

Correction of Diabetes Mellitus by Transplanting Minimal Mass of Syngeneic Islets Into Vascularized Small Intestinal Segment

Z. Kakabadze¹, S. Gupta², A. Pileggi³,
R. D. Molano³, C. Ricordi³, G. Shatirishvili⁴,
G. Loladze⁴, K. Mardaleishvili⁴, M. Kakabadze¹
and E. Berishvili^{1,4,5,*}

¹Department of Clinical Anatomy, Tbilisi State Medical University, and Division of Cell Transplantation, Georgian National Institute of Medical Research, DRI Federation, Tbilisi, Georgia

²Department of Medicine and Pathology, Diabetes Center, Marion Bessin Liver Research Center, Cancer Center, Gottesman Institute for Stem Cell Research and Regenerative Medicine, and Institute for Clinical and Translational Research, Albert Einstein College of Medicine, Bronx, NY

³Department of Surgery, Medicine, Microbiology and Immunology, and Biomedical Engineering, Cell Transplant Center, Diabetes Research Institute, University of Miami, DRI Federation, Miami, FL

⁴Mardaleishvili Medical Center, Tbilisi, Georgia

⁵Center of Tissue Engineering, Ilia State University, Tbilisi, Georgia

*Corresponding author: Ekaterine Berishvili,
eberishvili@gmail.com

Transplantation of mature islets into portal vein has been most effective thus far, although attrition of transplanted islets constitutes a major limitation, and alternative approaches are required. We analyzed the mechanisms by which islets engrafted, vascularized and functioned over the long term in the small intestinal submucosa. To determine engraftment, survival and function, 350 syngenic islets were transplanted into either intestinal segments or portal vein of diabetic rats. Islet reorganization, vascularization and function were analyzed by histological analysis, RT-PCR analysis as well as glycemic control over up to 1 year. Transplantation of syngeneic islets in marginal numbers successfully restored normoglycemia in diabetic rats. Transplantation of semi-pure islet preparation did not impair their engraftment, vascularization and function. Islets were morphologically intact and expressed insulin as well as glucagon over the year. Expression of angiogenic genes permitted revascularization of transplanted islets. We identified the expression of transcription factors required for maintenance of beta cells. These studies demonstrated that marginal mass of transplanted islets was sufficient to restore

euglycemia in streptozotocin-treated rats. These superior results were obtained despite use of an impure preparation of islets in animals with small intestinal segment. Our findings will help advance new horizons for cell therapy in patients with diabetes.

Keywords: Islets, pancreas, portal vein, small intestine, transplantation

Abbreviations: bFGF, basic fibroblast growth factor; DAB, 3,3'-diaminobenzidine; GLUT2, glucose transporter 2; H&E, hematoxylin and eosin; IBMIR, instant blood-mediated inflammatory reaction; IDF, International Diabetes Federation; Pax6, paired box protein; Pdx1, pancreatic and duodenal homeobox 1; α -SMA, α -smooth muscle actin; STZ, streptozotocin; TGF- α , transforming growth factor alpha; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor

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Introduction

Type-1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of pancreatic β cells with serious complications, such as retinopathy, nephropathy or ischemic heart disease (1). According to the database of International Diabetes Federation (IDF), 285 million people, that is 6.4% of the global population, currently suffer from diabetes and 438 million people are predicted to develop diabetes by 2030 (2,3). Although a majority of these individuals will develop type-2 diabetes, approximately 4 million patients with T1DM are estimated to die of diabetic complications annually. Therefore, despite various treatments, including longstanding availability of insulin, cell therapy for T1DM is of interest, especially for brittle or otherwise difficult-to-control disease, and for achieving better physiological control of blood sugar levels. To date, cell therapy with intraportal transplantation of pancreatic islets has been most useful for treating refractory cases of T1DM (4–6). However, intraportally transplanted islets have shown limited benefits in the long term because their engraftment and persistence have been suboptimal with early as well as later progressive attrition. The introduction of glucocorticoid-free immunosuppressive regimen by the Edmonton

group improved success of islet transplantation but restrictions remained (7,8), for example, needs for transplanting islets isolated from multiple donors, since islets isolated from a single pancreas are insufficient, or repeated infusion of islets (8). According to the Collaborative Islet Transplant Registry, second infusion of islets may provide greater islet function in the long term, but allosensitization to multiple donors could pose further potential problems for subsequent transplants of islets or other organs, for example, kidneys (9).

