Original Article

Donor Islet Endothelial Cells Participate in Formation of Functional Vessels Within Pancreatic Islet Grafts

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Pancreatic islet transplantation has emerged as a therapy for type 1 diabetes and is today performed using both freshly isolated and cultured islets. Islet blood vessels are disrupted during islet isolation; therefore, proper revascularization of the transplanted islets is of great importance for islet graft function and survival. We have studied intraislet endothelial cells after islet isolation, during islet culture, and following islet transplantation. By isolating islets from the transgenic Tie2-GFP (green fluorescent protein) mouse, characterized by an endothelial cell–specific expression of GFP, living endothelial cells could be studied in intact islets utilizing two-photon laser-scanning microscopy (TPLSM). Intraislet endothelial cells were found to survive islet transplantation but to rapidly disappear during islet culture. By transplanting freshly isolated Tie2-GFP islets and applying a novel ex vivo model for simultaneous perfusion and TPLSM imaging of the graft-bearing kidneys, GFP fluorescent endothelial cells were found to extensively contribute to vessels within the islet graft vasculature. Real-time imaging of the flow through the islet graft vasculature confirmed that the donor-derived vessels were functionally integrated. Hence, intraislet endothelial cells have the capability of participating in revascularization of pancreatic islets subsequent to transplantation. Therefore, preservation of intraislet endothelial cell mass may improve long-term graft function. *Diabetes* 54:2287–2293, 2005

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CLSM, confocal laser-scanning microscopy; ECGS, endothelial cell growth supplement; FGF, fibroblast growth factor; FITC, fluoroscein isothiocyanate; GRP, green fluorescent protein; TPLSM, two-photon laser-scanning microscopy; TRITC, tetrarhodamine isothiocyanate; VEGF, vascular endothelial growth factor.

After the recent establishment of the Edmonton Protocol (1), transplantation of pancreatic islets is today performed at clinics around the world. The Edmonton Protocol altered several parameters in the transplantation procedure, such as transplantation of freshly isolated islets instead of cultured islets, transplantation of a larger number of islets isolated from multiple islet donors, and a steroid-free immunosuppressive treatment of the transplant recipients (1). Despite the promising progress in islet transplantation, problems persist in obtaining long-term function of the islet graft (2). To solve these problems, it is of great importance to elucidate the mechanisms affecting islet graft function and survival. During the isolation procedure, the pancreatic islets are disrupted from the surrounding tissue and the connecting blood vessels. Therefore, revascularization of the islets following transplantation is important for the function and survival of the islet graft. Traditionally, both in experimental and clinical islet transplantation, the islets are cultured for several days between isolation and transplantation. The endothelial cells that remain in the islets after islet isolation, the intraislet endothelial cells, have been sparsely studied and shown to be lost after 7 days of islet culture (3,4). Transplanted islets have been considered to be an avascular tissue and have been described to be revascularized by vessels growing into the islets from the host organ (5). Following transplantation of cultured islets, the islet graft has been found to have a lower vascular density (6) and a reduced functionality, such as decreased blood flow (7,8) and oxygen tension (9,10), compared with islets localized in situ in the pancreas.

Studies of freshly isolated islets by Linn et al. (11) showed that intraislet endothelial cells exhibit an angiogenic capacity in vitro. In addition, by transplanting freshly isolated islets containing endothelial cells carrying the LacZ reporter gene, the same authors showed that donor islet endothelial cells might transfer and survive within the islet graft (11). Utilizing a similar approach, Brissova et al. (12) confirmed this finding and performed systemic injections of the endothelia-binding *L. esculentum* lectin in transplanted mice. *L. esculentum* lectin was found to colocalize with donor endothelial cells, identified by β-galactosidase staining, in tissue sections obtained from the islet grafts, and therefore donor endothelial cells were suggested to be in contact with the blood flow and to

contribute to the revascularization of transplanted islets (12). To date, all studies conducted to elucidate the origin of the islet graft vasculature have utilized histochemical methods (5,11,12) that require fixation and sectioning of the graft. This approach distorts the tissue morphology and drastically limits functional studies of the vasculature.

The cloning and first use of green fluorescent protein (GFP) as an intracellular reporter in living cells (13) laid the foundation for today's use of fluorescent proteins as intracellular reporters within transgenic mice (14). In parallel, the development of two-photon laser-scanning microscopy (TPLSM) has resulted in a microscopy technique that permits high-resolution fluorescence imaging within intact tissue (15). By utilizing laser-scanning microscopy, discrete optical sections can be captured within tissue in a temporal mode as time sequences or in a spatial mode as image axial (z)-stacks representing a volume. TPLSM facilitates a deeper penetration into tissue and a reduced photo-induced damage of living cells compared with confocal laser-scanning microscopy (CLSM). Therefore, TPLSM is suitable for imaging of living cells within multilayer tissue (16).

We investigated whether donor islet endothelial cells have a functional role in the revascularization of transplanted pancreatic islets by studying living endothelial cells within isolated donor islets and in the intact islet graft vasculature. The Tie2-GFP transgenic mouse, characterized by its endothelial cell–specific expression of GFP (17), was utilized as donor of pancreatic islets and facilitated the use of GFP as an endothelial cell marker in both living and fixed tissue. TPLSM was used to monitor the survival of intraislet endothelial cells following islet isolation and culture to determine their possible participation in the revascularization of freshly isolated or cultured pancreatic islets following transplantation. To enable both morphological and functional studies of donated endothelial cells within the intact islet graft, we developed a novel method for simultaneous ex vivo perfusion and imaging of transplanted islet cells under the kidney capsule. Following transplantation of Tie2-GFP islets, GFP fluorescent cells were found to form intact blood vessels in the islet graft vasculature. In addition, flow studies of the islet graft vasculature showed that the donor-derived vessels were integrated with the host-derived vessels and connected to the circulatory system of the host organ.

