Vascularization of Purified Pancreatic Islet-Like Cell Aggregates (Pseudoislets) After Syngeneic Transplantation

Carmela Beger, Vincenzo Cirulli, Peter Vajkoczy, Philippe A. Halban, and Michael D. Menger

To clarify whether avascular purified endocrine cell aggregates derived from islets of Langerhans (pseudoislets) revascularize similarly to what is known for intact pancreatic islet grafts, we studied the process of angiogenesis and revascularization of syngeneically transplanted pseudoislets using intravital fluorescence microscopy. Pseudoislets were composed of pure β-cells (B) or non–β-cells (NB), as well as of mixed β- and non–β-cells (B/NB; 70/30%) or nonsorted-cells (NC), and were transplanted into the dorsal skinfold of Syrian golden hamsters. Intact islet grafts served as controls. At day 6 after transplantation, microvascularization of all types of pseudoislets was found to be less than in controls, as indicated by a reduced number of transplants that contained newly formed microvessels (take-rate: B, 38.8; NB, 38.7; B/NB, 43.8; and NC, 40.3% vs. intact islet grafts, 71.9%; $P < 0.01$). Moreover, those pseudoislets that had developed a microvascular network revealed a significantly lower functional capillary density (145.8 ± 49.5 to 241.0 ± 47.5 cm^{-1} vs. intact islet grafts: 459.8 ± 65.6 cm^{-1}; $P < 0.05$). After 20 days, the take-rate of pseudoislets was still lower (B, 67.4; NB, 45.3; B/NB, 48.4; and NC, 64.2%) when compared with intact islet grafts (88%; $P < 0.05$); however, islet-like aggregates with vascularization now showed an islet-specific glomerulus-like network of capillaries with a functional capillary density (498.5 ± 49.1 to 601.4 ± 124.0 cm^{-1}) similar to that of intact islet grafts (644.3 ± 26.8 cm^{-1}). We conclude that the dissociation of pancreatic islets, followed by reaggregation of the purified endocrine cells to islet-like clusters (pseudoislets), delays the process of angiogenesis and revascularization after free transplantation; however, this does not influence the capacity to form an intact islet-specific microvasculature (angio-architecture), which appears to be independent from the cellular composition of pseudoislets. *Diabetes* **47:559–565, 1998**

From the Institute for Surgical Research (C.B., P.V.), University of Munich, Munich, Germany; Laboratoires de Recherche Louis Jeantet (V.C., P.A.H.), Centre Medical Universitaire, Geneva, Switzerland; the Department of Neurosurgery (P.V.), Klinikum Mannheim, University of Heidelberg, Mannheim; and the Institute for Clinical and Experimental Surgery (M.D.M), University of Saarland, Homburg/Saar, Germany.

Address correspondence and reprint requests to Dr. Michael D. Menger, Institute for Clinical and Experimental Surgery, University of Saarland, D-66421 Homburg/Saar, Germany. E-mail: exmdme@med-rz.uni-sb.de.

Received for publication 26 February 1997 and accepted in revised form 5 January 1998.

B, β-cells; B/NB, mixed endocrine cells containing 70% β- and 30% non–β-cells; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; IPI, intact pancreatic islet; KRBB, Krebs-Ringer bicarbonate buffer; NB, non–β-cells; NC, nonsorted cells.

Experimental studies have demonstrated that the transplantation of insulin-producing tissue has the potential to be a safe and simple procedure for curative treatment of diabetes (1). However, a variety of limitations compromises the success of islet transplantation in clinical practice (2). The major obstacles for long-term function of pancreatic islet grafts are the lack of nutritive blood supply due to inadequate vascularization (3) and endocrine-cell destruction induced by immunologic rejection (4).

Passenger leukocytes, in particular dendritic cells, are known as the primary effectors, initiating graft rejection (5,6). Moreover, endothelial cells, fibroblasts, and ductal structures, contained within isolated pancreatic islets, contribute to the sensitization for graft rejection (7,8). Therefore, removal or inactivation of such immunocompetent cells may prevent immunologic rejection and, thus, prolong graft survival and endocrine function (9–12).

The use of autofluorescence-activated cell sorting allows the purification of endocrine cells due to the separation of nonendocrine tissue and, in particular, the elimination of passenger leukocytes (13). During a culture period of a few days, the purified endocrine cells spontaneously reaggregate to islet-like clusters (pseudoislets) with an architecture typical of native islets (14,15). In vitro tests with such pseudoislets have demonstrated endocrine function comparable with that of intact islets (15).

Microvascularization of freely transplanted islets is one of the essential requirements for successful engraftment, guaranteeing sufficient nutritional blood supply to the tissue and establishing blood drainage for adequate liberation of the endocrine hormones. Complete vascularization of intact islets is achieved within a time period of 10–14 days after transplantation and is characterized by the development of a glomerulus-like network of capillaries similar to that observed in pancreatic islets in situ (16,17). However, little is known regarding whether the development of this islet-specific microvasculature depends on the intact structure and composition of the pancreatic islets, or whether it may also be achieved after islet dissociation and reaggregation of the purified endocrine cells (pseudoislet formation). To clarify this, we analyzed the process of angiogenesis and vascularization of syngeneically transplanted pseudoislets in vivo using fluorescence epi-illumination microscopy. Moreover, we studied the dependence of pseudoislet vascularization on endocrine cellular composition by the use of aggregates,

