Application of an Endothelialized Modular Construct for Islet Transplantation in Syngeneic and Allogeneic Immunosuppressed Rat Models

Rohini Gupta, Ph.D., and Michael V. Sefton, Sc.D.

Modular tissue engineering is a novel approach to assemble tissues with an inherent vascularization. In this article, we evaluated whether endothelialized module-driven vascularization enhances islet engraftment in diabetic rats. Two thousand islets were transplanted in the omental pouch of syngeneic and allogeneic immunosuppressed diabetic recipients as free islets, islets in collagen modules, or islets in endothelialized modules. Transplantation of islets in endothelialized modules significantly increased the vessel density compared with controls. Donor green fluorescent protein-positive endothelial cells (ECs) formed vessels in proximity to transplanted islets; donor vessels connected to host vasculature as the vessels included erythrocytes in their lumens and were supported by host smooth muscle cells by 21 days. Transplantation of 2000 islets reversed diabetes in two of five of syngeneic recipients until 60 days, although there was no apparent benefit to islet function of adding ECs relative to collagen modules without EC. However, there was a trend toward increased viability when islets were implanted in endothelialized modules compared with collagen modules at 21 days. Meanwhile, 2000 islets in allogeneic immunosuppressed recipients lowered blood glucose levels short term, but there was graft failure within 1 week. This study explored the simultaneous transplantation of primary ECs with islets in diabetic recipients. The endothelialized modular approach increased vessel density around transplanted islets. Further modulation (i.e., acceleration) of vessel maturation, is presumed necessary to improve islet engraftment.

Introduction

AT LAST REPORT, islet transplantation had treated 500 patients worldwide, and 60% of patients remain insulin independent for 1 year.^{1–3} However, a number of limitations exist with this procedure, including the need to transplant a large islet mass (isolated from two to three pancreases) for successful treatment. It is estimated that a large majority of the transplanted islets (>60%) fail to engraft^{1,4,5} due to a number of factors, including incompatibility of blood with the islet surface and insufficient oxygen and nutrient supply to engrafted islets.⁶ Islet engraftment may be improved by protecting the islets from the inflammatory and immune systems as suggested by new biomaterial strategies^{7,8} and by improving tissue vascularization.

Native islets are highly vascularized, contain a dense network of capillaries, and are located adjacent to blood vessels to efficiently secrete insulin⁹; much of this vasculature is lost during islet isolation and culture. Revascularization of islets after transplantation can take 7–14 days and in the immediate post-transplant period, islets are hypoxic and begin to fail.^{10–12} Even long-term revascularization is not

sufficient^{12,13} as islets continue to have significantly lower levels of vascular density, receive decreased blood flow, and have a lowered oxygen tension compared with endogenous islets. Improving islet vascularization may improve islet engraftment and lower the number of islets required to reverse diabetes. Consistent with this, studies in animal models have shown that increasing vessel density around transplanted islets by delivery of angiogenic growth factors and/or co-transplantation with mesenchymal stromal cells (MSCs) can improve islet survival and function.^{14–17}

Another way to achieve neo-vascularization is through transplantation of primary endothelial cells (ECs). Donor ECs drive new blood vessel formation that can support functional cells. For example, human umbilical vein ECs co-transplanted with supporting fibroblasts or mesenchymal precursor cells integrated with the host vasculature and promoted the survival of co-transplanted skeletal, skin, and human embryonic stem cell-derived myocardial tissue.^{18–22} Here we explore the use of primary rat EC-driven vascularization to support transplanted islets.

Endothelialized modular tissue engineering is a means of creating vascularized constructs with a mixed cell popula-

tion and.²³ Functional cells are embedded in sub-millimeter sized collagen rods, and the outside surface is coated with EC. These units are referred to as endothelialized modules. By varying the type of embedded cells in the individual modules, it is expected that complex modular tissue-engineered constructs can be used for different applications; HepG2 cells,²⁴ smooth muscle cells,²⁵ and cardiomyocytes²⁶ have been incorporated into modular constructs to date. Human ECs on the surface of the modules enabled a non-thrombogenic, vascularized construct *in vitro*²³ and drove primitive vessel formation in nude rats.²⁷ Moreover, transplantation of rat aortic EC (RAEC) covered modules in the omental pouch resulted in the formation of a stable, perfusable vascular construct in immunosuppressed allogeneic recipients.²⁸ Here, we explored the use of these RAEC endothelialized modules for supporting transplanted islets in both immunosuppressed allogeneic and syngeneic diabetic recipients. In the allogeneic transplantation model, both donor and recipients were of an outbred rat strain (Sprague-Dawley) and thus received immunosuppression therapy, as before.²⁶ Meanwhile, in the syngeneic (genetically similar) model, both donor and recipients were of an inbred strain (Lewis) and did not receive immunosuppression therapy. We hypothesized that co-transplanted EC will improve the vessel density surrounding the islets, which will in turn improve islet viability and/or function.

Materials and Methods

Cells

RAECs (Sprague-Dawley or Lewis) were purchased from VEC Technologies and maintained on 0.2% gelatin-coated flasks in MCDB-131 complete medium (VEC) with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Cells were used between passages 3 and 5. RAEC were transduced with HIV-1-based recombinant lentivirus vectors to engineer the stable expression of enhanced green fluorescent protein (eGFP) (kindly provided by Dr. Medin, University Health Network, Toronto, Canada) as described before.^{29,30} For cell transduction, RAEC were infected with recombinant LV-eGFP at a multiplicity of infection of 10 in the presence of 8 µg/mL protamine sulfate. Postinfection, eGFP-RAEC were cultured with a fresh medium for at least 2 days before use. Islets were isolated from male Sprague Dawley or Lewis rats as previously described at the University of Alberta.³¹ Islets were shipped overnight in RPMI-1640 medium (Invitrogen), and cultured for at least 1 day in a fresh medium before experimental use. Islets were maintained in RPMI-1640 medium with 10% FBS.