The loss of transplanted islets is driven by multiple mechanisms. Necessary procedures and processes for islet isolation, for example, enzymatic dissociation of pancreatic tissue and overnight culture, may decrease islet viability by anoikis (10) or stress-activated intracellular signaling (11). The microenvironment in portal vein may exacerbate these issues, due to vascular, immunologic (12,13) and other factors, such as lack of extracellular matrix components (14,15). These types of differences likely contribute to immediate or early loss of 50–70% of transplanted islets (16–18). Moreover, blockade of blood flow in the portal vein by transplanted islets serving as emboli likely induces hepatic ischemia and inflammatory responses with potential for further damage to transplanted islets (19). These problems require development of alternative implantation sites for pancreatic islets to improve therapeutic outcomes (20).

Previously, we reported that vascularized segments of small intestine, which is depleted of intestinal mucosa, can support transplanted pancreatic islets (21). Here, we considered that if vascularized segments of the small intestine provided superior microenvironment along with revascularization of transplanted islets, then hyperglycemia should be better corrected despite transplantation of fewer islets. In this study, we analyzed the mechanisms by which pancreatic islets engrafted, vascularized and functioned in vascularized small intestinal segments over the long term. This permitted evaluation of whether transplantation of fewer pancreatic islets was sufficient for glycemic control in rats with streptozotocin (STZ)-induced hyperglycemia. To reproduce the effects of impure preparations of islets that are often transplanted in humans, we examined the outcomes after transplanting nonhomogeneously purified preparations of syngeneic islets.

Methods

Animals

We obtained the inbred Lewis rats from Charles River Deutschland (Sulzfeld, Germany). The rats were 6–10 weeks old and weighed 180–300 g. The animals had unlimited access to tap water and pelleted food. All experimental procedures were approved by the Animal Ethics Committee of Georgian National Institute of Medical Research, and followed Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985).

Islet isolation and culture

Rat islets were isolated by enzymatic digestion (NB1 collagenase and neutral protease, Serva Electrophoresis GmbH, Heidelberg, Germany) followed by separation in discontinuous gradient of Ficoll (Biochrom, Berlin, Germany), as previously described (21). Islets were cultured free-floating (37°C, 5% CO₂) in 5 mL of CMRL 1066 culture medium (Biochrom) supplemented with 2 mM/L L-glutamine (Biochrom), 1000 U/mL–10 mg/mL penicillin–streptomycin (Sigma–Aldrich, Taufkirchen, Germany) and 10% fetal bovine serum (Biochrom) for 20–24 h before transplants.

Preparation of vascularized intestinal segments, transplantation of islets and assays of islet function in diabetic rats

The small intestinal segments were created as previously described (21). Briefly, a 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, and 350 islets resuspended in 20 µL each of 200 IU/mL thrombin and 40 U/mL fibrin glue (Tissel; Baxter AG, Vienna, Austria) were injected into the intestinal segment. The other end of the intestinal segment was then closed with 7-0 Prolene. In control animals, groups of 350 islets were infused via portal vein into the liver. Hemostasis was secured by compressing the injection site for 3–4 min.

Diabetes was induced in male rats by single intraperitoneal dose of 65 mg/kg STZ (Sigma–Aldrich, St. Louis, MO). Rats were used for studies when blood glucose was >350 mg/dL for 2 consecutive days.

For transplantation studies, nonfasting blood glucose was measured with portable glucometers daily during the first week, every other day during the first month and once weekly thereafter.

Normoglycemia was defined as blood glucose ≤200 mg/dL. Glucose tolerance test was performed in rats 6 months after islet transplantation. Rats were fasted overnight and given 2.0 g/kg glucose intraperitoneally with blood sampling at 0, 15, 30, 60 and 120 min. To exclude confounding by residual function of native pancreas, in several animals, graft-bearing intestinal segments were removed 7, 14, 30, 60, 270 and 360 days (n = 20) after islet transplants and nonfasting blood glucose was measured over subsequent days.

