Intraislet Endothelial Cells Contribute to Revascularization of Transplanted Pancreatic Islets

Marcela Brissova, Michael Fowler, Peter Wiebe, Alena Shostak, Masakazu Shiota, Aramandla Radhika, P. Charles Lin, Maureen Gannon, and Alvin C. Powers

Pancreatic islet transplantation is an emerging therapy for type 1 diabetes. To survive and function, transplanted islets must revascularize because islet isolation severs arterial and venous connections; the current paradigm is that islet revascularization originates from the transplant recipient. Because isolated islets retain intraislet endothelial cells, we determined whether these endothelial cells contribute to the revascularization using a murine model with tagged endothelial cells (lacZ knock-in to Flk-1/VEGFR2 gene) and using transplanted human islets. At 3–5 weeks after transplantation beneath the renal capsule, we found that islets were revascularized and that the transplant recipient vasculature indeed contributed to the revascularization process. Using the lacZ-tagged endothelial cell model, we found that intraislet endothelial cells not only survived after transplantation but became a functional part of revascularized islet graft. A similar contribution of intraislet endothelial cells was also seen with human islets transplanted into an immunodeficient mouse model. In the murine model, individual blood vessels within the islet graft consisted of donor or recipient endothelial cells or were a chimera of donor and recipient endothelial cells, indicating that both sources of endothelial cells contribute to the new vasculature. These observations suggest that interventions to activate, amplify, or sustain intraislet endothelial cells before and after transplantation may facilitate islet revascularization, enhance islet survival, and improve islet transplantation.

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RESEARCH DESIGN AND METHODS

Flk-1 (kinase insert domain-containing receptor, vascular endothelial growth factor receptor 2) heterozygote mice with lacZ-tagged endothelial cells (Flk-1\textsuperscript{wt/wt}) (13), C57BL/6 mice, and NOD-SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME). To identify the lacZ insert in the Flk-1 gene, \textit{lacZ} primers (stock no. 002288; for \textit{lacZ} inserts and strain C57BL/6) were genotyped by PCR using the following primers to detect the \textit{lacZ} insertion: 5’ primer: ATC TCT TGC AGC AGC TTG; 3’ primer: CGT GGC ATT CAT TCT TCT and the wild-type locus: 5’ primer: CAA ATG TTA CTT GTG TCT TG; 3’ primer: GTC AGT CGA GTG CAC ATG TT. Mouse islets from Flk-1\textsuperscript{wt/wt} donors and human islets were transplanted into an immuno-deficient NOD-SCID mouse model from The Jackson Laboratory (for additional information see http://jaxmice.jax.org/jaxmicedb.cgi?objtype=pricedetail&stock=001303).

Mouse islet isolation. Islets were isolated from Flk-1\textsuperscript{wt/wt} mice by dissection of the splenic portion of the pancreas followed by collagenase P digestion (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (20). To increase the yield of islets isolated from Flk-1\textsuperscript{wt/wt} mice, 3 ml collagenase P in Hank’s buffered saline (0.6 mg/ml) was first directly infused into the pancreas through the bile duct. Groups of two pancreata were then digested in 6.7 ml collagenase P (0.6 mg/ml) for 4–5 min at 37°C using a wrist-action shaker. Islets were handpicked under microscopic guidance and washed three times with 10 mmol/l PBS containing 1% mouse serum. Finally, 200–240 islets were suspended in 30 µl of the same solution and transplanted into mouse recipients immediately following the isolation procedure.

Human islets were obtained through the Juvenile Diabetes Foundation Human Islet Distribution Program and from Dr. David M. Harlan at the Transplantation and Autoimmunity Branch of the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health. After isolation, human islets were shipped in CMRL media by Diabetes and Digestive and Kidney Diseases of the National Institutes of Health. The cryosections were post-fixed with 0.2% glutaraldehyde/10 mmol/l PBS for 1 h and then equilibrated in 30% sucrose/10 mmol/l PBS overnight at 4°C. The tissues were embedded in a commercial freezing compound (Bio- Tek, Williston, VT) and then cut into 10-µm-thick serial sections. Digital images of the 10-µm-thick sections were obtained using a Zeiss LSM410 or LSM510 META confocal laser scanning microscope. Digital images were analyzed and three-dimensionally reconstructed using Meta- Morph 5.0 software (Universal Imaging, Downington, PA).