Module fabrication

Modules were prepared as before using bovine type 1 collagen (3.1 mg/mL, Cohesion Technologies)^{23–25}; islets were suspended in collagen solution (~2000 islets in 1.5 mL solution) and the mixture was gelled (37°C, 45 min) in sterile 0.71 mm ID polyethylene tubing (Intramedic). Tubing was cut into small pieces (~1.5 mm long × 0.6 mm diameter) and collected in RPMI-1640 medium. Collagen modules were separated from the outer tubing by gentle vortexing. In some cases, RAEC (2.5 × 10⁶) were seeded dynamically onto the surface of modules for 60 min on a low-speed shaker in

MCDB-131 complete medium. For co-culture studies, modules were immediately transferred into a mixture of 50% RPMI-1640 and 50% MCDB-131 medium and incubated overnight. The following day, unattached RAEC and medium were removed and islet-embedded endothelialized modules were maintained in RPMI-1640 medium (with 10% FBS) for 2–7 days.

In vitro EC staining

For VE-cadherin staining, modules were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton-X for 4 min. Modules were incubated with anti-rat VE Cadherin (Santa Cruz Biotechnology) at 1:50 dilution for 1 h, followed by a secondary Alexa Fluor 488 (Invitrogen) at 1:300 dilution for 30 min and counterstained with 1 µg/mL of Hoechst 33258 (Invitrogen) for 5 min at room temperature. For Live/Dead staining, samples were incubated with 1 µL of Calcein AM and 2 µL of ethidium homodimer-1 (Invitrogen) in 1 mL of phosphate-buffered saline (PBS) for 20 min at 37°C. Samples were washed with PBS and immediately observed with Zeiss LSM510 confocal microscope.

Static glucose challenge assay

Four samples of either free 20 IEQ (islet diameter standardized to 150 µm diameter³²) or 20 IEQ in endothelialized modules were equilibrated in low glucose (4.2 mM) Krebs-Ringer bicarbonate (KRB; Sigma-Aldrich) buffer with 0.25% bovine serum albumin (Sigma) for 1 h. The buffer was removed and islets were incubated in fresh low glucose KRB buffer for 1 h and then in high glucose (16.7 mM) KRB buffer with 0.25% bovine serum albumin for 1 h at 37°C. One hundred microliters aliquots was taken after low-glucose and high-glucose incubation and stored at -20°C. Samples were analyzed for rat insulin using an ELISA kit per manufacturer's instructions (Mercodia) by Mount Sinai Pathology Services. The stimulation index was the ratio of insulin concentration in high-glucose solution to that in low-glucose solution for each sample, and the average value for the group is reported ± standard error of the mean.

Diabetic animals

Adult male Sprague Dawley or Lewis rats (250–300 g) were purchased from Charles River Laboratories. They were individually housed and fed *ad libitum*. Animals were rendered diabetic with 50 mg/kg of streptozotocin (Sigma-Aldrich) freshly prepared in pH 4.5 citrate buffer (Sigma-Aldrich). Blood was sampled through the tail vein and measured with a glucometer (Onetouch Ultrasmart; Life-Scan). Animals with nonfasting blood glucose levels over 16 mM for two consecutive readings over a period of 5 days were considered diabetic. Sprague Dawley animals were immunosuppressed as follows: Tacrolimus (Astellas) was administered intramuscularly daily (day 1–6: 0.3 mg/kg, day 7–21: 0.2 mg/kg) in a saline, and Atorvastatin (Pfizer) was administered daily until 21 days via oral gavage at a dose of 0.5 mg/kg dissolved in sterile water. Lewis rats received no immunosuppressant drugs. Principles of laboratory animal care (National Institutes of Health [NIH] guidelines) were followed, and the study was approved by the University of Toronto animal care committee.

Omental pouch transplants

For transplantation, the greater omentum was spread out and 7-0 silk suture was run along the edges and the top to create a pouch with a small opening as before.²⁷ Two thousand islets in endothelialized modules, 2000 islets in collagen modules without EC, or 2000 free islets were suspended in PBS (~500 modules in 0.5 mL volume) and gently delivered by a sterilized 1 mL micropipette into the pouch and allowed to settle. The pouch was folded over and sutured to secure the modules inside. In most cases, animals were killed at 21 or 60 days after transplantation.

Metabolic follow-up

For blood glucose monitoring, blood was sampled bi-weekly through the tail vein for days 0–14 and weekly from days 14 to 60. Animals that were normoglycemic (blood glucose < 11.1 mM over a period of 5 days) were subjected to an oral glucose tolerance test at day 60. After 4 h of fasting, animals were gavaged with 2 mg/kg glucose and blood glucose was measured at 0, 15, 30, 60, 90, and 120 min. For insulin measurements, blood was collected weekly from the tail vein in microtubes (Sarstedt) and centrifuged (10,000 rpm) for 5 min, and serum was stored at –20°C. Serum insulin was measured using an ELISA kit per manufacturer's instructions (Mercodia) by Mount Sinai Pathology Services.