Histological assessment

Graft-bearing intestinal segments were collected from three or more rats per time point. Specimens were fixed in 10% formalin, embedded in paraffin, cut into 5 µm sections and stained with hematoxylin and eosin (H&E). Immunohistochemical staining for insulin and glucagon was performed to identify transplanted islets, for vascular endothelial growth factor (VEGF) to determine angiogenesis, for α-smooth muscle actin (α-SMA) to identify blood vessels and for von Willebrand Factor (vWF) to demonstrate endothelial cells. Primary antibodies were mouse monoclonal anti-insulin (1:1000), mouse monoclonal anti-glucagon (1:1000), rabbit anti-VEGF (1:100), mouse anti α-SMA (1:100) and rabbit anti-vWF (1:400) (Abcam plc, Cambridge, UK). After incubating with secondary rabbit or goat IgG antibody, peroxidase substrate solution containing 3,3'-diaminobenzidine (DAB) was used for visualization and counterstaining used hematoxylin (Abcam plc). Images were acquired using a Leica DMLB microscope (Leica Application Suite v.3.6.0, Wetzlar, Germany).

Vascular casts

The abdominal aorta was cannulated, blood was flushed out with normal saline and 3 mL of 30% latex in water (Nairit L3, Nairit, Yerevan, Armenia) was

injected. After filling of vessels in graft-bearing intestinal segments and pancreas, tissues of interest were removed and placed between two glass microscope slides for immediate observation. Vessels in intestinal walls and transplanted islets were evaluated under stereomicroscope (MBS-30, Moscow, Russia).

mRNA extraction and RT-PCR analysis

Total cellular RNA was isolated by RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNAs (1 μ g) were reverse transcribed and amplified in 20 μ L using 1 \times RT buffer, 1 μ L reverse transcriptase, 0.08 U RNase inhibitor and 1 μ M Oligo-dT primer and dNTPs (LongRange 2Step RT-PCR kit, Qiagen). Primers used for PCR analysis and annealing temperatures are summarized in Table 1. PCR was conducted with LongRange 2 Step RT-PCR kit (Qiagen). A 10 μ L portion of amplified product was resolved on 2% agarose gels (Sigma-Aldrich, Germany) containing 0.01% ethidium bromide.

Statistical analysis

Data were expressed as mean \pm SE as appropriate. The differences were considered significant when p-value was <0.05 utilizing the Mann-Whitney test.

Results

Assessment of posttransplant graft function

Diabetic rats (n = 50) with mean nonfasting blood sugar of 540 ± 92 mg/dL received 350 islets of 50% purity to establish animal groups with intraportal (n = 20) or small intestinal segment (n = 30) transplants. Islet function significantly differed between two groups. All animals with intestinal segment transplants returned to normoglycemic state within 3 weeks after transplantation (mean nonfasting glycemia before transplantation: 535 ± 63 mg/dL; post-islet transplantation 115 ± 25 mg/dL) and maintained function up to 1 year with glucose levels in normal range (Figure 1A). By contrast, rats with intraportal islet

transplantation remained diabetic (mean nonfasting glycemia: 550 ± 52 mg/dL) and because of deterioration of general condition and substantial weight loss, most of animals in this group were humanely killed after 6 weeks of follow-up.

It was noteworthy that removal of graft-bearing intestinal segments led to recurrence of hyperglycemia within 24 h (mean nonfasting glycemia before excision: 115 ± 25 mg/dL; after excision 440 ± 35 mg/dL; n = 30).

To evaluate islet function, we transplanted 800 islets into the portal vein of severely diabetic rats (mean nonfasting glycemia before transplantation: 560 ± 72 mg/dL; n = 6). Transplanted islets displayed functional activity and glycemia decreased in the first week. Transplants reached maximal function within 3–4 weeks (data not shown).

To demonstrate metabolic competence of islets transplanted into the small intestinal segment, glucose tolerance test was performed after 180 days (n = 6). These animals showed rapid return to normoglycemia after glucose bolus, which was similar to normal controls (Figure 1B), and indicated appropriate islet function.