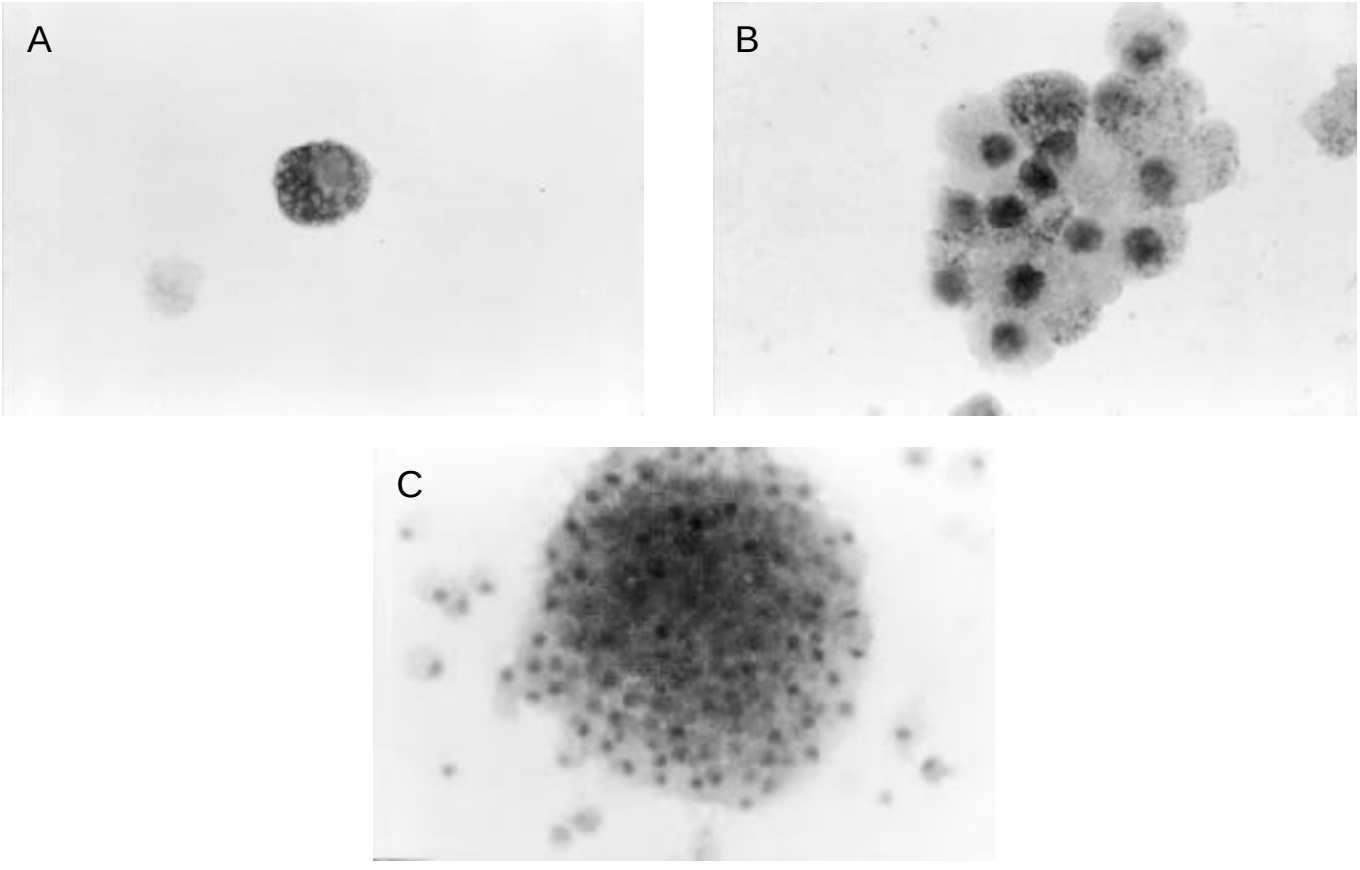

FIG. 1. Formation of islet-like aggregates of nonsorted endocrine cells. Single islet cells are identified directly after the isolation procedure (*A*). After overnight culture, small aggregates are formed (*B*), which grow to the typical pseudoislets with a size of ~100–150 µm within the 5-day culture period (*C*). β-cells are identified by indirect staining for insulin using a peroxidase method. Original magnification ×1,000 (*A*, *B*) and ×500 (*C*).

which were composed of purified β-cells (B) or non–β-cells (NB), as well as of mixed endocrine cells containing 70% β- and 30% non–β-cells (B/NB) or nonsorted cells (NC).

RESEARCH DESIGN AND METHODS

Islet isolation. Pancreatic islets were isolated from Syrian golden hamsters (80 g body weight) using modified collagenase digestion technique as described previously (18). Briefly, donor animals were anesthetized with pentobarbital (0.2 mg/100 g body wt intraperitoneally (Pentothal; Abbott, Cham, Switzerland). After laparotomy, the pancreas was exposed and distended by ductal infusion of 16 mg collagenase (type 1, catalogue no. C-0130; Sigma, St. Louis, MO), diluted in 6 ml ice-cold Hanks' balanced salt solution (HBSS), pH 7.2, containing 6 mmol/l $CaCl_2$. The distended pancreas was then immediately dissected out and stored at 4°C in a 50-ml plastic tube until all donor animals had been processed. Each pancreas was then washed twice with 50 ml of HBSS and prewarmed at 37°C. Digestion then continued under static conditions (37°C) for 20 min. The reaction was stopped by adding ice-cold HBSS containing 0.35% (wt/vol) bovine serum albumin (BSA, fraction V, catalogue no. A-2153; Sigma; HBSS-BSA). The digested pancreas was homogenized by five passages through a 14-gauge (3" length) syringe needle, and the homogenate was washed twice with buffer. Grossly undigested pancreatic tissue was removed by filtering through a plastic tea-strainer. The filtrate of each pancreas was split into two 25-ml plastic tubes and centrifuged for 10 s at 450*g*. The pellet was taken up in 10 ml Histopaque 1077 (Sigma) and overlaid with 10 ml HBSS-BSA, pH 7.4. The isolated pancreatic islets were finally hand-picked with a Gilson pipette under the stereomicroscope and kept at 4°C.

Preparation of islet cells. The freshly isolated islets were washed twice with Mg^{2+}-, Ca^{2+}-free phosphate-buffered saline containing 0.5 mmol/l EDTA, and they were resuspended into 1.5 ml Puck's buffer containing 0.16 mg/ml of trypsin (activity against casein 1:250) and 0.1 mmol/l EDTA. Digestion was carried out until only few doublet cells remained (6–7 min at 37°C [19]). The reaction was stopped by the addition of 10 ml ice-cold Krebs-Ringer bicarbonate buffer (KRBB), pH 7.4, containing 0.5% BSA, 2.5 mmol/l glucose, and 10 mmol/l HEPES. After centrifugation for 8 min at 4°C and 600*g*, cells were taken up in the same buffer to a final concentration of 3×10^6 cells/ml.

Autofluorescence-activated cell sorting of islet cells. Dispersed islet cells were analyzed in an Epis-V flow cytometer connected to a Multidisplay Acquisition Data System microcomputer (Courter Electronics, Hialeah, FL). Cellular autofluorescence, excited by an argon laser beam tuned to 488 nm at 500–600 mW output power, was plotted against forward light scatter, which relates to cellular size (13,18). At 2.5 mmol/l glucose, two islet cell populations became apparent when particle flavine adenine dinucleotide autofluorescence (510–550 nm) was plotted against light scatter. Sorting "windows" were then externally applied around both populations to deflect viable cells into one or the other collecting tube containing sterile KRBB-BSA. As described previously (18), one sorted population contains 93% non–β-cells (population 1), while the other population (population 2) mainly contains β-cells (95%). Four different cell composites were then selected for reaggregation by collecting the cells exclusively from population 1 (NB) or population 2 (B) or by reconstituting a population of cells composed of 70% β-cells and 30% non–β-cells (B/NB) or of all cells of populations 1 and 2 without further sorting (NC).