RESEARCH DESIGN AND METHODS

Tie2-GFP mice [STOCK Tg(TIE2GFP)287Sato/J] were purchased from The Jackson Laboratories (Bar Harbor, ME). Male athymic nude mice (B6;Cg/JBomTac-Foxn1^{nu}N3), aged 7–8 weeks, were purchased from Taconic M&B (Ry, Denmark). All experiments were approved by the local animal ethics committee at Karolinska Institutet.

Pancreatic islet isolation, culture, and transplantation. For pancreatic islet isolation, mice were starved overnight and killed. The abdominal side was opened, and 4–5 ml of 2 mg/ml collagenase (Collagenase A; Roche Diagnostics, Mannheim, Germany) was injected into the pancreas via the bile duct. The distended pancreas was removed and kept on ice for a maximum of 1 h before digestion at 37°C for 9 min. Following digestion, the pancreas was dissociated and washed with cold Hank's balanced salt solution before the islets were purified using a discontinuous gradient of Histopaque 1077 and 1119 (Sigma, St. Louis, MO), followed by handpicking. The islets were cultured in suspension in RPMI-1640 medium (Gibco BRL, Life Technologies, Gaithersburg, MD) supplemented with 10% (vol/vol) FCS (Gibco), 2 mmol/l L-glutamine (Sigma), 100 IU/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma). The culture medium was changed every 2nd day. Where indicated, the culture media was supplemented with either endothelial cell growth supplement (ECGS) (Sigma) at a final concentration of 100 μg/ml or both fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (Peprotech EC, London, U.K.) at final concentrations of 20 and 10 ng/ml, respectively. Athymic nude mice were used as transplant recipients to avoid any mechanism of graft rejection. At the time of transplantation, the recipient animal was anesthetized using inhalation anesthesia (Isoflurane; Abott Scandinavia, Solna, Sweden). An incision was made through the skin and the underlying muscle layer, and the left kidney was carefully extracted from the body cavity. A small cut was made through the kidney capsule, and 200–400 islets were placed in a pocket just under the capsule. The kidney was gently inserted back into the body cavity, and the animal was sutured. Before the anesthesia was discontinued, 0.1 ml/kg Temgesic (Schering-Plough, Brussels, Belgium) was subcutaneously injected to relieve postoperative pain.

Immunohistochemistry of isolated islets and islet grafts. Isolated islets were fixed in 4% formaldehyde for 15 min at 8°C. After fixation, islets were washed in PBS, incubated for 45 min in a 15% sucrose–PBS solution at 4°C before being embedded in Tissue-Tek OCT Compound (Sakura Finetek, Zoeterwoude, the Netherlands), frozen, and stored at −80°C. Pancreatic islet grafts were cut from the graft-bearing kidneys and fixed overnight in 4% formaldehyde at 4°C. Thereafter, the grafts were washed in PBS, incubated in a 15% sucrose–PBS solution at 4°C, embedded in Tissue-Tek OCT Compound (Sakura Finetek), frozen, and stored at −80°C. Ten-micrometer–thick sections of both islets and islet grafts were cut using a cryostat (Leica CM 3000; Leica Microsystems, Nussloch, Germany) and adhered to glass slides (Superfrost Plus; Menzel, Braunsweig, Germany). The sections were washed with Opti Max Wash Buffer (Biogenex, San Ramon, CA), which was used for all subsequent washings, before blocking with goat serum for 20 min (Biogenex). Primary antibodies were then applied for 1 h, using a ready-to-use polyclonal guinea pig anti-insulin antibody (Biogenex), a monoclonal rat anti-mouse CD31 antibody (BD Bioscience Pharmingen, San Diego, CA) at 1:50 dilution, and a rabbit polyclonal anti-GFP antibody (Molecular Probes, Eugene, OR) at 1:100 dilution. The sections were then washed before secondary antibodies, anti-rabbit Alexa Fluor 488, anti-rat Alexa Fluor 633, and anti–guinea pig Alexa Fluor 633 (Molecular Probes) were applied for 20 min at 1:200 dilutions. Finally, the sections were washed before being mounted with coverslips using ProLong Antifade (Molecular Probes).

Imaging and analysis of immunofluorescent stainings. The islet and islet graft sections were scanned with a Leica TCS-SP2-AOBS confocal laser scanner equipped with Argon and HeNe lasers connected to a Leica DMLFSA microscope (Leica Microsystems Heidelberg, Mannheim, Germany) using a Leica HCX PL APO CS 63x/1.2W objective. This microscope, in combination with different objectives, was utilized for all imaging applications. Native GFP fluorescence alone or in combination with Alexa Fluor 488 was excited at 488 nm, and emission light was collected between 495 and 525 nm. Simultaneously, Alexa Fluor 633 was excited at 633 nm and emission light collected between 644 and 670 nm. Image z-stacks of the tissue sections were captured, starting and ending at the uppermost- and lowermost-detectable level of fluorescence, respectively. The image stacks were analyzed as maximum projections without any threshold. All shown fluorescence images are projections of image stacks and have been subjected to changes in brightness and contrast for optimal visualization.