Histology and immunostaining

At explantation, the omental pouch was excised into 4% formalin (Sigma Aldrich) and fixed for 48 h. Tissue samples were embedded in paraffin wax blocks and 4-μm serial sections were cut at three levels (each level was 100 μm apart) within the block. Sections were processed and stained as before for hematoxylin and eosin (Fisher Scientific), Masson trichrome (Fisher), and various antibodies as outlined in Table 1.²⁷ All sections were viewed with a Zeiss Axiovert light microscope or an Olympus BX50 fluorescent microscope equipped with a CCD camera.

vWf-positive vessels surrounding the islets (vessels on the outside of islets) were counted in five representative sections at 200× (field of view area = 0.785 mm²) and the vessel density was converted to # vWf vessels/mm². Insulin-positive clusters were counted in the whole omentum tissue in three sections (5 μm thick, each level was 100 μm apart). The whole microscope slide (stained with anti-insulin) was digitized using the Aperio ScanScope XT and the area of positively stained cells was measured using the positive pixel algorithm in the Aperio ImageScope software. Data are reported as the average value for each group ± standard error of the mean.

Statistical analysis

A one-way ANOVA with Tukey *post hoc* analysis was used to compare means among multiple groups. For comparison between two groups, a two-sided *t*-test was used. Data were considered statistically significant at *p* < 0.05. All analysis was done with Statistica Version 5.1 software (Statsoft).

Results

In vitro characterization of islets in endothelialized modules

Islets were characterized *in vitro* after embedding in endothelialized modules (EC modules). With a live/dead assay, after 2 days in culture, free islets in culture and those embedded in modules with ECs were generally viable (Fig. 1a, b). By day 7, there were some dead cells associated with both free islets (Fig. 1c) and islets embedded in EC modules (Fig. 1d). After subjecting islets to a static glucose challenge, islets embedded in EC modules had a higher insulin stimulation index than free islets after 2 days in culture (*p* < 0.05, Fig. 1e). During the isolation procedure, islets lose contact with basement membrane and re-establishment of this contact has been shown to improve islet function.^{33,34} Perhaps embedding islets in collagen gels allowed a partial re-establishment of this contact to improve short-term function. However, after 7 days in culture, the insulin stimulation index was reduced in islets embedded in EC and the difference between them was not significant; this is consistent with reports from other groups that isolated rat (and human) islets lose responsiveness to glucose stimulation over time in culture.³⁵ For transplantation studies, free islets and islets in EC modules were transplanted after 2 days in culture to minimize the loss of islet viability and function.

A critical element of this study was the co-culture of islets with ECs; the effect of co-culture on Sprague-Dawley RAEC on islet-embedded modules was evaluated by VE-cadherin expression. VE-cadherin is a key EC junction marker and its presence determines EC contact integrity^{36,37} and may also mediate anti-apoptotic signaling in ECs.³⁶ In our hands, RAEC grow to confluence in 7 days on the surface of collagen modules (without embedded cells) and display strong VE cadherin expression (unpublished observations). When RAEC were initially seeded on islet embedded modules in RPMI-1640 medium, RAEC showed sparse coverage on the surface of the modules after 7 days (results not shown). A co-culture protocol was devised (detailed in the methods and used for all transplant studies) to improve coverage and with this protocol, RAEC completely covered the surface after 7 days in culture with strong and continuous VE cadherin expression (Fig. 1f).

TABLE 1. LIST OF ANTIBODIES AND THEIR TARGET ANTIGENS USED FOR IMMUNOSTAINING

Antibody	Supplier	Conditions	Target antigen
Insulin (18-0067)	Invitrogen, Burlington, Canada	1% pepsin pretreatment 1:100 for 1 h	Insulin
GFP (Ab6556)	Abcam, Cambridge, MA	Citrate pretreatment, 1:8000 for 1 h	GFP molecule–donor EC
Von Willebrand factor (CL20176A-R, vWf)	Cedarlane, Burlington, Canada	1:5000 overnight	All EC and platelets

GFP, green fluorescent protein; EC, endothelial cell.

FIG. 1. Free islets and islets in endothelialized modules (EC) in culture (RPMI-1640 medium) were characterized by (a–d) Live/Dead assay, (e) insulin stimulation index after a static glucose challenge, and (f) VE cadherin staining. (a) Free islets and (b) islets in EC modules were mostly alive (green) after 2 days in culture. (c) After 7 days in culture, a few dead (red) cells were seen in free islets and with (c) islets in EC modules. (e) Islets in EC modules had higher insulin stimulation index than free islets in culture. Insulin stimulation index was reduced at day 7 in both free islets and islets in EC modules. Data presented are average of the group $\pm$ SEM; 20 IEQ sample, $n=4$. (f) RAEC on the surface of islet embedded modules under co-culture conditions were characterized by VE cadherin staining at 7 days; RAEC were confluent at day 7. EC, endothelial cell; RAEC, rat aortic EC; SEM, standard error of the mean. Color images available online at www.liebertonline.com/tea

Transplanted islet vessel density

Vessels were counted 21 days after transplantation. Since revascularization of islets is known to take up to 2 weeks, vWf-positive vessels of various sizes formed in the vicinity of transplanted islets at day 21 (Fig. 2a–d). Approximately 25% more vessels were detected around the islets when islets were transplanted in EC modules compared with around free islets alone in the allogeneic model (Fig. 2e). Moreover, between days 14 and 21, there was a significant increase in vWf-positive vessel density around islets transplanted in EC modules (Fig. 2f).