RESULTS

Expression of endothelial cell markers in isolated islets and pancreas of Flk-1\textsuperscript{wt/lacZ} mice. Even though islet isolation severs arterial and venous connections, isolated islets retain their capillary network (Fig. 1A). Therefore, we asked whether these intrasplenic endothelial cells contribute to the revascularization of transplanted islets. To follow the fate of the intrasplenic endothelial cells, we used a model in which endothelial cells are tagged with lacZ (knock-in of lacZ to the Flk-1 locus termed Flk-1\textsuperscript{lacZ}). LacZ encodes the β-galactosidase enzyme. Figure 1B shows prominent X-gal staining (reflecting β-galactosidase activity) of islets in the whole-mount Flk-1\textsuperscript{wt/lacZ} pancreas. Pancreatic sections in Fig. 1C and D demonstrate that lacZ expression recapitulates expression of Flk-1. Similar to Flk-1 expression, there was a greater density of lacZ\textsuperscript{+} capillary structures in the islets compared with exocrine tissue, reflecting the higher vascularity of islets (Fig. 1C and D).
ately after islet isolation, wild-type islets (Flk-1 wt/wt) were transplanted into wild-type animals (Flk-1 wt/wt, n/H110053) (Fig. 1E–G) and mice that contain the endothelial lacZ tag (Flk-1wt/lacZ, n/H110054) (Fig. 1H–J). At the same time, murine islets containing tagged endothelial cells (Flk-1 wt/lacZ) were transplanted into mice that do not carry the endothelial cell marker (n/H1100510) (Fig. 1K–M). We used NOD-SCID mice as the recipient of Flk-1 wt/lacZ islets to avoid a possible immune reaction to endothelial cells expressing lacZ.

Kidneys bearing transplanted islets were retrieved 3–5 weeks after transplantation, a time in which the revascularization is completed, as shown by previous studies (9–16). In all three types of transplants, the islets were vascularized as detected by Flk-1 expression in vascular structures within and surrounding the islet graft (Fig. 1E, H, and K). As shown by detection of β-galactosidase activity (Fig. 1J and M), both donor and recipient endothelial cells were found within the islet graft area positive for insulin (Fig. 1I and L). Occasionally, there were a few lacZ− intraislet endothelial cells migrating further away from the transplant into kidney cortex (Fig. 1M). These data indicate that intraislet endothelial cells survive and possibly contribute to the revascularization process.

To determine whether intraislet endothelial cells participate in revascularization and to access the structure and composition of blood vessels in the revascularized grafts, 60-μm sections of the islet grafts were labeled for the mouse endothelial marker PECAM-1, which is ubiquitously expressed on the surface of all (both donor and recipient) endothelial cells. The sections were colabeled for lacZ-encoded β-galactosidase, which is only expressed by the endothelium of donor Flk-1 wt/lacZ islets (Fig. 1K, L, and M) and unlike PECAM-1 has a more cytoplasmic localization. In both mouse and human native islets and islet grafts, mouse PECAM-1 or human CD31 and Flk-1 are coexpressed in islet microvasculature (data not shown). Mounted sections were then subjected to optical section-
ing using a laser scanning confocal microscope. The three-dimensional reconstruction of optical sections through the islet grafts (Fig. 2) indicated the existence of two types of blood vessels in the revascularized islet graft: 1) capillaries formed predominantly of either donor or recipient endothelial cells directly connected to each other and 2) chimeric blood vessels formed from a mixture of donor and recipient endothelial cells (online data supplement 1C and D[available at http://diabetes.diabetesjournals.org]). By examining optical sections of the islet grafts in three dimensions, both donor and recipient endothelial cells were found to be components of tubular structures consistent with vessels that traversed throughout the islet graft (Fig. 2E and F and online data supplement 1C and D). To estimate the contribution of donor and recipient endothelial cells to the graft revascularization, we used MetaMorph software and calculated the volume of PECAM-1+/β-galactosidase+ endothelial cells in the insulin+ graft area. This calculation was applied to four different grafts, and three to six fields were examined per each graft. These data suggested that as much as 40 ± 3% (n = 18, range 18–68%) of endothelial cells in the revascularized graft originated from the donor islets.