Histopathological analysis

Histological analysis of graft-bearing intestinal segments at early time points (3–7 days, n = 3 each) demonstrated morphologically healthy islets. We observed pathologic changes in neither transplanted islets nor intestinal submucosa. We found no evidence of leukocyte infiltration in islets, 24–74 h after transplants. Abundant vascular structures were found both in the intestinal wall and adjacent to transplanted islets commencing from 7 days after transplantation and extending to long-term follow-up. At later time points of up to 1 year, well-organized

Table 1: Primers and PCR product sizes

Gene name	Primer name	Primer sequences	Annealing temperature ($^{\circ}$ C)	Size (bp)
PDX1	PDX1-sence	5-CGGACATCTCCCCATACG-3	55	169
	PDX1-antisence	5-AAAGGGAGATGAACGCGG-3		
PAX6	PAX6-sence	5-GTCTACCAGCCAATCCACAGC-3	55	494
	PAX6-antisence	5-CAGCCATTCTCTTTCTTTCCT-3		
GLUT2	GLUT2-sence	5-TGGGTTCCTCCAGTTCG-3	55	183
	GLUT2-antisence	5-AGGCGTCTGGTGTCTGATG-3		
Insulin	Insulin-sence	5-TCTTCTACACACCCATGTCCC-3	59	148
	Insulin-antisence	5-GGTGCAGCACTGATCCAC-3		
Glucagon	Glucagon-sence	5-ACCTAGACTCCCGCCGTG-3	55	245
	Glucagon-antisence	5-ATGTCTGCGCCCAAGTTC-3		
Somatostatin	Somatostatin-sence	5-ATGCTGTCTGCCGTCTC-3	55	194
	Somatostatin-antisence	5-TTCTGTCTGGTTGGGCTC-3		
TGFA	TGFA-sence	5-GACAAGTTGAACAAGAACCCTC-3	55	250
	TGFA-antisence	5-GTCATCCACCTAATACATAAG-3		
VEGF	VEGF-sence	5-AGGCTGCACCCACGACAGAAG-3	58	363
	VEGF-antisence	5-TCACCGCCTTGGCTTGTCAC-3		
bFGF	bFGF-sence	5-AAGCGGCTCTACTGCAAGAACG-3	58	344
	bFGF-antisence	5-TTCTGTCCAGCCCCGTTTTGG-3		

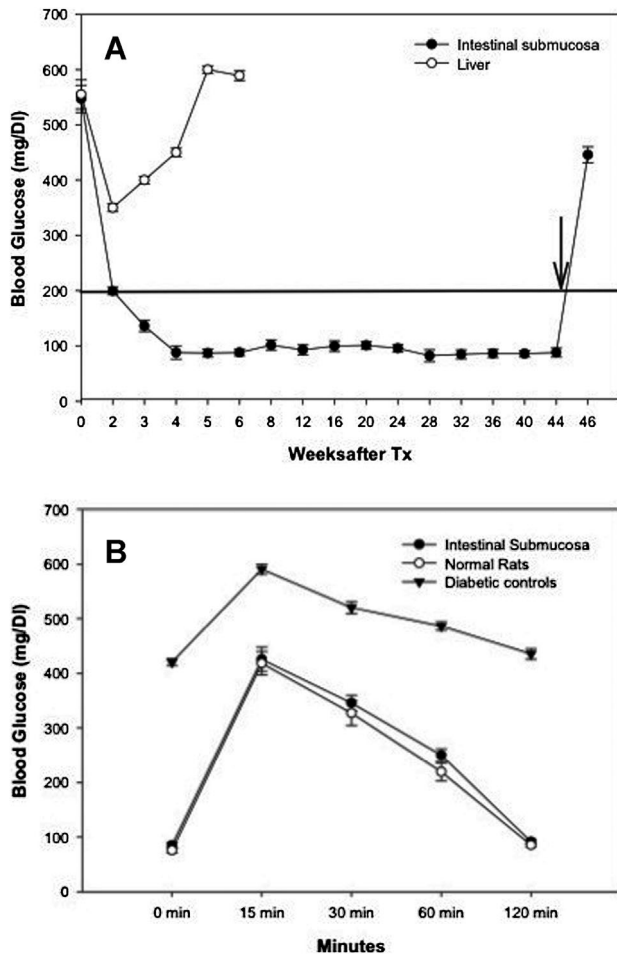


Figure 1: Glycemic control after islet transplantation. (A) Nonfasting blood glucose levels in Lewis rats with islet transplants in small intestinal segments (—●—) or intraportally (—○—). After islet transplantation in small intestinal segment, nonfasting blood glucose returned to normal levels (<200 mg/dL) over long term. By contrast, after intraportal islet transplantation, blood glucose levels remained elevated. The arrow indicates excision of islet-bearing grafts with increased blood glucose levels, indicating that functionally competent islets were present. (B) Glucose tolerance test 180 days after transplantation. The values are expressed as the mean ± SEM. Nondiabetic healthy rats (—○—) and STZ-treated diabetic rats (—▼—) are shown for comparison.

transplanted islet clusters were identified in intestinal segments (Figure 2A).