In allogeneic recipients, GFP-positive cells (donor RAEC) were prominent around the islets as early as day 7 and persisted until 21 days (Fig. 3). Donor RAEC migrated from

the surface of the modules (Fig. 3a) to form primitive vessels at day 14 (Fig. 3b). Donor-derived vessels were connected to host vasculature as indicated by erythrocytes in their lumen (Fig. 3b, c) and matured over time to be supported by host smooth muscle cells (Fig. 3d). GFP-positive vessels were only detected in the vicinity of islets and never in the intra-islet space, consistent with the observation that while the vessel density around the islets increased (Fig. 2) with EC transplantation, there was no change in the intra-islet vessel density (results not shown). Moreover, the proximity of the GFP-positive vessels to islets suggested that the donor-derived vessels may be directly supporting the transplanted islets.

Based on concerns that the tacrolimus used in the allogeneic rat model impaired islet survival,^{38,39} EC modules

FIG. 2. Vessel density (vWf-positive vessels, arrows) around islets (dashed outline). (a–d) Implanted modules could also be identified by a weak brown staining. There were more vWf-positive vessels (with a lumen) around islets when transplanted in EC modules in both (a–b) allogeneic recipients and (c, d) syngeneic recipients. (e) At 21 days, average vWf-positive vessel density was significantly higher when islets were transplanted in EC modules compared with control (free islets in allogeneic immunosuppressed and collagen modules in syngeneic recipients). (f) Average vWf-positive vessel density was significantly higher with transplantation of islets in EC modules at day 21 compared with day 14 in allogeneic recipients. Data presented are average value of 25 representative islets (5 islets/animal $\times$ 5 animals/group) $\pm$ SEM, $*p < 0.05$. Color images available online at www.liebertonline.com/tea

containing Lewis rat islets were also evaluated in syngeneic Lewis rats without immunosuppressants. The presence of the donor RAEC also resulted in a 20% increase in vWf-positive vessel density around the islets (Fig. 2e), relative to the control group (islets in modules but without RAEC). Interestingly, vessel density was also higher with the EC modules in the syngeneic group than with the allogeneic group. However, no GFP-positive donor EC was detected in the syngeneic group at day 21.

Transplanted islet viability

Insulin-positive islets were found at day 21 in all experimental conditions: after transplantation of free islets (Fig. 4a), islets in EC modules (Fig. 4b) in allogeneic immunosuppressed rats in syngeneic rats in modules without RAEC (Fig. 4c), and in EC modules in syngeneic rats (Fig. 4d). In cases where animals became normoglycemic, insulin-positive islets were also detected at 60 days regardless of

FIG. 3. GFP-RAEC around transplanted islets in the allogeneic model. (a–c) Islets transplanted in EC modules were double stained with an anti-insulin antibody (purple) to identify transplanted islets and an anti-GFP antibody (brown) to identify transplanted EC. (a) GFP-positive single cells (arrows) were near transplanted islets at day 7. (b) By day 14, GFP-positive cells formed vessels that included erythrocytes in their lumen and (c) at day 21, and these vessels grew larger in size. Scale bar = 25 μ m. (d) At day 21, GFP-positive vessels (green, arrows) were supported by smooth muscle α -actin-positive cells (orange). GFP, green fluorescent protein. Color images available online at www.liebertonline.com/tea

treatment (Fig. 4e, f). When free islets were used, they were found, at 21 days, distributed in clumps, randomly among the fat tissue in the omentum (Fig. 4a). Meanwhile, islets in modules remained inside the modules or were seen in the tissue spaces between individual modules (Fig. 4b–d). The presence of viable islets in EC modules is encouraging as others have reported that islets embedded in collagen gels were not viable in SCID mice at 28 days.⁴⁰

To quantify this observation, the area of all insulin-positive cells in three sections (5- μ m thick) was measured at 21 days after transplantation (Table 2). In allogeneic immunosuppressed animals, the area of insulin-positive cells was the same whether islets were transplanted alone or in EC modules. In syngeneic rats, the average area of insulin-positive cells was higher when islets were transplanted in EC modules rather than in collagen modules (no EC) but due to the large variation within the group, the difference was not statistically significant ($p=0.07$). Interestingly, the insulin-positive area in the syngeneic group with EC modules was significantly higher than either group in the allogeneic rats. The lower islet viability in allogeneic rats is presumed to be a result of the toxic effects of the daily administered tacrolimus.^{38,39}

Transplanted islet function

In allogeneic immunosuppressed rats, there was an immediate decrease in blood glucose with transplantation of 2000 free islets or islets in EC modules (Fig. 5a). However, blood glucose continued to rise over time and within a week, glucose levels in both groups were similar to the levels before transplantation, indicating that transplanted islets failed during this period (Fig. 5a). This level of graft failure was not

unexpected as the mass required for successful reversal in allogeneic immunosuppressed rats is 15,000 IEQ/kg as compared with our minimal mass implant of 8000 IEQ/kg.³⁹ In the syngeneic rats, two of the five animals transplanted with 2000 islets in collagen modules (Fig. 5b) and two of the five animals transplanted with 2000 islets in EC modules had a significant decrease in blood glucose levels, which was maintained until the end of the study (60 days), although one of the animals in the EC module group remained slightly hyperglycemic (blood glucose levels in this recipient were less than in the “failed” implants) (Fig. 5c). Although blood glucose level decreased immediately after transplantation, normal (<11.1 mM) blood glucose levels were generally achieved only after 21 days in this subset of four animals. A similar result was seen in a pilot study in which one of three syngeneic animals treated with free islets also achieved normoglycemia for some of the experimental period (results not shown).