Imaging and analysis of intact isolated islets. At the time point for imaging, one islet was transferred from culture to a petri dish containing PBS, in which the whole islet was imaged using a dipping objective (Leica HCX APOL 40x/0.80W). Two-photon excitation was achieved using a Ti:Sapphire laser (Tsunami; Spectra-Physics, Mountain View, CA) for ~100 fs excitation at ~82 MHz. GFP was excited at 900 nm, and emission light was collected and separated onto two nondescanned detectors using a dicronic mirror (RSP560) and emission filters for fluorescein isothiocyanate (FITC) (BP 525/50) and tetrarhodamine isothiocyanate (TRITC) (BP 610/75) fluorescence. Image z-stacks, starting at the uppermost-detectable fluorescence of the islet and ending when the GFP fluorescence signal from the islet center was lost, were captured. Every image was captured with a 1-μm step interval, and the stacks usually corresponded to a physical distance of ~60 μm. Before the image analysis was performed, bright spots of non-GFP fluorescence appearing in both the FITC and the TRITC channel were removed from the FITC channel by subtracting with the fluorescence from the TRITC channel. All images shown have been subjected to changes in brightness and contrast for optimal visualization.

Calculation of vascular density in Tie2-GFP islets. The vascular density was defined as the number of blood vessels (i.e., distinct regions of endothelial cells) per tissue area. In the intact islets scanned with TPLSM, the vascular density was calculated from the GFP fluorescent cells. The vascular density of each islet was determined as the mean value of the vascular density calculated in three different optical sections captured at 15-, 30-, and 45-μm depth into the islet. One such optical section captured at 15-μm depth is shown in Fig.

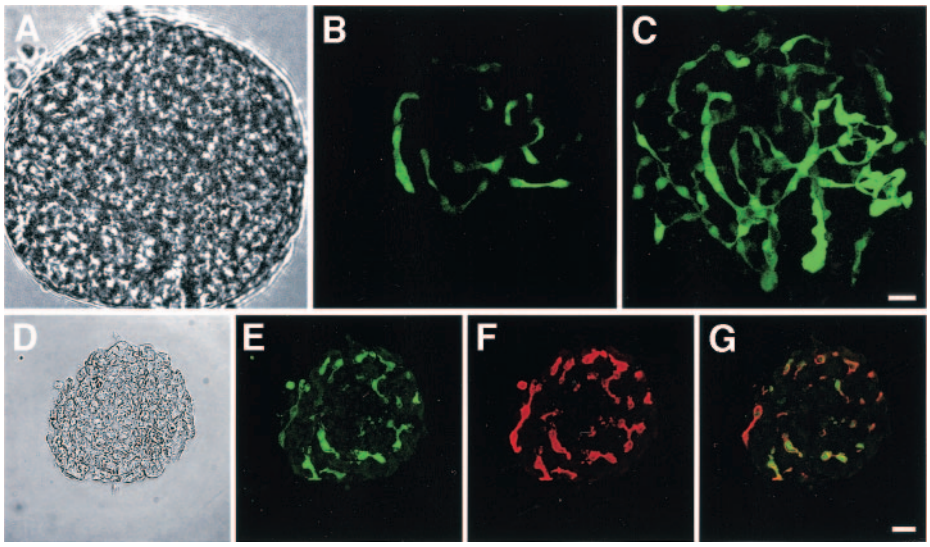

FIG. 1. GFP serves as an endothelial cell marker in pancreatic Tie2-GFP islets. Images from a freshly isolated Tie2-GFP islet captured by TPLSM are displayed (*A–C*). *A*: Islet in transmission light. *B*: GFP fluorescence captured from one optical section at 15-μm depth into the islet. *C*: Projection of GFP fluorescence captured from 58 sections, corresponding to 58 μm (see online appendix 1) (n = 19 islets). One representative section of a freshly isolated Tie2-GFP islet stained with antibodies against GFP and CD31 is shown (*D–G*). The section is shown in transmission light (*D*), stainings of GFP (*E*) and CD31 (*F*), and an overlay image of the two stainings (*G*) (n = 156 islet sections). The scale bars are representative of each picture row and correspond to 20 μm.

1*B*. In the islet sections, the vascular density was calculated from the combined GFP and CD31 staining in the image projections (Fig. 1*G*). To normalize for the methodological difference, the values obtained for the vascular density with each method were divided with the values obtained at day 0.

Ex vivo perfusion of islet grafts. All grafts were studied between 3 and 7 weeks after transplantation, a time period when the revascularization of the transplanted islets is complete (8,18). The preparations enabling perfusion of the graft-bearing kidney were modified from Korsgren et al. (19). At the time point for imaging, the graft-carrying recipient animal was anesthetized using inhalation anesthesia (Isoflurane) and placed on a heating pad. The abdominal cavity was cut open and the left kidney together with the aorta and the caval vein were made visible by moving the overlaying organs to the side and wrapping them in moist compresses. The left kidney together with the aorta and the caval vein were carefully prepared free from surrounding tissue. Thereafter, the branching vessels in the regions above and below the renal vessels were electrically coagulated and cut. The aorta and the right renal vessels were ligated with a thread above the branch of the left renal vessels. The lower part of the aorta was cut and cannulated with a thin plastic catheter connected to a running perfusion system; subsequently, the vena cava was cut open and cannulated with a plastic catheter. The catheters, acting as in- and outlet for the perfusion buffer, were kept in place with two threads. The kidney preparation was then cut free from the aorta and the vena cava before it was transferred to a custom-made chamber and placed on soft and moist compresses. A coverslip was carefully placed on top of the kidney, covering the region of the islet graft. Finally, the chamber was attached to the microscope stage and kept at 37°C. The time from induction of anesthesia to the start of imaging was ~30 min. The kidney preparation was constantly perfused with a buffer containing (in mmol/l) 118.5 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 5 HEPES, and 3 glucose, as well as 2 mg/ml BSA. The buffer was equilibrated to pH 7.3 by gassing with 95/5% O_2/CO_2 gas before it was loaded into a pressure-regulated multichannel perfusion system (AutoMate Scientific, San Francisco, CA). The O_2/CO_2 gas was used to pressurize the perfusion system and to set the flow rate at 200–300 μl/min. Texas Red-Dextran (70 kDa; Molecular Probes) was added using a syringe pump (Univentor Limited, Zejtun, Malta), as indicated during continuous perfusion.