Clusters of transplanted islets were readily seen throughout the 1-year period with insulin and glucagon expression, which was similar to islets in intact pancreas (data not shown) (Figure 2B and C). Of note, glucagon staining showed typical localization in the islet periphery.

Revascularization of transplanted islets

To evaluate angiogenesis and tissue revascularization, graft-bearing intestinal segments were stained for VEGF

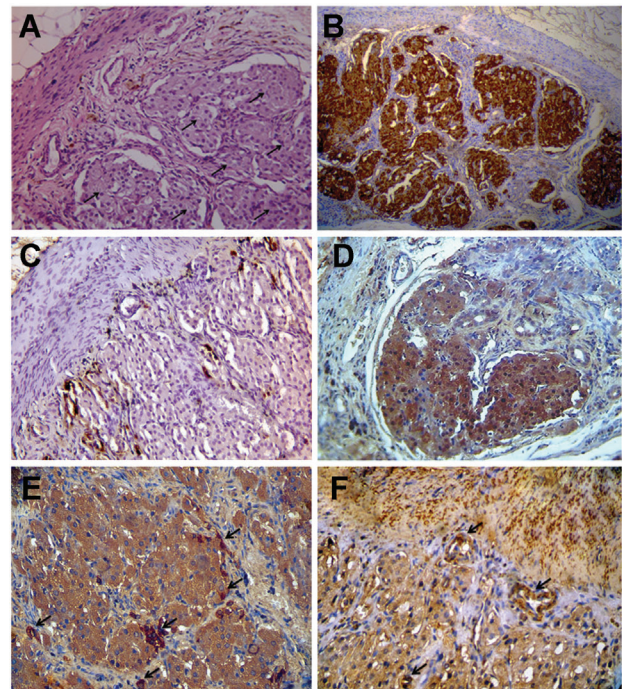


Figure 2: Histopathological evaluation, functional activity and revascularization of transplanted islets in intestinal segment. (A) Graft-bearing intestinal segment showing well-organized islet clusters (arrows), along with multiple vascular structures, after 1 year. H&E staining. (B and C) Immunostaining for insulin (B) and glucagon (C) in transplanted islets as indicated by DAB staining. (D) Expression of VEGF-A in transplanted islets, intestinal submucosa and adjacent extracellular matrix. (E) Staining for endothelial marker, von Willerbrand factor, demonstrated endothelial cells (arrows) within transplanted islets. (F) α -SMA immunostaining showing mature blood vessels (arrows) in transplanted islets and intestinal submucosa. Original magnification $\times 200$.

and α -SMA. VEGF was well expressed in the intestinal segment including in islets and intestinal submucosa (Figure 2D). Staining for vWF showed endothelial cells lining islets during long-term follow-up, thereby confirming presence of vascular structures (Figure 2E). Immunostaining for α -SMA showed that numerous blood vessels were present in intestinal submucosa and also in areas adjacent to transplanted islets (Figure 2F).

These findings of vasculogenesis in transplanted islets were confirmed by vascular casts of graft-bearing intestinal segments, which were obtained by antegrade filling of arteries via abdominal aorta and retrograde filling of veins by the portal vein. Arterial and venous networks originating from proximal arterioles and venules in intestinal submucosa were detected (Figure 3A and B). Moreover, transplanted islets were directly vascularized with arterioles emanating from the wall of intestinal segment (Figure 3C). The overall extent of vascularization and direction of blood

flow in transplanted islets were similar to those in intact pancreas from healthy control rats (Figure 3D).

