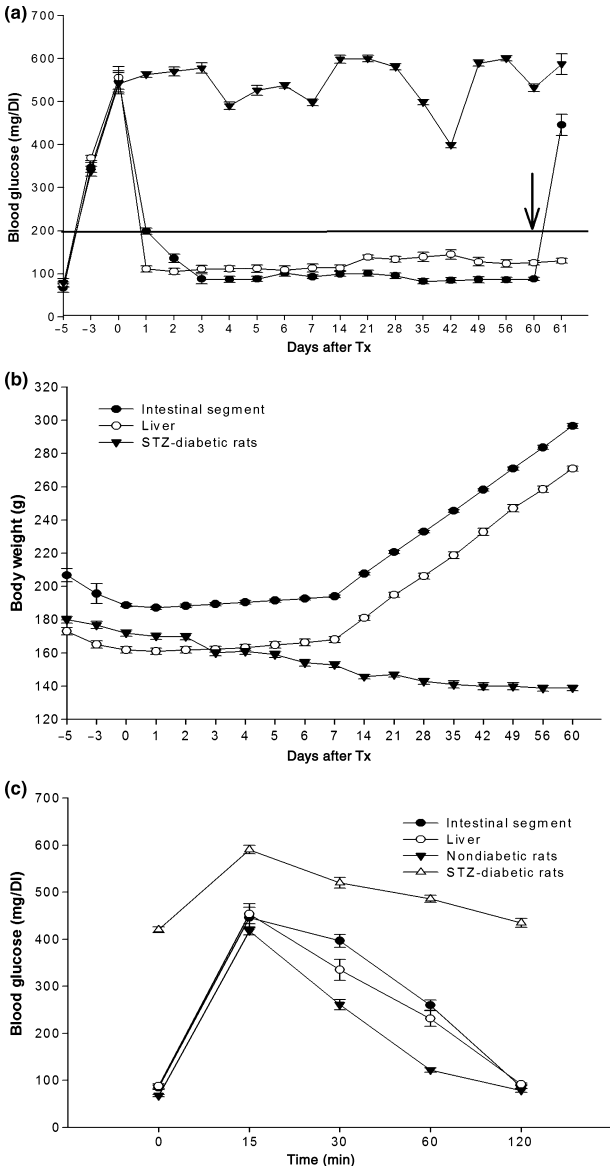

Figure 4 Regulation of blood glucose levels and body weight in diabetic rats after islet transplantation. (a) Shown are animals with small intestinal islet transplants (Group 1, -●-) and intraportal islet transplants (Group 2, -○-) to indicate return of blood glucose levels to <200 mg/dl in both groups. Control STZ-treated diabetic rats without islet transplantation (-▼-) are shown for comparison. Note that hyperglycemia promptly recurred when islet-bearing intestinal segments were removed in animals (arrow), $P < 0.05$, which confirmed that glycemic control was due to transplanted islets. (b) Showing gains in body weight in treated animals after improvement in hyperglycemia. (c) Shows normalization of glucose tolerance tests in diabetic rats after transplantation of islets into either intestinal segment (-●-) or portal vein (-○-). Nondiabetic healthy rats (-▼-) and control STZ-treated diabetic rats (-△-) are shown for comparison.

metabolites in the liver [18,19,20,21] may cause toxicity to β-cells, enhancing islet graft loss, as observed in clinical trials [22,23]. In contrast, extrahepatic sites for islet implantation may be a significant alternative, as indicated by results of intramuscular islet autotransplantation [24]. In contrast, transplantation of islets beneath the kidney capsule in human patients has not produced convincing beneficial results. It should be noteworthy that the space under the kidney capsule, while adequate for experimental studies in rodents, seems to be insufficient for humans, and limited vascularization in this site leads to generally inadequate oxygenation of islets, especially in the deeper parts of the graft [25,26]. Certainly, subcutaneous implantation of islets is technically simple, although this produced limited graft survival [27]. Similarly, survival of transplanted islets was limited in the spleen, despite its vascularized nature [4]. Although studies in animal models examined the utility of islet transplantation under the gastric submucosa and subserosa [8,28], more data are needed to understand the potential of these locations.

Our interest in vascularized small intestinal segments was stimulated by previous experience of transplanted liver tissue [13]. These studies showed that several growth factors required for angiogenesis and vasculogenesis were expressed in the intestinal wall of the vascularized segment, including vascular endothelial growth factor, fibroblast growth factor-2, hepatocyte growth factor, and transforming growth factor-β, along with several angiogenesis-related genes. This findings suggested that mucosal denudation of the intestinal segment will facilitate oxygen supply through revascularization of engrafted islets. The findings of rapid islet reorganization, including development of vessels and ECM system in the grafted tissue, correlated with long-term islet survival and post-transplant function. The microenvironment as located in intestinal segments seems to provide expression of trophic growth factors, nutrients and antiapoptotic signals as indicated by the absence of apoptosis in transplanted islets. In the past, numerous studies described the benefits of ECM on islet survival and islet functional capacity [29]. Recently, pancreatic islets were found to attach well to the small intestinal submucosa, leading to improved survival over prolonged periods [30], which was in agreement with our findings, as small intestinal submucosa is naturally enriched in collagen types I, III and VI, glycosaminoglycans (hyaluronic acid, chondroitin sulfate A and B, heparin, heparan sulfate), proteoglycans, fibronectin, etc. Therefore, we considered that the small intestinal segment fabricated from autologous tissues *in vivo* provided opportunities for graft vascularization, survival and function of transplanted islets.

In conclusion, we demonstrate that pancreatic islets can be successfully transplanted into small intestinal segments. This should encourage further development of this model to enhance islet neogenesis, survival, and

function. The tissue-engineering approach described here could eventually be considered for cell therapy in diabetes mellitus.

Authorship

ZK and EB: research design and performance of the research. ZK, SG, DB, and EB: writing of the manuscript and data analysis.

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