Islet cell culture. After sorting, islet cells were seeded into 100-mm nonadherent petri dishes (catalog no. 1007; Falcon, Oxnard, CA) at a concentration of 3×10^4 cells/ml in Dulbecco's minimum essential medium (Gibco catalogue no. 074–01600T) containing 5% fetal calf serum and 8.3 mmol/l glucose. The cells were maintained in culture at 37°C, 95% O_2/5% CO_2, for 5 days. The culture medium was changed every 2nd day. As previously demonstrated for rat islet cells (14,15,20), dissociated hamster islet cells reaggregate to small clusters after overnight culture and to typical pseudoislets with a diameter of 100–150 µm after a 5-day culture period (Fig. 1).

Chamber preparation and pseudoislet transplantation. The hamster dorsal skinfold chamber was used as the host site for islet transplantation. The cham-

ber and its implantation procedure have been described in detail previously (3,17). Briefly, with the animals under Nembutal anesthesia (50 mg/kg body wt intraperitoneally; Abbott, North Chicago, IL), two symmetrical titanium frames were implanted into the dorsal skinfold of 6- to 8-week-old Syrian golden hamsters in such a fashion that they sandwiched the extended skinfold. One layer of skin and the retractor muscle were completely removed in a circular area of 15 mm in diameter. The remaining thin layer of skin muscle (musculus cutaneous maximus) and the subcutaneous tissue were then covered with a removable cover slip, which was incorporated into one of the titanium frames. For islet transplantation, the cover glass of the skinfold chamber was removed, and 8 to 12 islets/pseudoislets were placed on the striated muscle tissue within the chamber. As demonstrated in previous studies, the skinfold preparation is a valid model for the intravital microscopic study of the microvasculature of freely transplanted pancreatic islets (21). The technique allows for quantitative analyses of all microvascular segments of individual islet grafts, including the supplying arterioles, nutritive capillaries, and postcapillary venules (22).

Intravital fluorescence microscopy. For intravital microscopy, the awake animals were immobilized in a plexiglass tube, and the skinfold preparations were attached to the microscope stage. To study the process of vascularization, contrast enhancement was achieved by intravenous injection of 0.1 ml of 5% FITC (fluorescein isothiocyanate)-dextran 150.000 (Sigma), which stains the plasma and remains intravascularly due to its high molecular weight. Intravenous injection of 0. l ml (0.5%) rhodamine-6G (Sigma) enabled the evaluation of endothelial diffusion characteristics by analyzing the extravasation of the fluorescent dye. Analyses of the microcirculation were performed by means of epi-illumination fluorescence microscopy at days 6, 10, 14, and 20 after transplantation using a modified Leitz Orthoplan microscope with a 100 W, HBO mercury lamp and a Ploemo-Pak illuminator with an $I_{2/3}$ blue and a N2 green filter block (Leica, Wetzlar, Germany). The observations were recorded by means of a charge-coupled device video camera (FK 6990; Prospective Measurements, San Diego, CA) and were transferred to a video system for off-line evaluation.

Quantitation of the vascularization process included the determination of the take-rate of the islet grafts, the size of the microvascular network, its functional capillary density, and the islets' individual capillary diameters. The take-rate was assessed as the number of pseudoislets presenting with newly formed microvessels and is given in percent of all islets transplanted. The size of the pseudoislets' microvascular network and its functional capillary density were analyzed in all islets that showed newly formed microvessels. The size of the microvascular network was measured planimetrically and is given in square millimeters. The functional capillary density, defined as the length of red-cell perfused capillaries per islet area, was analyzed pythagoreically (23) and is presented in reciprocal centimeters. The three-dimensional aspect was included by focusing through the islet grafts. Capillary diameters were measured perpendicularly to the path of the microvessels and are given in micrometers. Analyses were performed using a computer-assisted image analysis system (CAMAS; Zeintl, Heidelberg, Germany).

Experimental protocol. The process of angiogenesis and vascularization was studied in syngeneically transplanted pseudoislets (islet-like aggregates), consisting of purified pancreatic endocrine cells with different cellular composition. Purified B (n = 85) and NB (n = 75) aggregates were transplanted into the dorsal skinfold chambers of nine animals in each group. In the study groups with mixed endocrine cell aggregates, 64 B/NB pseudoislets were transplanted into chamber preparations of eight animals, while 77 NC pseudoislets were grafted into another eight animals. Fifty-seven intact pancreatic islets (IPIs), transplanted into the dorsal skinfold chambers of five animals, served as controls. In all groups, the process of vascularization was analyzed at days 6, 10, 14, and 20 after transplantation by means of repeated intravital fluorescence microscopy.

Immunohistochemical staining. Cytospin preparations of dissociated islet cells and aggregates were made for immunohistochemical staining on the day of islet-cell preparation and after 3 and 5 days of cell culture, respectively.

After intravital microscopy at day 20, the tissue containing the islet/pseudoislet grafts was excised and fixed in 10% formaldehyde for light microscopy and immunohistochemical staining. After 24 h, preparations were dehydrated through a graded series of alcohol, embedded in paraffin and sectioned at 3-µm thickness. Intracellular content of insular hormones was demonstrated immunohistochemically (peroxidase technique) by means of polyclonal anti-insulin, anti-glucagon, and anti-somatostatin antibodies (17,24). For the detection of intracellular insulin, the dewaxed sections were incubated with a polyclonal guinea pig anti-porcine insulin antibody (1:200; Dako, Hamburg, Germany). Cross-reactivity of the anti-porcine insulin antibody was confirmed by Western blotting. A peroxidase-conjugated rabbit anti-guinea pig IgG (1:50; Dako) was used as secondary antibody. Intracellular glucagon and somatostatin were detected by a polyclonal rabbit anti-porcine glucagon (1:50; Milab, Malmo, Sweden) and a polyclonal rabbit anti-human somatostatin (1:200; Dako) antibody, respectively. Specimens were reacted serially with a biotinylated secondary mouse anti-rabbit IgG and an avidin-biotin-horseradish peroxidase complex (both diluted according to the manufacturer's instructions; Vector Laboratories, Burlingame, CA). Finally, all specimens were stained with 3-amino-9-ethylcarbazole (Sigma), DMSO, and H_2O_2 (Merck, Darmstadt, Germany) and were counterstained with hemalaun (Merck). Controls were performed without primary and secondary antibodies to exclude nonspecific staining.