Regulation of gene expression in intestinal submucosa and transplanted islets

Tissue samples were taken from rats after 1 month and 1 year (n = 8) following islet transplantation. The intestinal and islet portions of grafts were collected separately by careful dissection. We found the expression of trophic growth factors, for example, transforming growth factor alpha (TGF- α) and basic fibroblast growth factor (bFGF), in intestinal submucosa and also transplanted islets (Figure 4). Among angiogenesis regulatory genes, VEGF was expressed in intestinal submucosa and transplanted islets (Figure 4). Transcription factors, for example, Pdx1 and Pax6, which regulate expression of β cell genes, were expressed in transplanted islets. Similarly, transplanted islets expressed insulin, glucagon and somatostatin (Figure 4). The glucose transporter 2 (GLUT2) was expressed in transplanted islets as well as in the intestinal submucosa (Figure 4).

Discussion

Although intraportal islet transplantation represents an effective therapy for patients with T1DM, there are numerous challenges currently facing the clinical application of islet transplantation that need to be overcome in order to expand its indication. The first is extensive loss of islets immediately after intraportal transplantation, which

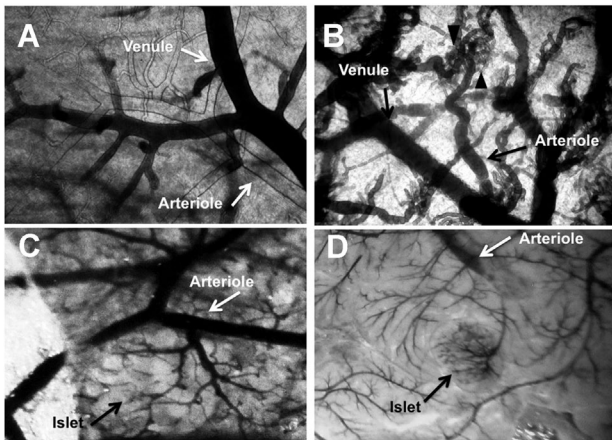


Figure 3: Vascular casting of graft-bearing intestinal segment. (A) Vascular casting immediately after islet transplantation showed truncation of submucosal vessels in intestinal segment. (B) Formation of capillary networks 12 days after transplantation (arrowheads). (C) Transplanted islets were revascularized with capillaries arising from adjacent arterioles. The vascularization of transplanted islets was similar to that of islets in intact pancreas (D).

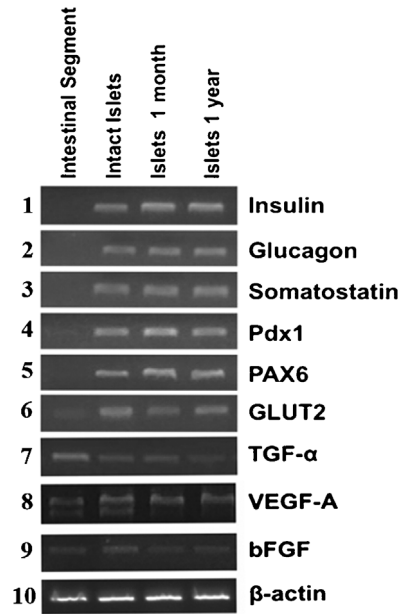


Figure 4: RT-PCR for genes encoding beta cell transcription factors, endocrine hormones and angiogenic genes. Lanes 1–4 indicate source of tissue, including transplanted islets at given times.

requires transplantation of islets from more than one donor pancreas or repeated islet transplantation (22,23). Mechanisms underlying losses of transplanted islets include exposure of islets to portal blood with an instant blood-mediated inflammatory reaction (IBMIR) and acute destruction of transplanted islets (23). In addition, transplanted islets may produce localized ischemia within the liver, with activation of monocytes/macrophages (Kupffer cells). These Kupffer cells play central roles in hepatic inflammatory responses and secrete an array of substances, including arachidonic acid metabolites, cytokines and peptides, that can directly affect survival of intraportal islets (24,25). Furthermore, Kupffer cells play fundamental roles in initiation/amplification of immune responses via antigen processing and presentation. Indeed, depletion of macrophages reduced inflammation after embolization of islets and inert beads into portal vein (26). Thus, alternative implantation sites with appropriate microenvironments supporting islet engraftment and function have been of considerable interest, for example, the omental pouch (27), intramuscular (28,29), intra-bone marrow (30), intrapancreatic (31,32), intra-ocular (33), an isolated venous sac (34) as well as prevascularized implantable devices (35–37).