Animals that appeared normoglycemic at 60 days were subjected to an oral glucose tolerance test. Compared with nondiabetic control animals, all treated animals displayed slower glucose clearance, but by the end of the test all animals returned to near normoglycaemic levels (Fig. 6). The slower glucose clearance in treated animals has been noted in other studies with transplantation into the omentum.³¹ It was suggested that islets are not innervated as well in the omentum, which may explain the slow response to an increase in serum glucose.⁴¹

Islet function was also assessed by measuring nonfasting serum insulin levels in diabetic recipients. Animals transplanted with 2000 islets (free, in collagen modules or in EC modules) had higher serum insulin levels at 21 days as compared with sham-treated animals (Fig. 7). This is

FIG. 4. Anti-insulin antibody was used to identify transplanted islets at 21 days in (a, b) immune-suppressed allogeneic and in (c, d) syngeneic recipients and (e, f) 60 days; arrows show examples of insulin-positive islets and modules are highlighted by dashed outline. Viable islets were seen in all cases. Scale bar=100 μ m. Color images available online at www.liebertonline.com/tea

consistent with the observation that at least some islets were viable (insulin positive) in transplanted animals at 21 days in all groups (Fig. 5). In allogeneic immunosuppressed recipients, there was a slight increase (not statistically significant) in serum insulin levels when islets were transplanted in EC modules as compared with free islets, but the serum insulin levels were still lower than nondiabetic controls (Fig. 7a). Syngeneic recipients transplanted with islets in EC modules

had serum insulin levels that were similar to that transplanted with islets in modules without EC and to nondiabetic controls; those all three groups were higher than sham-treated diabetic animals (Fig. 7b). However, there was large variation within these groups: animals that had lower glucose levels (two out of five recipients for each of the islet treated groups) had higher serum insulin levels than those that continued to remain hyperglycemic (Fig. 7b).

TABLE 2. AVERAGE INSULIN-POSITIVE AREA (mm^2) FOR DIABETIC ANIMALS TRANSPLANTED WITH 2000 ISLETS 21 DAYS AFTER TRANSPLANTATION

Group	Average insulin-positive area (mm^2)	SEM	N
Allogeneic—Free islets	0.148	0.036	5
Allogeneic—Islets in EC modules	0.149	0.008	5
Syngeneic—Islets in collagen modules	0.477	0.137	4
Syngeneic—Islets in EC modules	0.854	0.258	4

SEM, standard error of the mean.

FIG. 5. (a) Average blood glucose levels in diabetic immunosuppressed allogeneic recipients that underwent sham surgery, or were transplanted with 2000 free islets or islets in EC modules. There was a sharp decrease in blood glucose levels 1 day after transplantation, but all animals became hyperglycemic again within 1 week; there was no difference between animals transplanted with free islets or islets in EC modules. Data presented are average of blood glucose levels $\pm$ SEM; sham ($n=2$), allogeneic groups ($n=5$). (b) Blood glucose levels of individual syngeneic recipients that were transplanted with (b) islets in collagen modules (no EC) and (c) islets in EC modules. Only two of five recipients of either group had reductions in glucose levels and remained low until 60 days.

FIG. 6. Blood glucose levels after an oral glucose challenge in two nondiabetic controls and the four syngeneic "normoglycemic" recipients 60 days after transplantation. The transplanted animals had increased blood glucose levels 30 min after glucose administration as compared with non-diabetic controls. By the end of the test, all animals returned to near "normoglycemic" levels.

Discussion

We have shown that islets may be embedded in endothelialized modules and delivery of islets in EC modules is a means of improving the vessel density around transplanted islets. However, the increased vessel density did not improve the ability of 2000 islets to restore or maintain normoglycemia in diabetic animals.

There was a significant increase in vessel density around islets when transplanted in EC modules as compared with both free islets and islets in collagen modules (no EC) (Fig. 2). Donor RAEC (GFP positive) appeared to be responsible for the increase in vascular density as they formed vessels that both integrated with the host vasculature and were supported by host smooth muscle cells (Fig. 3). Donor vessels formed in proximity to transplanted islets, suggesting a likely interaction between the co-transplanted islets and RAEC. It is also likely that donor RAEC secreted angiogenic growth factors to further promote host angiogenesis. In syngeneic recipients, although there was an increased vessel density at 21 days, oddly no donor GFP-positive RAEC were detected, suggesting that the increased vessel density consisted solely of host-derived vessels. After transplantation, islets are hypoxic and secrete angiogenic growth factors, vascular endothelial growth factor (VEGF-A) in particular, to promote angiogenesis.⁴² For example, islet-secreted VEGF has been implicated for migration of intra-islet mouse EC to form chimeric vessels with host EC.^{43–45} Similarly, in our model, islet secreted factors may have promoted co-transplanted EC-derived vascularization.