Statistical analysis. Data are presented as means ± SE. After analysis of variance, comparison between the groups was performed by the use of Dunnett's test. Differences of take-rates were calculated by χ^2 test. Analyses were performed using Sigma-Stat (Jandel Corporation, San Rafael, CA); differences were considered significant at a level of $P < 0.05$.

RESULTS

Islet vascularization. Six days after syngeneic transplantation, 72% (41/57) of the intact pancreatic islets showed newly formed microvessels with blood perfusion along interconnections between the individual capillaries (Fig. 2*A*). At day 20, the take-rate amounted to 88% (50/57; Fig. 3). The size of the islets' microvascular network and the functional capillary density increased during the first 10 days after transplantation and presented with values of ~0.05 mm^2 and 600 cm^{-1} when the process of vascularization was completed (Table 1, Fig. 4). The angio-architecture of the completely vascularized islet grafts was characterized by an individual supplying vessel, which pierced into the center of the graft, a glomerulus-like network of capillaries with core-to-mantle perfusion orientation and blood drainage by distinct venules, as well as islet-muscle intercapillary anastomoses (Fig. 2*B*). Following the course of vessels by focusing through the islet graft, in vivo microscopic analysis confirmed that the newly formed microvessels did not only surround the grafts but passed through the tissue under investigation. In parallel, light microscopy demonstrated that microvessels surround but also pierce through the grafts (Fig. 2*C*).

Analysis of rhodamine fluorescence showed increased microvascular permeability within the islet grafts when compared with host striated muscle tissue, indicating specific endothelial diffusion characteristics of the newly formed islet grafts' microvasculature. Immunohistochemistry revealed an intact architecture of grafted islets, with insulin-positive cells located primarily in the core of the islets and glucagon- and somatostatin-positive cells distributed in the islets' periphery.

Pseudoislet vascularization. Six days after syngeneic transplantation, the process of vascularization of all types of pseudoislet grafts was found to be less developed when compared with that of intact islet grafts. This was indicated by a significantly ($P < 0.01$) reduced number of transplants that contained newly formed microvessels (Fig. 3). Moreover, in most aggregates, which presented with signs of vascularization after 6 days, only a few capillary sprouts were observed (Fig. 5*A*), and functional capillary density was found to be markedly ($P < 0.05$) reduced, with values of only 146–241 cm^{-1} (intact islet grafts, 459.8 ± 65.6 cm^{-1}), regardless of the β-cell or non–β-cell proportions (Fig. 4). After 20 days, the take-rate of the pseudoislet grafts was still lower when compared with that of intact islet grafts ($P < 0.05$; Fig. 3); however, islet-like aggregates with vascularization now showed an islet-specific glomerulus-like network of capillaries with an individual arteriolar supply, core-to-mantle perfusion orientation, and a functional capillary density similar to that of intact islet grafts (Figs. 4 and 5*B*). The size of the pseudoislets' microvascular network and the diameters of the newly formed capillaries ranged between 0.038 and 0.068 mm^2, and 5.6 and 7.7 µm,