Role of intraislet endothelial cells in revascularization process of transplanted human islets. Human islets, transplanted under the kidney capsule of NOD-SCID mice (n = 4), were analyzed for the presence of the donor and recipient endothelial cells using species-specific antibodies to the endothelium-specific marker CD31. Sixty-micron sections of the human islet grafts were stained for mouse CD31 (PECAM-1) and human CD31. Mounted sections were then subjected to optical sectioning using a laser scanning confocal microscope. The three-dimensional reconstruction of optical sections through the islet grafts indicated the existence of two types of blood vessels in the revascularized islet grafts: 1) capillaries formed predominantly of either donor or recipient endothelial cells directly connected to each other and 2) chimeric blood vessels formed from a mixture of donor and recipient endothelial cells (online data supplement 1C and D). By examining optical sections of the islet grafts in three dimensions, both donor and recipient endothelial cells were found to be components of tubular structures consistent with vessels that traversed throughout the islet graft (Fig. 2E and F and online data supplement 1C and D). To estimate the contribution of donor and recipient endothelial cells to the graft revascularization, we used MetaMorph software and calculated the volume of PECAM-1+/β-galactosidase+ endothelial cells in the insulin+ graft area. This calculation was applied to four different grafts, and three to six fields were examined per each graft. These data suggested that as much as 40 ± 3% (n = 18, range 18–68%) of endothelial cells in the revascularized graft originated from the donor islets.

FIG. 2. Three-dimensional reconstruction of vasculature within the revascularized graft of Flk-1wt/lacZ islets transplanted into NOD-SCID recipient. Sixty-micron sections of the kidneys bearing islet grafts were optically sectioned and three-dimensionally reconstructed. Both donor and recipient endothelial cells were labeled for PECAM-1 (green), and β-galactosidase antibody was used to visualize donor-specific endothelial cells (red, [β-gal]). Islet graft was identified by staining for insulin (blue, Ins). A–F (F is an enlargement of the highlighted area in E): Representation of two different fields of view in the same islet graft, 40× magnification. D: A merge of the images in A–C. E and F: Only endothelial cell markers are shown. The dashed line in A–E shows a boundary between graft and kidney cortex (kidney cortex is to the right of dashed line). White arrows point to blood vessels derived from either donor or recipient endothelial cells, and magenta arrows point to chimeric blood vessels. (See also online data supplement 1A–D.)
grafts (Fig. 3) indicated that human intraislet endothelial cells, similar to the murine islets, survive after transplantation. In contrast to mouse intraislet endothelial cells, the human intraislet endothelial cells did not form chimeric blood vessels. Moreover, examination of human islet grafts in three dimensions revealed fewer possible connections of human endothelial cells with the host vasculature (Fig. 3D and E) than was seen in mouse islet grafts. The human intraislet endothelial cells also appeared to more readily migrate into the kidney cortex (Fig. 3F) than was observed in transplants of murine Flk-1wt/lacZ islets (Fig. 1M).

**Do intraislet endothelial cells become a part of functional vasculature in revascularized graft?** Even though intraislet endothelial cells survived transplantation and were a part of vascular-like structures within the revascularized islet grafts, it was not known whether they integrated into a functional blood vessel network of the revascularized graft. To address this question, mice that received transplants of Flk-1wt/lacZ islets were infused with fluorescein isothiocyanate–conjugated tomato lectin (n = 4), which binds with high affinity to the surface of mouse endothelial cells (21). Lectin infusion, a well-established technique, has been widely used in the area of angiogenesis (22). For example, many investigators (22) feel that it is the technique of choice for identifying functional blood vessels since the only way lectin can reach the endothelial cells is via the circulation. Furthermore, it has been shown
previously that \textit{L. Esculentum} lectin, which is utilized in our studies, binds uniformly to the luminal surface of murine endothelial cells in arteries, veins, and capillaries and is colocalized with CD31 in functional blood vessels (22). We confirmed the uniformity of \textit{L. Esculentum} lectin binding in both pancreas and kidney. Optical sections of the lectin-perfused mouse tissue specimens acquired with a laser scanning confocal microscope show that the lectin signal is localized in the luminal lining of blood vessels (online data supplement 2A and B).