Ex vivo imaging of islet grafts. Using 10–20× magnifying lenses (Leica 10x/0.3 PL FLUOTAR, 20x/0.7IMM HC PL APO), the islet grafts could be easily distinguished under the kidney capsule with the eye, and images that covered large surface areas (1.2 × 1.2 mm) could be captured with CLSM (Fig. 3*A* and *B*). To localize and visualize the graft with high-magnification lenses, we developed a method to identify the transplanted pancreatic islet cells via their ability to reflect light. Since the endocrine cells of the pancreatic islets are densely packed with hormone-containing vesicles, they reflect light to a high extent. In contrast, the surrounding proximal tubule of the kidney consists of liquid-filled channels with only thin layers of supporting cells and therefore reflects light poorly. For CLSM imaging, GFP was excited at 488 nm and emission light was collected between 495 and 525 nm simultaneously with illumination at 633 nm and collection of reflection light between 630 and 635 nm. As a result, the transplanted endocrine cells could be easily discriminated from the kidney tissue, and in addition, the reflection image of the endocrine cells could be captured in parallel with the GFP fluorescence without any need for labeling of the endocrine cells (Fig. 3*A* and *C* and online appendix 2 [available at http://diabetes.diabetesjournals.org]). For TPLSM imaging, GFP and Texas Red-Dextran were excited at 900 nm and emission light collected using the described FITC and TRITC filters. For image z-stacks, series of images were captured with 1-μm distance in between each image. During the imaging of time sequences, continuous scanning was applied and one image scanned approximately every 3rd s.

Statistical analysis. All values are given as means ± SE. Student's unpaired t test was used, and $P < 0.05$ was considered statistically significant for all comparisons.

RESULTS

Intraislet endothelial cells persist after islet isolation. Pancreatic islets were isolated from Tie2-GFP transgenic mice to investigate whether GFP was expressed in intraislet endothelial cells. TPLSM imaging of intact Tie2-GFP islets revealed the existence of a large number of GFP fluorescent cells with slender and outstretched morphology (Fig. 1*B* and *C*), which were arranged in continuous intraislet networks (online appendix 1). To verify the endothelial origin of the GFP-expressing cells, Tie2-GFP islets were fixed, cut, and stained with antibodies against GFP and the CD31 protein, which was used as an endothelial cell marker. GFP staining was abundant throughout the whole cell, whereas CD31 staining was primarily localized to the cell membrane with no nuclear and less cytosolic staining (Fig. 1*E* and *F*). From the immunostainings, it could be concluded that all GFP-expressing cells also expressed CD31 (Fig. 1*G*), confirming an endothelial origin. To investigate the percentage of intraislet endothelial cells that expressed GFP, pancreatic islets were isolated from seven Tie2-GFP mice, and 156 islet sections were stained against GFP and CD31. The percentage of cells expressing both markers was analyzed, and 81% of the intraislet CD31-positive cells were concluded to express GFP.

The majority of intraislet endothelial cells rapidly disappear during islet culture. To investigate the fate of intraislet endothelial cells during culture, intact Tie2-GFP islets were imaged with TPLSM at different time points following islet isolation. After 1 day of culture, 45.2 ± 7.5% (n = 7) of the GFP-expressing cells remained in the islets, and after 2 days of culture, the number of GFP-expressing cells was reduced to 14.3 ± 2.5% (n = 16) compared with the isolation day. The number of GFP-expressing cells