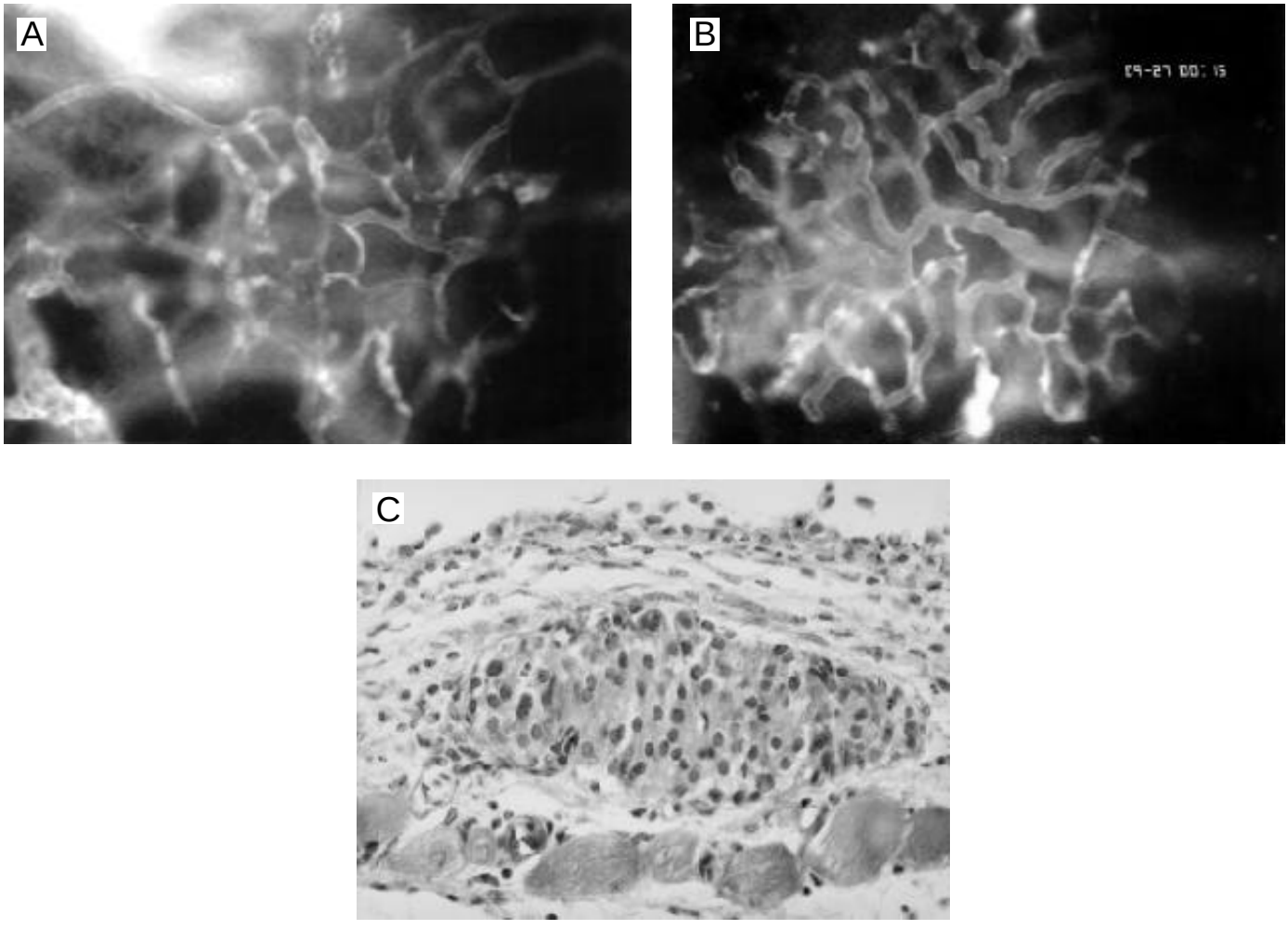

FIG. 2. Vascularization of intact syngeneic islet grafts. Vascularization of an intact islet graft after syngeneic transplantation into the dorsal skinfold chamber of a Syrian golden hamster. *A*: At day 6 after transplantation, newly formed microvessels connect with each other and form a network of capillaries, which is already blood perfused. *B*: At day 20 after transplantation, vascularization is completed, demonstrating the typical glomerulus-like network of capillaries, known for pancreatic islets in situ. Intravital fluorescence microscopy; contrast enhancement by FITC-dextran 150.000; original magnification ×250. *C*: Light microscopy of intact islet graft (transplanted onto striated muscle tissue of the dorsal skinfold chamber) demonstrating newly formed microvessels surrounding the graft as well as piercing into the grafted tissue (microvessels are easily identified by the presence of erythrocytes). Hematoxylin-eosin staining; original magnification ×250.

respectively. These sizes were not significantly different from those of intact islet grafts (Tables 1 and 2).

Comparison of the four pseudoislet groups revealed that, apart from a slightly higher take-rate of B and NC aggregates at day 20 after transplantation (Fig. 3), there were no significant differences in the process of vascularization of the four variations of endocrine-cell aggregates. Moreover, analysis of rhodamine fluorescence showed the islet-specific increased microvascular permeability within all types of pseudoislets, independent of their cellular composition.

Light microscopy demonstrated spatial distribution of microvessels similar to that in intact islet grafts. Immunohistochemistry of intracellular insulin, glucagon, and somatostatin at day 20 after transplantation revealed positive staining for insulin in the center and for glucagon and somatostatin in the periphery of pseudoislets composed of mixed endocrine cells (B/NB and NC). Pure B and NB aggregates revealed a dense staining for merely insulin or glucagon and somatostatin, respectively.

DISCUSSION

The major finding of our study is that the dissociation of isolated pancreatic islets, followed by reaggregation of the purified endocrine cells to islet-like clusters (pseudoislets), delays the process of angiogenesis and vascularization after free transplantation but does not disable the formation of an intact islet-specific microvasculature (angio-architecture). This indicates that pseudoislets survive by revascularization. The angio-architecture of the glomerulus-like network of capillaries of pseudoislet grafts is similar to that of intact islet grafts and corresponds to the microvascular architecture described for pancreatic islets in situ (25), including also an individual supplying vessel and a distinct venular drainage system. The arrangement of the microvessels has to be considered as specific for pancreatic islets because this type of glomerulus-like arrangement is not observed in the microvasculature of other tissues in situ or after free transplantation (26–29).

Experimental studies have shown that intact islets as well as pseudoislets have to be considered avascular at the time

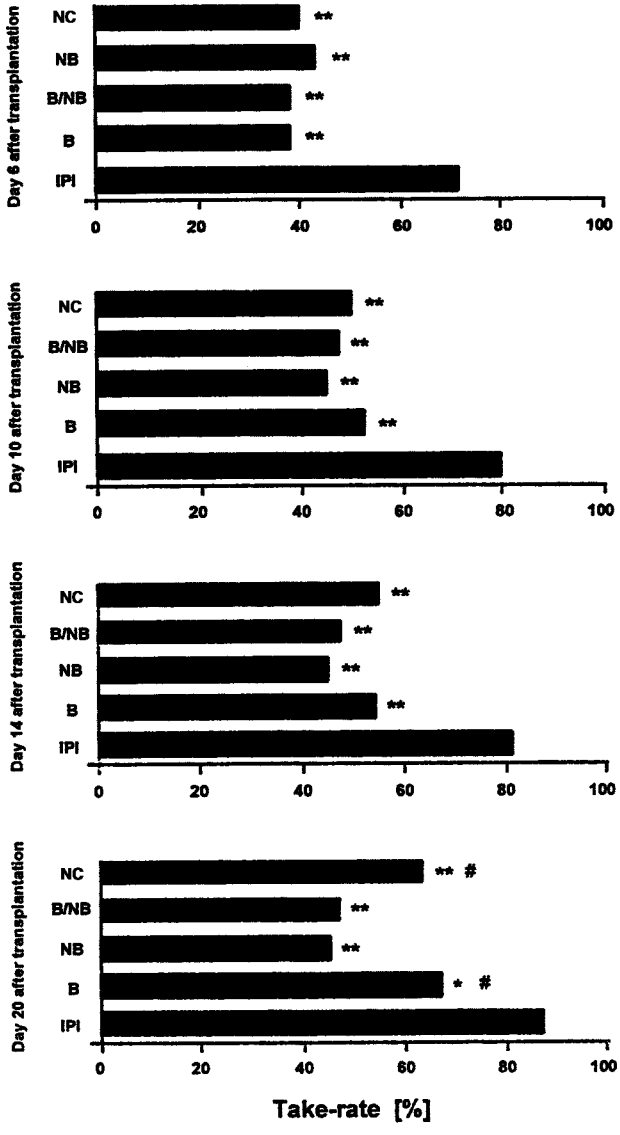

FIG. 3. Take-rate of pseudoislet and intact islet grafts. Take-rate of pseudoislets and intact islets at days 6, 10, 14, and 20 after syngeneic transplantation into the dorsal skinfold chamber of Syrian golden hamsters. Pseudoislets were composed of purified β-cells (B), purified non–β-cells (NB), 70% β-/30% non–β-cells (B/NB), or nonsorted cells (NC). IPIs served as controls. Take-rate is given in percent of all islets/pseudoislets transplanted. χ^2 test; *$P < 0.05$; **$P < 0.01$ vs. IPI; #$P < 0.05$ vs. NB.

of transplantation and are revascularized by endothelial cells of host origin (30,31). The demonstration of a specific diaphragmal fenestration of these endothelial cells in both types of transplants (30,31) indicates morphological differentiation and represents the ultrastructural correlate for the islet-specific diffusion function (rhodamine extravasation), which corresponds well to the endothelial diffusion characteristics known for pancreatic islets in situ (32).

