Decreased Vascular Density in Mouse Pancreatic Islets After Transplantation

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An adequate revascularization is crucial for islet survival and function after transplantation. Previous studies have suggested that islet revascularization is concluded within 14 days after transplantation. We investigated if the vascular density of transplanted islets and endogenous pancreatic islets differs. Cultured islets were syngeneically transplanted into the kidney, liver, or spleen of C57BL/6 mice. One month later, the graft-bearing organ was removed, and histological specimens were prepared and stained for endothelium with the lectin Bandeiraea simplicifolia. Pancrea from nontransplanted control animals were prepared similarly. Uniform staining of endothelium with the lectin Bandeiraea simplicifolia was detected in connective tissue surrounding intra-islet capillaries. A much lower vascular density was detected in connective tissue surrounding implanted microspheres of a size similar to the islets, which suggests that the islets per se induced blood vessel formation in their vicinity. We conclude that the vascular density in revascularized transplanted islets is markedly decreased compared with endogenous islets. This has potential implications for islet graft metabolism and function. *Diabetes* 51:1362–1366, 2002

The recent application of a new treatment regimen, the so-called Edmonton protocol, has markedly improved the outcome of clinical islet transplantation (1,2). When applying this protocol, however, transplantation of a large number of islets (>9,000 islet equivalent [IEQ]/kg body wt) has been shown to be necessary to achieve insulin independence. Because of the limited availability of islet tissue, this severely restricts the number of patients who may be treated with islet transplantation. Methods to reduce the number of islets needed to cure a diabetic individual are therefore warranted.

Endogenous pancreatic islets have a unique glomerular-like angioarchitecture with a high blood perfusion of 5–7 ml · min⁻¹ · g⁻¹ tissue (3,4). This secures an optimal delivery of oxygen and nutrients to islet cells and ensures an adequate dispersal of secreted hormones. When islets are isolated and cultured before transplantation, the islet endothelium dedifferentiates or degenerates (5). A rapid revascularization is therefore crucial for islet function after transplantation, and this has been shown to occur within 7–14 days (6,7). However, the extent of revascularization has not been thoroughly studied, and recent experiments on islets transplanted to the renal, splenic, or hepatic subcapsular space have suggested that this process is insufficient to achieve optimal oxygenation of the transplanted islets (8–10).

The aim of the present study was to compare the vascular density of endogenous pancreatic islets to that of mouse islets syngeneically implanted into different organs. Measurements were performed 1 month posttransplantation, i.e., at a time point when transplanted islets have become fully revascularized. Connective tissue was found to constitute a substantial part of the transplant and to surround individual islets in grafts consisting of several islets (islets implanted to the kidney and spleen). Therefore, the vascular densities in this connective tissue and the transplanted islets of these grafts were determined separately.

**RESEARCH DESIGN AND METHODS**

**Animals.** Inbred, male C57BL/6 mice weighing 25–30 g were purchased from B&K Universal (Sollentuna, Sweden). The animals had free access to tap water and pelleted food throughout the course of the study. All experiments were approved by the local animal ethics committee at Uppsala University. Islet isolation, culture, and transplantation. Pancreatic islets were prepared by collagenase digestion, as previously described (11). The isolated islets were cultured free-floating in groups of 150 for 3–4 days in 5 ml culture medium consisting of RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with l-glutamine (Sigma-Aldrich), benzylpenicillin (100 units/ml) (Roche Diagnostics Scandinavia, Bromma, Sweden), streptomycin (0.1 mg/ml; Sigma-Aldrich), and 10% (vol/vol) fetal calf serum (Sigma-Aldrich). The culture medium was changed every second day. After culture, 250–350 islets were either packed in a bracing pipette and implanted beneath the renal capsule of the left kidney or packed in a butterfly needle (25 gauge) and injected into the splenic parenchyma or portal vein (12). All recipients were anesthetized intraperitoneally with avertin: 0.02 ml/g body wt of a 2.5% (vol/vol) solution consisting of 10 g of 97% (vol/vol) 2,2,2-tribromo-ethanol (Sigma-Aldrich) in 10 ml 2-methyl-2-butanol (Kemila, Stockholm, Sweden).