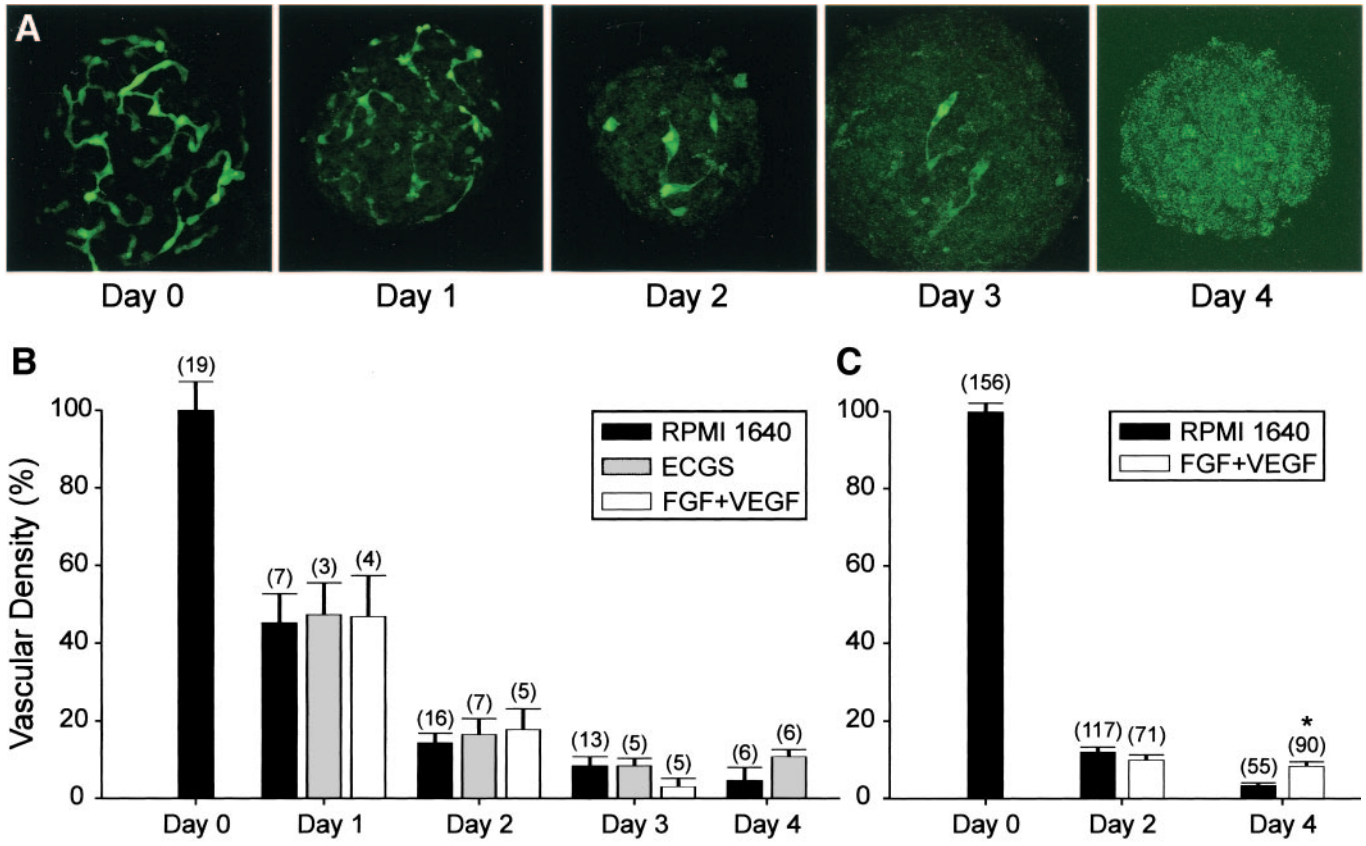

FIG. 2. Rapidly decreased intraislet endothelial cell content during islet culture. Tie2-GFP islets were imaged with TPLSM at different time points during islet culture in RPMI-1640 medium. *A*: GFP fluorescence of representative islets at the indicated time points. Note that at day 4, the weak cellular autofluorescence is enhanced during the image processing due to the absence of a strong GFP signal and therefore appears brighter compared with the other time points. The endothelial cell mitogenic factors, ECGS alone or FGF together with VEGF, were added during islet culture, and the intraislet endothelial cell content was measured and calculated as vascular density. *B*: Relative vascular density is presented as means ± SE together with the analyzed islet number at each respective time point. Freshly isolated and cultured islets were also fixed and immunostained with GFP and CD31 antibodies. *C*: Relative vascular density is presented as means ± SE together with the number of analyzed islet sections at the investigated time points.

decreased further during the culture, with 8.4 ± 2.4% (n = 13) remaining after 3 days and 4.6 ± 3.4% (n = 6) remaining after 4 days of culture (Fig. 2*A* and *B*). To verify that the loss of GFP fluorescence corresponded to the disappearance of endothelial cells, Tie2-GFP islet sections from islets cultured for 2 and 4 days were stained with GFP and CD31 antibodies. Both endothelial cell markers decreased at a similar rate and pattern during islet culture (Fig. 2*C*), indeed suggesting that the intraislet endothelial cell population drastically decreased during culture. The endothelial cell mitogenic factors, ECGS or FGF together with VEGF, were added to the culture medium, and GFP fluorescence was studied in intact islets and in fixed islet sections together with CD31 expression. Although a slightly higher number of endothelial cells was observed after 4 days of islet culture in the presence of FGF and VEGF compared with the control situation (Fig. 2*C*), neither of these two mitogenic factors had any effect on the drastic disappearance of endothelial cells (Fig. 2*B* and *C*). After 4 days of culture, in both the presence and absence of FGF and VEGF, 31–46% of the surviving intraislet endothelial cells stained positively for GFP but not for CD31 (data not shown).

Donor islet endothelial cells form vessel structures in the islet graft. To investigate whether donor intraislet endothelial cells have the potential to participate in the formation of the islet graft vasculature, freshly isolated Tie2-GFP islets were transplanted under the kidney capsule of nude mice. From low-magnification images of the islet grafts, GFP-expressing donor endothelial cells were found among the transplanted islet cells, both as single cells as well as cell aggregates resembling vessel-like structures of several-hundred micrometers in length (Fig. 3*A* and *B*). High-resolution imaging showed that single-donor endothelial cells lined up between endocrine cells (Fig. 3*C*). Detailed investigations of the vessel-like structures formed by GFP fluorescent cells revealed intact vessels composed of a large number of donor cells (Fig. 3*D*). Although the cell boundaries between the GFP-expressing cells were difficult to determine, the nuclear localization of GFP made it possible to appreciate the number of cells taking part in a vessel structure (Fig. 3*D*). The connections between GFP-expressing cells in the fluorescent vessels could be followed by analyzing image z-stacks (online appendix 2), and the intact vessel morphology could be visualized by three-dimensional reconstructions (online appendix 4). Donor islet endothelial cells were found to exist as single cells in 9 of 10 studied grafts and to participate in the formation of vessel structures in 7 of 10 studied grafts.