The process of capillary formation and vascularization of endocrine-cell aggregates composed of purified β-cells was not different from that observed in pseudoislets composed of purified non–β-cells and was comparable with revascularization of pseudoislets composed of mixed endocrine cells, i.e., B/NB and NC cells, respectively. This indicates that the formation of the microvasculature is not dependent on a particular endocrine-cell composition.

Vascularization of freely transplanted endocrine tissue may involve different growth factors and other cytokines. Although we cannot discern from the present study which individual factors initiate adequate pseudoislet vascularization, our results suggest that the factors responsible for the process of angiogenesis may either be expressed by both B and NB cells (because the formation of new microvessels was not found to be dependent on the cellular composition of the pseudoislet grafts) or may even be derived from nonendocrine cellular or structural components of the host tissue.

The processes of islet dissociation, endocrine-cell purification, and reaggregation did not prevent the formation of an islet-specific angio-architecture after free transplantation. Revascularization, however, was found markedly delayed when compared with that of intact islet grafts. This could be due to the removal of immunocompetent cell types (dendritic cells and resident islet macrophages) and other structural components (endothelial cells, pericytes, fibroblasts, ductal cells) from the islets before reaggregation. These cellular and structural components are well known to secrete growth factors and other cytokines, thus contributing to the process of angiogenesis (33–35). In addition, they may themselves initiate angiogenesis by responding to various biochemical stresses that are imposed on the graft in vivo after transplantation. Thus, lack of those cellular and structural components may be responsible for the delayed graft vascularization observed in the present study.

Graft rejection is one of the major limitations of successful clinical application of islet transplantation (4). Rejection is initiated by immunocompetent nonendocrine cells within the pancreatic islets (5–8). Various immunosuppressive treat-

TABLE 1
Size of the microvascular network (mm^2) of pseudoislet and islet grafts

Group	Day 6	Day 10	Day 14	Day 20
B	0.017 ± 0.004	0.028 ± 0.005	0.047 ± 0.008	0.067 ± 0.010
NB	0.029 ± 0.009	0.048 ± 0.015	0.039 ± 0.014	0.053 ± 0.010
B/NB	0.014 ± 0.005	0.033 ± 0.010	0.036 ± 0.007	0.038 ± 0.005
NC	0.015 ± 0.005	0.032 ± 0.007	0.047 ± 0.009	0.068 ± 0.010
IPI	0.027 ± 0.003	0.041 ± 0.005	0.047 ± 0.003	0.056 ± 0.002

Data are means ± SE. Values were obtained at days 6, 10, 14, and 20 after syngeneic transplantation into the dorsal skinfold chamber of Syrian golden hamsters. Pseudoislets were composed of purified β-cells (B), purified non–β-cells (NB), 70% β-/30% non–β-cells (B/NB), or nonsorted cells (NC). IPIs served as controls.

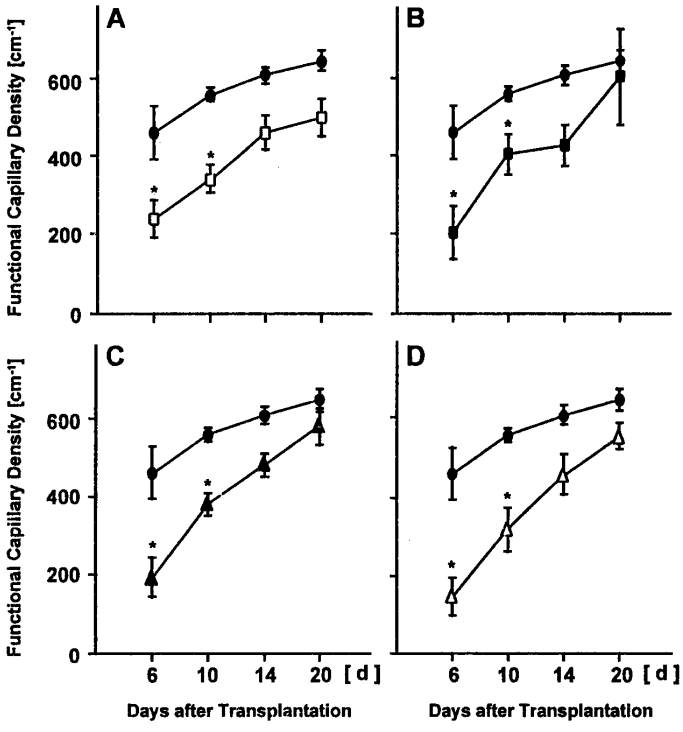

FIG. 4. Functional capillary density of pseudoislet and intact islet grafts. Functional capillary density of pseudoislets and intact islets at days 6, 10, 14, and 20 after syngeneic transplantation into the dorsal skinfold chamber of Syrian golden hamsters. Pseudoislets were composed of purified β-cells (*A*; □), purified non–β-cells (*B*; ■), 70% β-/30% non–β-cells (*C*; ▲), or nonsorted cells (*D*; △). IPIs served as controls (●). Means ± SE; ANOVA, Dunnett's test; **P* < 0.05 vs. IPIs (●).