The absence of an apparent effect of vascularization on islet function was unexpected. Others have reported improved islet engraftment when vessel density is increased by VEGF delivery or by co-transplantation with adult stem cells. Elevated VEGF production in islets has been shown to increase vessel density and improve glucose control short term.^{14,17} Exogenous VEGF delivery has also been shown to improve glucose control after islet transplantation likely

FIG. 7. Non-fasting serum insulin levels of (a) allogeneic or (b) syngeneic diabetic recipients at 21 days after transplantation with 2000 free islets, islets in EC modules or islets in collagen modules. For each group, serum levels for individual animal are marked by (◆) and the group average is represented as (–). (a) Allogeneic immunosuppressed recipients that received islets in EC modules had slightly higher serum insulin levels than animals with free islets but not as high as the nondiabetic control group. (b) In syngeneic recipients, transplantation of 2000 islets in EC modules significantly ($p < 0.05$) increased the serum insulin levels at day 21 as compared with diabetic shams, and serum levels were equivalent to nondiabetic controls and to those receiving islets in collagen modules.

through multiple mechanisms that include improved vascularization and antiapoptotic signaling.¹⁵ Also, co-transplantation of islets with bone marrow-derived MSCs improved vessel density and lowered blood glucose levels until 39 days; MSCs secreted high levels of VEGF in culture.³² Additionally, MSC improved long-term survival of a suboptimal number of islets and reversed diabetes in allogeneic rats; the role of MSC in modulating the immune and inflammation response was shown to be a key factor in promoting islet engraftment.⁴⁶ These studies suggested that increased vascular density can improve transplanted islet engraftment. However, rather than just increase vessel density for transplanted islets, these reported therapies likely benefited transplanted islets through multiple mechanisms. VEGF delivery also prevented islet apoptosis through antiapoptotic signaling and transplantation of MSC modulated

the host inflammation/immune response. Therefore, while an increase in vessel density alone may promote short-term islet viability, it is likely that a combination of effects is required to promote eventual islet engraftment (i.e., long-term islet function).

There may be several reasons for the lack of improvement in islet engraftment with transplantation in EC modules. The significant improvement of vascular density with transplantation of islets in EC modules likely occurred a few weeks after transplantation. We noted that in allogeneic rats, vessel density was much lower at day 14 compared with day 21 (Fig. 2h) and donor EC derived vessels developed and fully matured by day 21 (Fig. 3). This is consistent with our previous studies where complete vascularization took 2–3 weeks after transplantation of EC modules in allogeneic immune-suppressed recipients.²⁸ In that study, we showed that donor RAEC migrated from the surface of the modules to form primitive vessels within 1 week; however, donor vessel maturation (erythrocytes in lumen, supporting host smooth muscle cells, perfusable) and overall vessel density (both host and donor derived) peaked at 21 days. The immature vessels in the early transplant period likely did not support islets and presumably, the majority of transplanted islets were lost during this time. Thus, an important goal is to accelerate vascularization, for example, by adding MSCs to the modules (article in preparation). An alternative approach would be to create a mature vasculature first and then add islets (free or in collagen gel) to the prevascularized site, as has been done by those creating an “artificial fat pad.”⁴⁷ We will pursue this idea using EC modules without islets to create a pre-vascularized implant site. It is our premise that with accelerated vessel maturation, endothelialized modules would improve islet function.

Additionally, the administration of immunosuppressants to allogeneic rats increased islet toxicity as evidenced by the significantly lower number of insulin-positive cells in allogeneic rats as compared with syngeneic rats (Table 2). The increased islet toxicity may have resulted in a large loss of islet mass regardless of vascularization; previous studies with VEGF delivery and MSC co-transplantation have not evaluated islet function in the presence of immunosuppressants. Immunosuppressant toxicity was not relevant to the syngeneic model and the observed increase in the number of insulin-positive stained cells with transplantation in EC modules in syngeneic recipients was encouraging (Table 2).

On the other hand, we had expected 2000 syngeneic islets in the omental pouch to be sufficient to restore normoglycemia.³¹ Insulin secretion assays before implantation (Fig. 1e) indicated that islets were functional, but this assay may have missed some damage (e.g., that associated with overnight shipment), which would only become apparent after implantation. Furthermore, there may have been protocol differences in the preparation of the pouch or in preparing the islets for transplantation that may have affected outcomes.

Conclusions

We have demonstrated that transplantation of islets in endothelialized modules improved peri-islet vascular density in both allogeneic immune-suppressed and syngeneic diabetic recipients. However, no significant improvements in islet function, as measured by blood glucose levels, were

noted with transplantation of islets in endothelialized modules compared with controls (free islets) in allogeneic animals and controls (islets in collagen modules without EC) in syngeneic animals. This study was the first exploration of primary islet transplantation with endothelialized modules, but further improvement in vascularization is presumed to be necessary to enhance islet engraftment.

Acknowledgments

The authors acknowledge the financial support of the U.S. National Institutes of Health (EB001013), the Canadian Institutes of Health Research (MOP-89864), and Natural Sciences and Engineering Research Council of Canada (Postgraduate scholarship). We are grateful to Chuen Lo and his technical expertise in animal surgeries and for Dr. Mark Cattral of the Toronto General Hospital for his assistance in garnering research support for this work. Also, we thank Chyan-Jang Lee (Dr. J. Medin) for generation of the eGFP-RAEC, Deb Dixon (Dr. Rajotte lab) for islet isolations, Mount Sinai Services for Insulin ELISA assay, and Toronto General Hospital's Pathology research group for all histology and immunostaining services.

Disclosure Statement

No competing financial interests exist.