Donor islet endothelial cells form functional vessels in the islet graft vasculature. To analyze the functional

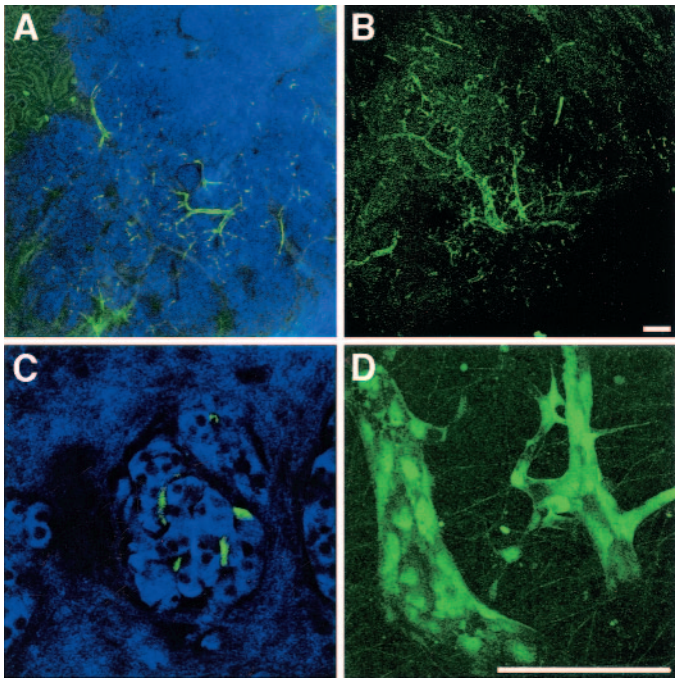

FIG. 3. Donor islet endothelial cells form vessel structures in the islet graft. Projections of image stacks captured ex vivo using CLSM and TPLSM of Tie2-GFP islet grafts are displayed (*A–D*). Using low-magnification lenses, GFP fluorescent endothelial cells of donor origin (green) were found both as single cells and as cell aggregates resembling vessel-like structures within the islet grafts (*A* and *B*). The donor endothelial cells could be visualized together with the reflection from the transplanted endocrine cells (blue) (*A* and *C*). In *A*, the autofluorescence (green) from the kidney proximal tubule cells can also be seen in the *upper left corner*. High-magnification imaging was used to depict GFP fluorescent cells among the endocrine cells of a former islet surrounded by extracellular matrix (*C*) and two vessel structures composed of donor endothelial cells (*D*). GFP fluorescent cells were found as single cells in 9 of 10 studied grafts and to form vessels in 7 of 10 grafts. The scale bars are representative of each picture row and correspond to 100 μm.

role of the vessels formed by the donor endothelial cells, we performed real-time imaging studies of the buffer flow through the islet graft vasculature. The red fluorophore Texas Red-Dextran was added to the perfusion buffer simultaneous with time sequence imaging of one optical section within islet grafts. Figure 4*A–D* shows one optical section captured within a Tie2-GFP graft during a 40-s pulse of Texas Red-Dextran. Green vessel structures and scattered GFP fluorescent cells can be seen within the chosen section before the arrival of Texas Red-Dextran (Fig. 4*A*). During the pulse, Texas Red-Dextran could be observed to gradually fill both fluorescent and nonfluorescent vessels (Fig. 4*B* and *C*) before it was cleared away (Fig. 4*D* and online appendix 3). From the Texas Red-Dextran perfusions through the islet grafts, seven different vessel areas in three different grafts, it could be concluded that the dye was transported through the vessels of donor and host origin in a similar manner. To visualize the islet graft vasculature, Texas Red-Dextran was continuously perfused through the graft-bearing kidney as image z-stacks were captured with TPLSM. One optical section, extracted from such a z-stack, shows GFP fluorescent vessels as an overlay upon the reflection image of endocrine cells captured by CLSM (Fig. 4*E*). The same section is shown during constant perfusion with Texas Red-Dextran, and the total vessel density among the transplanted cells is depicted (Fig. 4*F*). A projection of the entire image z-stack, which covers a physical distance of 42 μm, displays the Texas Red-Dextran–filled islet graft vasculature consisting of both fluorescent and nonfluorescent vessels (Fig. 4*E* and online appendix 4). To confirm that the GFP fluorescent cells found in the islet grafts displayed an endothelial cell phenotype and that they localized among the endocrine cells, the Tie2-GFP islet grafts were fixed following ex vivo imaging, cut, and