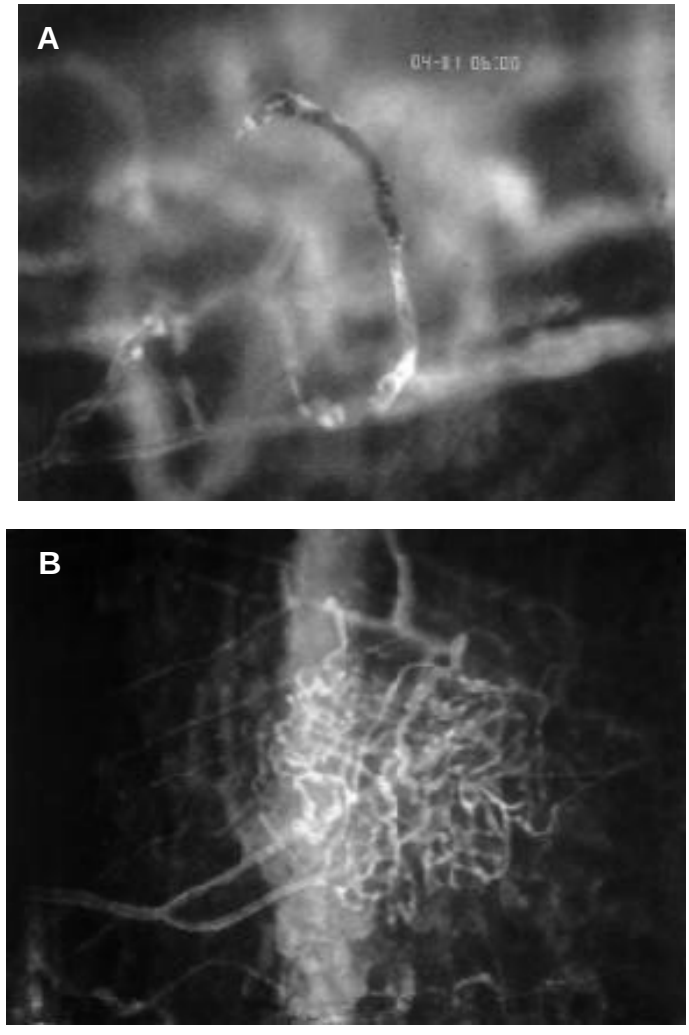

FIG. 5. Vascularization of syngeneic pseudoislet grafts. *A*: Vascularization of a pseudoislet graft, composed of nonsorted cells, at day 6 after syngeneic transplantation into the dorsal skinfold chamber of a Syrian golden hamster. Note the capillary sprout formation, which originates from the nutritive striated muscle capillaries of the host tissue. Interconnections of newly formed microvessels are not observed. *B*: Microvasculature of the same pseudoislet graft as demonstrated in *A* at day 20 after transplantation. The process of vascularization is completed, with a glomerulus-like network of capillaries similar to that demonstrated for intact islet grafts (see Fig. 2*B*). Intravital fluorescence microscopy; contrast enhancement by FITC-dextran 150.000. Original magnifications ×250 and ×100, respectively.

ments have been applied to inhibit graft rejection but could not provide long-term function of intact islet grafts (26–38). This failure is in part explained by drug-related inhibitory effects on the process of revascularization of the transplanted tissue (39). Islet dissociation and purification of the endocrine-cell population via cell sorting have been suggested as an alternative to the transplantation of intact islets (40). The process of purification allows the transplantation of a population of mainly endocrine cells, excluding nonendocrine cells, in particular, immunocompetent "passenger leukocytes." As demonstrated in the present study, transplantation of aggregates, consisting of

TABLE 2
Capillary diameters (μm) of pseudoislet and islet grafts

Group	Day 6	Day 10	Day 14	Day 20
B	7.58 ± 0.46	7.59 ± 0.32	6.99 ± 0.27	7.66 ± 0.57
NB	7.00 ± 0.66	6.56 ± 0.28	6.86 ± 0.43	7.16 ± 0.60
B/NB	6.53 ± 0.44	6.20 ± 0.45	6.19 ± 0.25	5.55 ± 0.29
NC	6.37 ± 0.42	7.05 ± 0.37	6.02 ± 0.19	6.21 ± 0.16
IPI	6.48 ± 0.34	6.67 ± 0.15	6.39 ± 0.24	6.43 ± 0.08

Data are means ± SE. Values were obtained at days 6, 10, 14, and 20 after syngeneic transplantation into the dorsal skinfold chamber of Syrian golden hamsters. Pseudoislets were composed of purified β-cells (B), purified non–β-cells (NB), 70% β-/30% non–β-cells (B/NB), or nonsorted cells (NC). IPIs served as controls.

these purified endocrine cells (pseudoislets), leads to formation of a microvascular network comparable to that of intact islet grafts and therefore fulfills one of the essential requirements for adequate graft function. In fact, pseudoislets that are depleted of nonendocrine immunocompetent cells may attenuate graft rejection after allogeneic or xenogeneic transplantation or may at least allow reduction of host immunosuppression, leading to improved long-term function and favorable clinical outcome. This view is supported by recent studies demonstrating diminished graft rejection after allogeneic pseudoislet transplantation (41,42).

Taken together, we conclude that the process of vascularization of pseudoislet syngrafts is delayed when compared with intact islet grafts; however, independent of their cellular composition, pseudoislets are able to form an islet-specific microvasculature that guarantees survival by sufficient nutritional blood supply to tissue and adequate blood drainage for liberation of endocrine hormones. Therefore, transplantation of pseudoislets may become a suitable alternative to islet transplantation in diabetes.

ACKNOWLEDGMENTS

This work was supported by grant no. 32-40427.94 of the Swiss National Science Fund (P.A.M.), by grant no. BMH 4-CT95-0875 of the European Community, and by a fellowship from the Juvenile Diabetes Foundation International (V.C.).

We gratefully acknowledge the excellent technical assistance of Elke Schütze and Beate Wolf.