The lectin-perfused islet grafts were additionally analyzed for expression of insulin and \(\beta\)-galactosidase by immunocytochemistry and laser scanning confocal microscopy (Fig. 4) (online data supplement 3A–F). The series of optical sections was acquired at a 0.5-\(\mu\)m interval in the axial (\(z\)) dimension and an appropriate pinhole setting to alleviate any concerns about overlapping signals from the layers of the specimen above and below the given focal plane. Individual optical sections of the islet grafts demonstrate that in the same focal plane, \(\beta\)-galactosidase, which marks intraislet endothelial cells, is colocalized with lectin, thus proving unequivocally the functionality of these \(\beta\)-galactosidase-positive blood vessels. The presence of cells double positive for lectin and \(\beta\)-galactosidase indeed proves that blood flows through donor-derived blood vessels in revascularized mouse islet grafts (Fig. 4B and D). Immunocytochemistry of the lectin-perfused specimens required several modifications to avoid lectin leaching during the staining procedure (see RESEARCH DESIGN AND METHODS) and underestimated the number of \(\beta\)-galactosidase\(^+\) endothelial cells (especially in cells with lower \(\beta\)-galactosidase expression). This was based on a comparison of \(\beta\)-galactosidase\(^+\) cells in consecutive sections stained with either \(\beta\)-galactosidase antibody (modified immunocytochemistry procedure) or using an enzymatic reaction of \(\beta\)-galactosidase with X-gal substrate. Because there were fewer \(\beta\)-galactosidase\(^+\) cells detected by immunocytochemistry compared with enzymatic reaction, we did not feel it was appropriate to estimate the ratio of \(\beta\)-galactosidase\(^+\) cells to cells double positive for \(\beta\)-galactosidase and lectin\(^+\). We did find a few cells positive for \(\beta\)-galactosidase and negative for lectin, and, for example, one such cell appeared in Fig. 4B (arrow) (online data supplement 3C and F). These \(\beta\)-galactosidase\(^+\)-lectin cells could be either intraislet endothelial cells that survived the transplantation but did not establish a lumen or proliferating donor-derived endothelial cells.

In the human islet grafts perfused with \textit{L. Esculentum} lectin, we did not find human endothelial cells double positive for human CD31 and lectin. While binding of \textit{L. Esculentum} lectin to mouse endothelium is well documented (22), it is unknown if this lectin binds to human endothelial cells. The human CD31\(^+\) cells remained negative for the lectin even when the sections of human islet grafts were subsequently stained with \textit{L. Esculentum} lectin (a standard method to assess if a lectin binds to endothelial cells). These observations indicate that human intraislet endothelial cells may lack the \(N\)-acetyl-\(l\)-glucosamine oligomer moieties that are recognized by \textit{L. Esculentum} lectin. The formation of tubular structures by
human endothelial cells is evident from the three-dimen-
sional projections in Fig. 3; however, it is still possible that
they were incompletely connected to the recipient’s vas-
culature.

DISCUSSION
Transplanted pancreatic islets, unlike transplanted solid
organs, must revascularize to survive and function, and
this process of angiogenesis is likely an important factor
that ultimately determines whether an islet transplant
secretes sufficient insulin to reverse diabetes. The reasons
for the current requirement of islets from two pancreata
for islet transplantation to reverse diabetes are uncertain,
but this limitation is a major obstacle to advancing islet
transplantation. While continued focus on improving im-
umunosuppression after islet transplantation is crucial, a
better understanding of the molecular events of islet
engraftment or revascularization could lead to a dramatic
increase in islet survival and improve islet transplantation
as a therapy for type 1 diabetes. In contrast to the current
thinking that revascularization is solely a function of the
transplant recipient (10,13,16–18), our results show that
intraislet endothelial cells from the donor survive after
transplantation and play an important role in the revascu-
larization process. The survival of intraislet endothelial
cells was recently reported by investigators (19) using the
transgenic Tie2-lacZ mouse model, but this report did not
examine whether these cells can contribute to functional
vasculature.