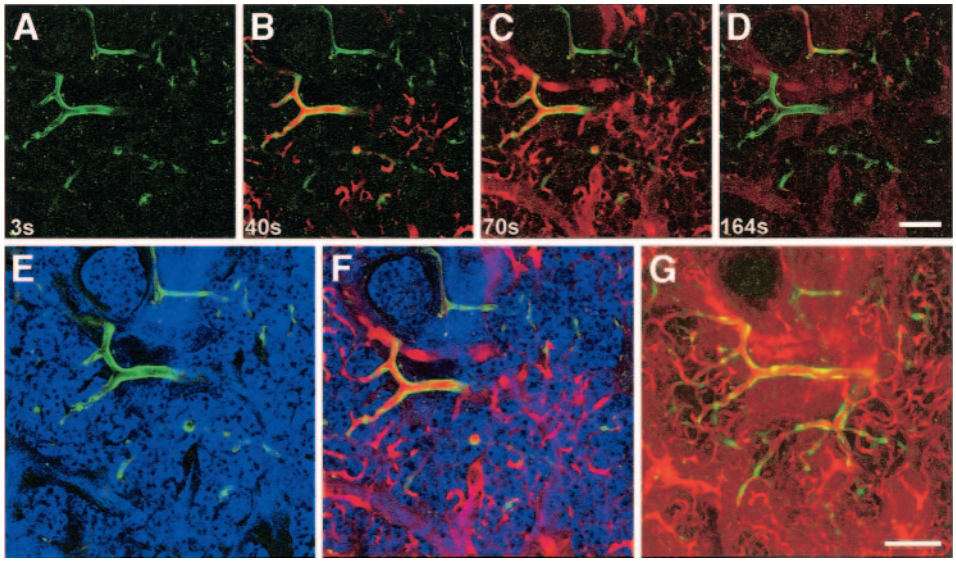

FIG. 4. Donor islet endothelial cells form functional blood vessels in the islet graft vasculature. During ex vivo TPLSM imaging of Tie2-GFP islet grafts, the red fluorophore Texas Red-Dextran was added to the perfusion buffer. A time sequence of images was captured from one optical section in an islet graft during the addition of a 40-s pulse of Texas Red-Dextran, and extracted images from different time points are displayed as indicated (*A–D*). *A*: Chosen graft area with GFP-expressing cells (green). During the perfusion, Texas Red-Dextran (red) gradually filled (*B* and *C*) both fluorescent (green) and nonfluorescent vessel before it was gradually flushed away (*D* and online appendix 3). To visualize the islet graft vasculature, Texas Red-Dextran was continuously added to the perfusion buffer during the collection of an image z-stack. Images before (*E*) and during (*F*) Texas Red-Dextran perfusion are displayed together with the reflection from the transplanted endocrine cells (blue). A projection of the graft vasculature made from 42 optical sections, corresponding to a distance of 42 μm, is shown (*G* and online appendix 4) ($n = 7$ different vessel areas in three grafts). The scale bars are representative for each picture row and correspond to 80 μm.

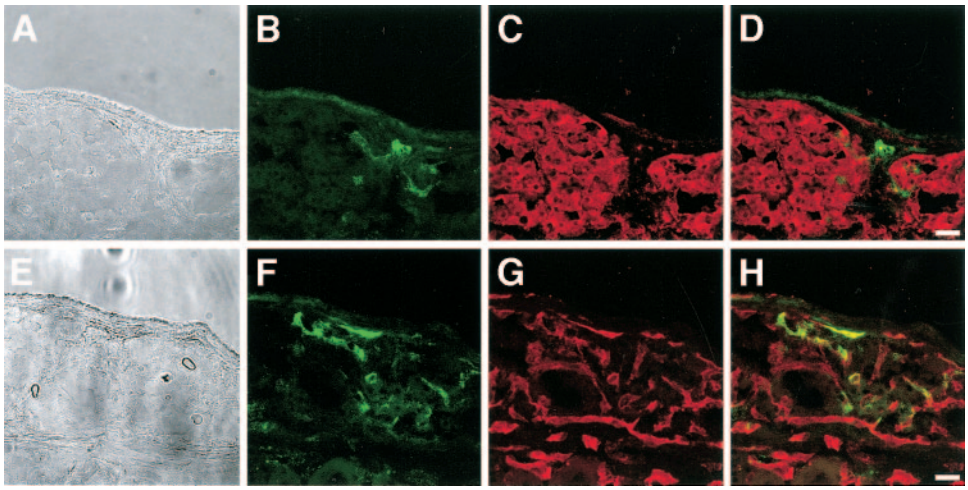

FIG. 5. Immunostaining of islet graft sections. Tie2-GFP islet grafts sections were immunostained with antibodies against GFP and CD31. One representative section stained against insulin is shown in transmission light (*A*), GFP fluorescence (*B*), insulin staining (*C*), and one overlay image of the signal from GFP and insulin (*D*). One representative section stained against CD31 is shown in transmission light (*E*), GFP fluorescence (*F*), CD31 staining (*G*), and one overlay image of the signal from GFP and CD31 (*H*) (n = 19 sections from two grafts). The scale bars are representative of each picture row and correspond to 20 μm.

stained with antibodies against CD31 and insulin. The GFP-expressing cells were found to localize among transplanted β-cells in the islet graft sections (Fig. 5*B–D*) and to express CD31 (Fig. 5*E–G*). In total, 19 tissue sections from two Tie2-GFP islet grafts were analyzed, and GFP fluorescent cells were found in all sections.