REFERENCES

1. Federlin KF: Islet transplantation: the connection of experiment and clinic exemplified by the transplantation of islets of Langerhans. *Exp Clin Endocrinol* 101:334–345, 1993
2. Brunicardi FC, Mullen Y: Issues in clinical islet transplantation. *Pancreas* 9:281–290, 1994
3. Menger MD, Vajkoczy P, Leiderer R, Jäger S, Messmer K: Influence of experimental hyperglycemia on microvascular blood perfusion of pancreatic islet isografts. *J Clin Invest* 90:1361–1369, 1992
4. Gray DWR, Morris PJ: Prospects for pancreatic islet transplantation. *World J Surg* 10:410–421, 1986
5. Lechler RI, Batchelor JR: Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J Exp Med* 155:31–41, 1982
6. Steinman RM, Gutchinov B, Witmer MD, Nussenzweig MC: Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J Exp Med* 157:613–627, 1983
7. Lacy PE, Davie JM, Finke EH: Transplantation of insulin-producing tissue. *Am J Med* 70:589–594, 1981
8. Steiniger B, Hiller WFA, Klempnauer J: Identical pattern of acute rejection after isolated islet and vascularized whole-pancreas transplantation in rat. *Am J Pathol* 137:93–102, 1990
9. Bartlett ST, Naji A, Silvers WK, Barker CF: Influence of culturing on the functioning of major-histocompatibility-complex-compatible and incompatible islet grafts in diabetic mice. *Transplantation* 36:687–690, 1983
10. Faustman DL, Steinman RM, Gebel HM, Hauptfeld V, Davie JM, Lacy PE: Prevention of rejection of murine islet allografts by pretreatment with anti-dendritic cell antibody. *Proc Natl Acad Sci USA* 81:3864–3868, 1984
11. Kedinger M, Haffen K, Grenier J, Eloy R: In vitro culture reduces immunogenicity of pancreatic endocrine cells. *Nature* 2709:736–738, 1977
12. Lacy PE, Davie JM, Finke EH: Prolongation of islet allograft survival following in vitro culture (24° C) and a single injection of ALS. *Science* 204:312–313, 1979
13. Van de Winkel M, Pipeleers D: Autofluorescence-activated cell sorting of pancreatic islet cells: purification of insulin-containing B-cells according to glucose-induced changes in cellular redox state. *Biochem Biophys Res Commun* 107:525–532, 1982
14. Halban PA, Powers SL, George KL, Bonner-Weir S: Spontaneous reassociation of dispersed adult rat pancreatic islet cells into aggregates with three-dimensional architecture typical of native islets. *Diabetes* 36:783–790, 1987
15. Hopcroft DW, Mason DR, Scott RS: Insulin secretion from perifused rat pancreatic pseudoislets. *In Vitro Cell Dev Biol* 21:421–427, 1985
16. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K: Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 38 (Suppl. 1):199–201, 1989
17. Menger MD, Jäger S, Walter P, Hammersen F, Messmer K: A novel technique for studies on the microvasculature of transplanted islets of Langerhans in vivo. *Int J Microcirc Clin Exp* 9:103–117, 1990
18. Rouiller DG, Cirulli V, Halban PA: Differences in aggregation properties and levels of the neural adhesion molecule (NCAM) between islet cell types. *Exp Cell Res* 191:305–312, 1990
19. Cirulli V, Halban PA, Rouiller DG: Tumor necrosis factor-alpha modifies adhesion properties of rat islet B cells. *J Clin Invest* 91:1868–1876, 1993
20. Tze WJ, Tail T: Preparation of pseudoislets for morphological and functional studies. *Transplantation* 34:228–231, 1982
21. Menger MD, Lehr H-A: Scope and perspectives of intravital microscopy: bridge over from in vitro to in vivo. *Immunol Today* 14:519–522, 1993
22. Menger MD, Hammersen F, Messmer K: The microvasculature of free pancreatic islet grafts. In *Pathways in Applied Immunology*. Messmer K, Stein M, Eds. Heidelberg, Springer, Germany, 1991, p. 109–126
23. Zeintl H, Tompkins WR, Messmer K, Intaglietta M: Static and dynamic microcirculatory video image analysis applied to clinical investigations. *Prog Appl Microcirc* 11:1–10, 1986
24. Menger MD, Vajkoczy P, Beger C, Messmer K: Orientation of microvascular blood flow in pancreatic islet isografts. *J Clin Invest* 93:2280–2285, 1994
25. Bonner-Weir S, Orci L: New perspectives on the microvasculature of the islets of Langerhans in the rat. *Diabetes* 31:883–889, 1982
26. Funk W, Endrich B, Messmer K: A novel method for follow-up studies of the microcirculation in non-malignant tissue implants. *Res Exp Med* 186:259–270, 1986
27. Foitzik T, Funk W, Roth H, Messmer K: Splenic implants: influence of particle size and fibrin fixation on vascularization and angioarchitecture. *Pediatr Surg Int* 4:263–268, 1988
28. Leunig M, Yuan F, Berk DA, Gerweck LE, Jain RK: Angiogenesis and growth of isografted bone: quantitative in vivo assay in nude mice. *Lab Invest* 71:300–307, 1994
29. Menger MD, Hammersen F, Messmer K: In vivo assessment of neovascularization and incorporation of prosthetic vascular biografts. *Thorac Cardiovasc Surg* 40:19–25, 1992
30. Hart TK, Pino RM: Pseudoislet vascularization: induction of diaphragm-fenestrated endothelia from the hepatic sinusoids. *Lab Invest* 54:304–313, 1986
31. Vajkoczy P, Olofsson AM, Lehr H-A, Leiderer R, Hammersen F, Arfors K-E, Menger MD: Histogenesis and ultrastructure of pancreatic islet graft microvasculature: evidence for graft vascularization by endothelial cells of host origin. *Am J Pathol* 146:1397–1405, 1995
32. Kusterer K, Beck O, Enghofer O, Usadel K-H: In-vivo staining of the islets of Langerhans for microcirculatory investigations (Abstract). *Int J Microcirc Clin Exp* 11:S201, 1992
33. Risau W: Mechanisms of angiogenesis. *Nature* 386:671–674, 1997
34. Beck L Jr, D'Amore PA: Vascular development: cellular and molecular regulation. *FASEB J* 11:365–373, 1997
35. Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD: The codependence of angiogenesis and chronic inflammation. *FASEB J* 11:457–465, 1997
36. Bell PR, Wood RF, Peters M, Nash JR: Comparison of various methods of chemical immunosuppression in islet cell transplantation. *Transplant Proc* 12:291–293, 1980
37. Reece-Smith H, Du TD, McShane P, Morris PJ: Prolonged survival of pancreatic islet allografts transplanted beneath the renal capsule. *Transplantation* 31:305–306, 1981
38. Kneteman NM, Alderson D, Scharp DW: Long-term normoglycemia in pancreatectomized dogs following pancreatic islet allotransplantation and cyclosporine immunosuppression. *Transplantation* 44:595–599, 1987
39. Menger MD, Wolf B, Höbel R, Schorlemmer HU, Messmer K: Microvascular phenomena during pancreatic islet graft rejection. *Langenbecks Arch Chir* 376:214–221, 1991
40. Pipeleers DG, Pipeleers-Marichal M, Hannaert JC, Berghmans M, In't Veld PA, Rozing J, Van de Winkel M, Gepts W: Transplantation of purified islet cells in diabetic rats. I. Standardization of islet cell grafts. *Diabetes* 40:908–919, 1991
41. Pipeleers DG, Pipeleers-Marichal M, Vanbrabandt B, Duys S: Transplantation of purified islet cells in diabetic rats. II. Immunogenicity of allografted islet beta-cells. *Diabetes* 40:920–930, 1991
42. Pipeleers-Marichal M, Ling Z-D, Pipeleers DG: Transplantation of purified islet cells in diabetic rats. III. Immunosuppressive effect of cyclosporine. *Diabetes* 40:931–938, 1991

Author Queries (please see Q in margin and underlined text)

Q1: **<<Au: What does "their" refer to here? Islets?>>**
Q2: FIG. 1., page 2. Okay to join the two phrases with "and"? per ADA style.
"Pseudoislet formation and formation of islet-like aggregates of nonsorted endocrine cells. Single islet cells are identified directly after the isolation procedure (*A*). "