Three-dimensional reconstructions of optical sections
through islet grafts allowed a more detailed examination
of blood vessels within the revascularized mouse and
human islet grafts. In mouse Flk-1wt/lacZ islet transplants,
donor and recipient endothelial cells cooperated effect-
ively during the process of angiogenesis in that some
vessels were lined with donor or recipient endothelial
cells, while other vessels were a chimera of donor and
recipient endothelial cells. In contrast, there was less
interaction between human intraislet endothelial cells and
recipient mouse endothelial cells. Examination of optical
sections in three dimensions did not demonstrate chimeric
blood vessels in the human islet grafts and revealed fewer
possible connections of mouse and human endothelial
cells. The limited interaction of mouse and human endo-
thelial cells may reflect species differences in endothelial
cells. This may also explain the relatively extensive migra-
tion of human endothelial cells into the kidney cortex,
which was only occasionally found in mouse Flk-1wt/lacZ
islet transplants. In addition, human islets (unlike those of
mice) were procured from brain-dead donors and cultured
longer, which may have influenced their intraislet endo-
thelial cell biology.

Even though the presence of vascular connections be-
tween capillaries derived from donor and recipient endo-
thelial cells seemed possible based on three-dimensional
projections of islet grafts, it does not prove that these
vessel-like structures are functional. Infusion of endothe-
lum-binding lectin in the mouse islet–transplant model
demonstrated that blood flows through donor-derived capillaries and,
thus, for the first time, showed that the
intraislet endothelial cells have a capacity to integrate into
the functional vasculature within the revascularized graft.

These results have several implications for the handling of
pancreatic islets destined for transplantation. Pancreas
procurement and islet isolation procedures must not only
strive to maximize the health of islet endocrine cells, but
must now also consider the effects on endothelial cell
health and survival. In contrast to the original Edmonton
procedure, many human islet transplantation centers cur-
rently culture pancreatic islets for several days before
transplantation; the effect of such culture on endothelial
cell health and survival after transplantation is unknown
and warrants further study. The model system in which
intraislet endothelial cells are tagged with lacZ should
allow one to address such questions. In addition, our
transplantation studies with both mouse and human islets
did not utilize immunosuppression; the effect of immuno-
suppressive agents on endothelial cell health and islet
revascularization is not known and warrants further study.

Since islet revascularization may be a limiting factor in
islet survival and since transplanted islets have reduced
density compared with islets in the pancreas
(10,13,23), the capacity of intraislet endothelial cells to
integrate into functional graft vasculature suggests a num-
ber of avenues of investigation to enhance the revascu-
larization process. For example, can intraislet endothelial
cells be activated or primed ex vivo (pretransplantation)
to accelerate angiogenesis? Will inclusion of exogenous
endothelial cells with transplanted islets promote islet
engraftment and revascularization? Emerging evidence
supports a direct interaction between pancreatic islet cells
and endothelial cells. Melton et al. (24) recently suggested
that the developing aorta was essential for the initiation of
endoctrine cell differentiation during pancreas develop-
ment. These investigators also found that transgenic over-
exression of the angiogenic factor, vascular endothelial
growth factor (VEGF), under the control of a pancreas-
specific promoter from the pancreatic duodenal home-
obox-1 (PDX-1) gene increased islet size. When
combined with the current results, this raises the possibil-
ity that intraislet endothelial cells and the newly formed
islet vasculature may nurture transplanted islet cells in
addition to the reestablishment of islet blood flow. Future
studies are needed to address this hypothesis.

Because of the shortage of organ donors and the large
number of patients with type 1 diabetes, investigators are
developing alternative sources of insulin-producing cells
such as stem cells and genetically engineered cells. Like
transplanted pancreatic islets, such cell transplants will
likely need to become vascularized. Our studies would
suggest that inclusion of exogenous endothelial cells with
such insulin-producing cells may facilitate the survival and
function of these surrogate islet cells. A better understand-
ing of how transplanted cells survive and become vascu-
larized will also be relevant to the development of cell
transplantation therapy for other diseases.

Our studies also give rise to a number of questions about
the molecular events of islet engraftment. For example, we
transplanted islets beneath the renal capsule, whereas in
human transplantation, islets are infused into the portal
vein and embolize in the liver. Whether the angiogenic
and revascularization processes are identical in the liver
and beneath the renal capsule is unknown. What signals initi-
ate angiogenesis after islet transplantation? Are islet cells
the source of such signals? What are the steps of islet engraftment and capillary sprouting, from breakdown of the basement membrane of preexisting vessels within and surrounding the islet graft to endothelial cell migration? The current findings and future research to better understand the molecular events of islet engraftment should enhance islet revascularization, improve islet survival after transplantation, reduce the number of pancreatic islets required to reverse diabetes, and improve the outcome of islet transplantation in type 1 diabetes.

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