References

- Merani, S., and Shapiro, A.M. Current status of pancreatic islet transplantation. *Clin Sci (Lond)* **110**, 611, 2006.
- Alejandro, R., Barton, F.B., Hering, B.J., and Wease, S. 2008 Update from the Collaborative Islet Transplant Registry. *Transplantation* **86**, 1783, 2008.
- Fiorina, P., Shapiro, A.M.J., Ricordi, C., and Secchi, A. The clinical impact of islet transplantation. *Am J Transplant* **8**, 1990, 2008.
- Shapiro, A.M., Ricordi, C., Hering, B.J., Auchincloss, H., Lindblad, R., Robertson, R.P., *et al.* International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* **355**, 1318, 2006.
- Balamurugan, A.N., Bottino, R., Giannoukakis, N., and Smetanka, C. Prospective and challenges of islet transplantation for the therapy of autoimmune diabetes. *Pancreas* **32**, 231, 2006.
- Barshes, N.R., Wyllie, S., and Goss, J.A. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. *J Leukoc Biol* **77**, 587, 2005.
- Kizilel, S., Scavone, A., Liu, X., Nothias, J.M., Ostrega, D., Witkowski, P., *et al.* Encapsulation of pancreatic islets within nano-thin functional polyethylene glycol coatings for enhanced insulin secretion. *Tissue Eng Part A* **16**, 2217, 2010.
- Lin, C.C., and Anseth, K.S. Glucagon-like peptide-1 functionalized PEG hydrogels promote survival and function of encapsulated pancreatic beta-cells. *Biomacromolecules* **10**, 2460, 2009.
- Konstantinova, I., and Lammert, E. Microvascular development: learning from pancreatic islets. *Bioessays* **26**, 1069, 2004.
- Olsson, R., and Carlsson, P.O. The pancreatic islet endothelial cell: emerging roles in islet function and disease. *Int J Biochem Cell Biol* **38**, 710, 2006.
- Carlsson, P.O., Palm, F., Andersson, A., and Liss, P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes* **50**, 489, 2001.
- Mattsson, G., Jansson, L., and Carlsson, P.O. Decreased vascular density in mouse pancreatic islets after transplantation. *Diabetes* **51**, 1362, 2002.
- Mattsson, G., Jansson, L., Nordin, A., and Carlsson, P.O. Impaired revascularization of transplanted mouse pancreatic islets is chronic and glucose-independent. *Transplantation* **75**, 736, 2003.
- Lai, Y., Schneider, D., Kizszun, A., Hauck-Schmalenberger, I., Breier, G., Brandhorst, D., *et al.* Vascular endothelial growth factor increases functional beta-cell mass by improvement of angiogenesis of isolated human and murine pancreatic islets. *Transplantation* **79**, 1530, 2005.
- Stagner, J., Mokshagundam, S., Wyler, K., Samols, E., Rilo, H., Stagner, M., *et al.* Beta-cell sparing in transplanted islets by vascular endothelial growth factor. *Transplant Proc* **36**, 1178, 2004.
- Stendahl, J.C., Wang, L.J., Chow, L.W., Kaufman, D.B., and Stupp, S.I. Growth factor delivery from self-assembling nanofibers to facilitate islet transplantation. *Transplantation* **86**, 478, 2008.
- Zhang, N., Richter, A., Suriawinata, J., Harbaran, S., Altomonte, J., Cong, L., *et al.* Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. *Diabetes* **53**, 963, 2004.
- Koike, N., Fukumura, D., Gralla, O., Au, P., Schechner, J.S., and Jain, R.K. Creation of long-lasting blood vessels. *Nature* **428**, 138, 2004.
- Merx, M.W., Zerneck, A., Liehn, E.A., Schuh, A., Skobel, E., Butzbach, B., *et al.* Transplantation of human umbilical vein endothelial cells improves left ventricular function in a rat model of myocardial infarction. *Basic Res Cardiol* **100**, 208, 2005.
- Shepherd, B.R., Enis, D.R., Wang, F., Suarez, Y., Pober, J.S., and Schechner, J.S. Vascularization and engraftment of a human skin substitute using circulating progenitor cell-derived endothelial cells. *FASEB J* **20**, 1739, 2006.
- Stevens, K.R., Kreutziger, K.L., Dupras, S.K., Korte, F.S., Regnier, M., Muskheli, V., *et al.* Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. *Proc Natl Acad Sci U S A* **106**, 16568, 2009.
- Lesman, A., Habib, M., Caspi, O., Gepstein, A., Arbel, G., Levenberg, S., *et al.* Transplantation of a tissue-engineered human vascularized cardiac muscle. *Tissue Eng Part A* **16**, 115, 2010.
- McGuigan, A.P., and Sefton, M.V. Vascularized organoid engineered by modular assembly enables blood perfusion. *Proc Natl Acad Sci U S A* **103**, 11461, 2006.
- McGuigan, A.P., and Sefton, M.V. Design and fabrication of sub-mm-sized modules containing encapsulated cells for modular tissue engineering. *Tissue Eng* **13**, 1069, 2007.
- Leung, B.M., and Sefton, M.V. A modular tissue engineering construct containing smooth muscle cells and endothelial cells. *Ann Biomed Eng* **35**, 2039, 2007.
- Leung, B., and Sefton, M.V. A modular approach to cardiac tissue engineering. *Tissue Eng Part A* **16**, 3207, 2010.
- Gupta, R., van Rooijen, N., and Sefton, M.V. Fate of endothelialized modular constructs implanted in an omental pouch in nude rats. *Tissue Eng Part A* **15**, 2875, 2009.