**Preparation of histological sections and staining procedures.** One month posttransplantation, the transplanted animals were killed by cervical dislocation, and the graft-bearing organ was removed. Separate, nontransplanted C57BL/6 mice of the same age were also killed, but only their pancreas was removed. The organs were fixed in 10% (vol/vol) neutral buffered formalin for 24 h, dehydrated in ethanol, and embedded in paraffin. Sections, 5 μm thick, were mounted on glass slides. The slides were pretreated with neuraminidase type X (Sigma-Aldrich), and the microvascular endothelium was stained with the lectin Bandeiraea simplicifolia (BS-1; Sigma-Aldrich), as previously described (13). Briefly, the sections were incubated with normal goat serum (NGS) (Dakopatts, Glostrup, Denmark) for 1 h at room tempera-

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BS-1, Bandeiraea simplicifolia; BSA, bovine serum albumin; NGS, normal goat serum; TBS, Tris-buffered saline.
tured (20°C). Biotinylated BS-1 was applied to the sections, which were incubated at 4°C overnight. The slides were washed in Tris-buffered saline (TBS) (3 × 5 min) and incubated with StreptABComplex (Dakopatts) for 30 min at 20°C. The slides were washed again, and new fuchsin substrate system (Dakopatts) was applied to the slides and developed. To exclude endogenous alkaline phosphatase activity in the new fuchsin substrate system, 1 mol/l levamisole (Sigma-Aldrich), dissolved in redistilled water, was added before development. The slides were counterstained with hematoxylin. Negative control sections were incubated with NBC (Dakopatts) diluted in TBS containing 0.1% bovine serum albumin (BSA) (dilution 1:20) instead of BS-1.

To find the islet grafts in the liver sections, the latter were also stained with antibodies against insulin. The slides were washed, incubated for 10 min with 8% (wt/vol) hydrogen peroxide in TBS, and washed again. The sections were thereafter incubated with normal swine serum (Dakopatts) diluted 1:20 with TBS containing 0.1% (wt/vol) BSA for 1 h. Primary antibodies against insulin (ICN Biomedicals, Aurora, OH), diluted in TBS containing 0.1% (wt/vol) BSA, were applied to the slides for 1 h. The slides were washed (TBS; 3 × 5 min) and incubated for 30 min with secondary swine anti-rabbit antibody (Dakopatts) diluted 1:100 in TBS. The slides were then washed again. Rabbit peroxidase anti-peroxidase antibody (Dakopatts), diluted 1:100 in TBS, was applied to the slides for 30 min. The slides were washed and developed with Vector SG substrate kit (Immunkemi, Jäla, Sweden) for 8 min, washed again, and new fuchsin substrate system (Dakopatts) was applied to the slides and developed. To exclude endogenous tissue was similar in the kidney (36 ± 2%; n = 6) and spleen (27 ± 2%; n = 6). The intraorally transplanted islets were also surrounded by connective tissue. Because most of these islets were entrapped in perportal areas, it was impossible to decide whether the connective tissue belonged to the graft or to the liver stroma. Connective tissue was also formed around the microspheres implanted to the renal subcapsular space and constituted 57 ± 2% (n = 6) of these grafts.

Vascular density. The vascular density of the transplanted islets was decreased compared with that of the endogenous pancreatic islets, irrespective of the implantation organ (Fig. 2). Islets transplanted into the spleen had a lower vascular density than islets transplanted beneath the renal capsule or into the liver (Fig. 2). In the connective tissue stroma of grafts implanted into kidney or spleen, the number of capillaries was markedly higher than in the endocrine parts of these grafts (Fig. 3). The density of intra-graft stromal capillaries appeared to be higher in the immediate vicinity of the islets at all three implantation sites.

A markedly lower number of capillaries was found in the connective tissue surrounding microspheres implanted to the renal subcapsular space compared with connective tissue surrounding implanted islets (185 ± 30 vs. 3,854 ± 516 capillaries/mm²; n = 6 in both groups; P < 0.05).

DISCUSSION

Previous studies have shown that revascularization of transplanted islets is initiated within 2–4 days and is completed within 14 days after implantation (6,7,17–20). Although vascular density has been estimated in transplanted islets (6,20,21), there have been no quantitative studies comparing the actual number of blood vessels in transplanted and endogenous islets. The present study shows that vascular density in transplanted islets is decreased compared with endogenous islets 1 month after implantation. This decrease is present at all investigated implantation sites, although it is more pronounced in islets implanted into the spleen than in islets implanted into the liver or kidney. Our data therefore suggest that the revascularization of transplanted islets is impaired, at least quantitatively, irrespective of the implantation site.