In 2001, Sageshima et al (38) investigated the potential of intestinal subserosa as a place for islet transplantation. The islets were transplanted in clusters and resulted in normoglycemia in rats. Recently, the utility of islet transplantation under the gastric submucosa was also examined in large animal models (39–41).

In 2003, we reported that isolated small intestinal segment denuded from mucosa is a suitable site for hepatic fragment transplantation (42,43). Recently, we have shown that also islet cells can successfully engraft into Small Intestinal Submucosa (SIS) used as a vascularized reservoir (21). However, previously we did not elucidate mechanisms of islet engraftment and reorganization and efficacy of impure islets, especially when transplanted in low numbers.

Our findings established that use of semi-pure islet preparation did not impair islet engraftment, vascularization and function. It may be that presence of additional cells could have benefited islet engraftment and long-term maintenance of graft function and survival. For instance, presence of endothelial cells or mesenchymal cells in such preparations could have aided angiogenesis and revascularization of transplanted islets. Our histological studies revealed that transplanted islets were morphologically intact and expressed insulin as well as glucagon over the 1-year study period. We considered that the evidence of revascularization of islets in small intestinal segment was noteworthy. Expression of angiogenic genes, as confirmed by RT-PCR analysis, permitted revascularization of transplanted islets. This revascularization process should be of crucial importance for survival of transplanted islets. For instance, pretreating islets as well as the implantation site with proangiogenic factors, such as VEGF and/or FGF, was shown to accelerate islet revascularization (44). Recent studies showed that VEGF-A was responsible for high Endothelial Cell (EC) numbers in islets (45,46), and 10-fold fewer endothelial fenestrae were observed in VEGF-A-deficient islets (47). These findings suggest that suitable capillary network is required for secretory function of islets, which would have benefited from islet vascularization in the intestinal segment. Similarly, direct vascularization of transplanted islets should have contributed to their engraftment, survival and function in the SIS. Such vascularization through sprouting of new vessels cannot obviously be expected to occur in the portal vein. Other regulators of islet graft survival and reorganization should include extracellular matrix components, which are abundantly present in small intestinal submucosa, and collagens types I, III and VI, glycosaminoglycans, proteoglycans and fibronectin among others.

Moreover, we identified the expression in the SIS of transcription factors required for maintenance of β cells, including Pdx1 and Pax6. Therefore, it should be appropriate to consider that survival, engraftment and excellent long-term function of transplanted islets resulted from superior revascularization and angiogenic signaling. This possibility is in agreement with previous descriptions of trophic and cytoprotective properties in SIS for islet cells *ex vivo* (48). Isolation and demucosation of intestinal segment give the possibility to disperse islets throughout submucosal layer, which is essential for adequate gas exchange, islet engraftment and prompt vascularization. Importantly, the

relatively large surface area obtained would adequately accommodate relatively large volumes of impure islet preparations that are currently used for autologous and allogeneic transplants that are otherwise concerning for intrahepatic implantation.

Although creation of intestinal segments requires surgical methods, we did not observe the evidence of intestinal perforation or other complications in rats. Reanastomosed small intestine appeared healthy without any evidence of gastrointestinal obstruction.

Therefore, these studies of islet transplantation in small intestine segment suggest that this approach should be worthy of further development. Such studies should strengthen the rationale for cell therapy in people with islet transplantation in small intestinal segment. The safety issues have to be carefully investigated in large animal models. Nevertheless, studies in small animals indicate that this site provides excellent conditions for survival and engraftment of implanted islets, without inflammatory and fibrotic components.

Our findings offer new horizons for developing effective cell therapy applications to restore β cell function in patients with diabetes.

Authors' Contributions

Z.K. contributed to designing the study, performed experiments and analyzed data. A.P. researched and analyzed the data and revised the manuscript. S.G. contributed to designing the study, the data analysis and revising the manuscript. C.R. contributed to data analysis and revised the manuscript. R.D.M., K.M., G.S., M.K. and G.L. researched and analyzed the data. E.B. designed the study, performed experiments, researched and analyzed data and drafted and finalized the manuscript. All the authors gave final approval to the submission of the manuscript.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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