DISCUSSION

By utilizing TPLSM for imaging of isolated Tie2-GFP islets, both the morphology and the number of intraislet endothelial cells within the intact islets could be analyzed. A large number of endothelial cells existed within the freshly isolated islets, and the arrangement of the intraislet vascular network could be visualized by three-dimensional reconstructions. Immunostaining of islet sections confirmed the endothelial phenotype of GFP-expressing cells and showed that 81% of the intraislet endothelial cells expressed GFP. These data show that pancreatic islets isolated from the Tie2-GFP transgenic mouse represent a relevant and unique model for studies of living intraislet endothelial cells. The vascular density calculated from sections of freshly isolated islets correlated closely with the vascular density obtained with a similar methodology from islets localized in situ in the pancreas (6). Altogether, this indicates that the majority of intraislet endothelial cells survive the islet isolation procedure. Arriving at this conclusion, the survival of intraislet endothelial cells during islet culture was investigated. During the first 2 days of islet culture, the number of GFP-expressing cells was found to decrease by >80%, and from 3 days of culture and onwards, only 4–8% of the intraislet endothelial cells remained. These results, which correlate with the reported absence of intraislet endothelial cells in mouse (3) and rat (4) islets after 7 days of culture, clearly show that islet culture results in a rapid and radical decrease of intraislet endothelial cell content. Previous studies have shown that intraislet endothelial cells might be outgrown on collagen during stimulation with ECGS (20) and into a fibrin matrix when stimulated with FGF and VEGF (11). It has also been suggested that intraislet endothelial cells form cords extending from islets during islet suspension culture when the endothelial growth factor α, a truncated form of basic FGF, is added to the culture medium (21). To investigate if the survival rate of intraislet endothelial cells could be improved during islet culture by the presence of endothelial cell mitogenic factors, Tie2-GFP islets were cultured with the addition of either ECGS or FGF in combination with VEGF to the culture media. Neither of these two conditions affected the rapid and drastic loss of endothelial cells. This may suggest that intraislet endothelial cells in addition to mitogenic stimuli also require adherence to an extracellular matrix for survival. The mechanism behind the loss of endothelial cells was not investigated in detail, but cell death is probably a highly contributing factor. However, during islet culture, a population of cells expressing GFP but not CD31 appeared. This indicates that some endothelial cells change their gene expression over culture time, suggesting that dedifferentiation might be a contributing factor in the disappearance of cells displaying endothelial cell markers.

To facilitate functional studies of transplanted intraislet endothelial cells and the islet graft vasculature, we developed a novel model permitting simultaneous ex vivo perfusion and imaging of the islet graft located under the kidney capsule. The perfused graft–bearing kidney model has previously been successfully utilized for studies of insulin release from transplanted islets (19,22). We have now modified this model so that fluorescent reporters expressed by transplanted islet cells or included in the perfusion buffer can be imaged in the intact and living islet graft with laser-scanning microscopy techniques. In addition, we have developed a technique using reflected light to identify the subcapsular transplanted endocrine cells without any prerequisite of cell labeling. Following transplantation of freshly isolated Tie2-GFP islets, GFP fluorescent cells were identified in the islet grafts confirming previous findings of transferred and surviving donor endothelial cells within the islet graft (11,12). By using low-magnification lenses, unique images could be captured showing extensive vessel-like structures formed by donated endothelial cells in the islet grafts. Detailed morphological analysis of these vessel structures revealed that donor endothelial cells have the capacity to form intact vessels that contribute to the islet graft vasculature. By adding the fluorescent dye Texas Red-Dextran to the perfusion of the graft-bearing kidneys, the flow through the islet vasculature could be studied, and real-time imaging confirmed perfusion of the donor-derived vessels. Furthermore, three-dimensional reconstructions of the perfused islet graft vasculature showed that the donor-

derived vessels were integrated with host-derived vessels in the islet graft vasculature. Interestingly, from the images captured during Texas Red-Dextran perfusion of islet grafts, the transplanted islets appeared to be vascularized to a higher extent than expected. Further studies are required to quantify this observation and to evaluate how it correlates with previous results obtained by immunostaining. From the ex vivo studies of the islet grafts, we conclude that donor islet endothelial cells have the capacity to participate in the formation of functional blood vessels within the islet graft vasculature.

Transplantation of freshly isolated islets that contain an extensive number of endothelial cells, in contrast to cultured islets, may result in a significantly improved vascularization of the transplanted islets. This concept is supported by results from Brissova et al. (12), which indicate that as much as 40% of the islet graft vasculature are derived from the donor endothelial cells following transplantation of freshly isolated islets. Improved vascularization of the transplanted islets may in turn lead to enhanced endocrine function and survival of the islet graft. This idea is supported by a recent study by Olsson and Carlsson (23). The authors, without being able to discriminate between endothelial cells of host or donor origin, made the observation that islet grafts derived from freshly isolated islets have a higher vascular density and oxygen tension as well as an increased capacity to cure chemically induced diabetes compared with islet grafts derived from cultured islets (23). However, within this study, no difference in insulin content was found between the grafts derived from freshly isolated or cultured islets 1 month following transplantation. Therefore, it may be speculated that islet culture should be avoided in association with transplantation in order to preserve intraislet endothelial cell mass and thereby obtain a better vascularization and function of the transplanted islets. However, future studies should quantitatively clarify the contribution of host- and donor-derived endothelial cells to the islet graft vasculature following islet transplantation and determine whether the improved endocrine function of islet grafts composed by freshly isolated islets is due to an enhanced revascularization contributed by donor endothelial cells.

In conclusion, we have developed a novel model for ex vivo perfusion and imaging studies of transplanted islet cells. Utilizing this model, we have shown for the first time that donor islet endothelial cells participate in the formation of functional blood vessels within the islet graft vasculature. Functional imaging studies of transplanted islet cells ex vivo, and in the future in vivo, will contribute to the understanding of the mechanisms underlying insufficient islet graft function and survival.

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