28. Chamberlain, M.D., Gupta, R., and Sefton, M.V. Chimeric vessel tissue engineering driven by endothelialized modules in immunosuppressed Sprague-Dawley rats. *Tissue Eng Part A* **17**, 151, 2010.
29. Yoshimitsu, M., Higuchi, K., Ramsbair, S., Nonaka, T., Rasaiah, V.I., Siatskas, C., *et al.* Efficient correction of Fabry mice and patient cells mediated by lentiviral transduction of hematopoietic stem/progenitor cells. *Gene Ther* **14**, 256, 2007.
30. Yoshimitsu, M., Sato, T., Tao, K., Walia, J.S., Rasaiah, V.I., Sleep, G.T., *et al.* Bioluminescent imaging of a marking transgene and correction of Fabry mice by neonatal injection of recombinant lentiviral vectors. *Proc Natl Acad Sci U S A* **101**, 16909, 2004.
31. Kin, T., Korbitt, G.S., and Rajotte, R.V. Survival and metabolic function of syngeneic rat islet grafts transplanted in the omental pouch. *Am J Transplant* **3**, 281, 2003.
32. Ricordi, C., Gray, D.W., Hering, B.J., Kaufman, D.B., Warnock, G.L., Kneteman, N.M., *et al.* Islet isolation assessment in man and large animals. *Acta Diabetol Lat* **27**, 185, 1990.
33. Lucasclerc, C., Massart, C., Campion, J.P., Launois, B., and Nicol, M. Long-term culture of human pancreatic-islets in an extracellular-matrix - morphological and metabolic effects. *Mol Cell Endocrinol* **94**, 9, 1993.
34. Wang, R.N., and Rosenberg, L. Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *J Endocrinol* **163**, 181, 1999.
35. Holmes, M.A., Clayton, H.A., Chadwick, D.R., Bell, P.R., London, N.J., and James, R.F. Functional studies of rat, porcine, and human pancreatic islets cultured in ten commercially available media. *Transplantation* **60**, 854, 1995.
36. Vestweber, D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol* **28**, 223, 2008.
37. Wu, J.Q., and Sheibani, N. Modulation of VE-cadherin and PECAM-1 mediated cell-cell adhesions by mitogen-activated protein kinases. *J Cell Biochem* **90**, 121, 2003.
38. Larsen, J.L., Bennett, R.G., Burkman, T., Ramirez, A.L., Yamamoto, S., Gulizia, J., *et al.* Tacrolimus and sirolimus cause insulin resistance in normal sprague dawley rats. *Transplantation* **82**, 466, 2006.
39. Lopez-Talavera, J.C., Garcia-Ocana, A., Sipula, I., Takane, K.K., Cozar-Castellano, I., and Stewart, A.F. Hepatocyte growth factor gene therapy for pancreatic islets in diabetes: reducing the minimal islet transplant mass required in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. *Endocrinology* **145**, 467, 2004.
40. Hiscox, A.M., Stone, A.L., Limesand, S., Hoying, J.B., and Williams, S.K. An islet-stabilizing implant constructed using a preformed vasculature. *Tissue Eng Part A* **14**, 433, 2008.
41. Merani, S., Toso, C., Emamaullee, J., and Shapiro, A.M. Optimal implantation site for pancreatic islet transplantation. *Br J Surg* **95**, 1449, 2008.
42. Brissova, M., Shostak, A., Shiota, M., Wiebe, P.O., Poffenberger, G., Kantz, J., *et al.* Pancreatic islet production of vascular endothelial growth factor—a is essential for islet vascularization, revascularization, and function. *Diabetes* **55**, 2974, 2006.
43. Nyqvist, D., Kohler, M., Wahlstedt, H., and Berggren, P.O. Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. *Diabetes* **54**, 2287, 2005.
44. Linn, T., Schneider, K., Hammes, H.P., Preissner, K.T., Brandhorst, H., Morgenstern, E., *et al.* Angiogenic capacity of endothelial cells in islets of Langerhans. *Faseb J* **17**, 881, 2003.
45. Brissova, M., Fowler, M., Wiebe, P., Shostak, A., Shiota, M., Radhika, A., *et al.* Intra-islet endothelial cells contribute to revascularization of transplanted pancreatic islets. *Diabetes* **53**, 1318, 2004.
46. Solari, M.G., Srinivasan, S., Boumaza, I., Unadkat, J., Harb, G., Garcia-Ocana, A., *et al.* Marginal mass islet transplantation with autologous mesenchymal stem cells promotes long-term islet allograft survival and sustained normoglycemia. *J Autoimmun* **32**, 116, 2009.
47. Fumimoto, Y., Matsuyama, A., Komoda, H., Okura, H., Lee, C.M., Nagao, A., *et al.* Creation of a rich subcutaneous vascular network with implanted adipose tissue-derived stromal cells and adipose tissue enhances subcutaneous grafting of islets in diabetic mice. *Tissue Eng Part C Methods* **15**, 437, 2009.

Address correspondence to:

Michael Sefton, Sc.D.

Institute of Biomaterials and Biomedical Engineering
and Department of Chemical Engineering and Applied Chemistry

University of Toronto

164 College St.

Room 407

Toronto

Canada M5S 3G9

E-mail: michael.sefton@utoronto.ca

Received: September 13, 2010

Accepted: March 28, 2011

Online Publication Date: May 6, 2011