We have recently evaluated various markers for microvascular endothelial cells in tissue samples from rodents and found that the lectin BS-1, which binds to α-D-galactosyl residues on endothelial cells (22), enabled us to detect these cells in all tissues examined (13). Of particular interest was the finding that BS-1 consistently stained endothelium in endogenous pancreatic islets and in syngeneically transplanted rat and mouse pancreatic islets irrespective of implantation site. As found previously, the microvessels of the intraorapal and renal subcapsular islet grafts were distinctly stained with BS-1 in the present study. The blood vessels within the islet grafts implanted to the spleen were, however, more faintly stained. The reason is unknown, but may reflect revascularization from the unique vascular system that occurs in the spleen. Unknown confounding factors for the staining technique

RESULTS

Staining and morphology of endogenous and transplanted islets. Endothelium in all tissue samples was stained with the lectin BS-1 (Fig. 1). No structures corresponding to unstained microvascular blood vessels could be identified in any of the investigated sections. The endothelial cells of endogenous pancreatic islets and islets transplanted under the renal capsule or into the liver were strongly stained. However, endothelium of the intrasplenic islet grafts was less intensely stained. The insulin-, glucagon-, or somatostatin-positive cells in endogenous islets did not stain with BS-1.

The fraction of the islet grafts consisting of connective tissue was similar in the kidney (36 ± 2%; n = 6) and spleen (27 ± 2%; n = 6). The intraorally transplanted islets were also surrounded by connective tissue. Because most of these islets were entrapped in perportal areas, it was impossible to decide whether the connective tissue belonged to the graft or to the liver stroma. Connective tissue was also formed around the microspheres implanted to the renal subcapsular space and constituted 57 ± 2% (n = 6) of these grafts.

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may also be present within the spleen. Despite the more faint staining, the endothelium within the intrasplenically grafted islets and their surrounding connective tissue could be discerned. A further evaluation of the staining specificity of BS-1 was added in the present study, by applying staining with BS-1 and antibodies for insulin, glucagon, or somatostatin in the same pancreatic slides. The rationale for this was that expression of α-galactosyl epitopes on neonatal porcine islet cells has previously been shown (23). No staining of any of these islet endocrine cells with BS-1 was detected, which is consistent with the findings that these epitopes are not expressed in adult pig (24) or adult mouse (22) pancreatic tissue.

We did not quantitatively compare the vascular density in the surface and interior regions of the renal subcapsular grafts, but there were no obvious regional differences in vascular density within these grafts. This result is in line with previous findings of an absence of oxygen-tension gradients in such islet grafts (8). We have previously observed that both blood perfusion and oxygen tension are similar in renal subcapsular islet grafts curing a diabetic animal and in islet grafts implanted to normoglycemic recipients (10). It is therefore anticipated that similar results would have been obtained if cured diabetic recipients had been investigated in the present study rather than normoglycemic recipients. However, this awaits further study.

Besides their main endocrine constituent, islet grafts consist of blood vessels, connective tissue, and, at least initially, a few contaminating exocrine cells. The amount of connective tissue found within the grafts varies between implantation sites, and most likely depends on, at least partially, a foreign body reaction. Both the endocrine and stromal compartments of islet grafts contribute to graft blood flow as measured by, for example, laser-Doppler flowmetry. The oxygenation of the hormone-producing cells, however, depends only on closely adjacent capillaries, i.e., mainly (or only) blood vessels in the endocrine parts of the grafts. We therefore decided to evaluate the endocrine and stromal compartments separately for vascular density. Surprisingly, whereas the vascular density in transplanted islets per se was low, a large number of blood vessels were found in the connective tissue surrounding the renal subcapsular and intrasplenic islets. To elucidate whether this reflected inherent properties of a normal foreign body reaction or a compensation for the low revascularization of the endocrine parts of the grafts, plastic microspheres, rather than islets, were im-
planted in separate animals. Interestingly, no accumulation of newly formed blood vessels was seen in the foreign body reaction of connective tissue surrounding the implanted microspheres. Thus, an angiogenic response initiated by the cells within the transplanted islets seems to induce a compensatory increase in vascular density in the surrounding connective tissue. Indeed, the predominant location of capillaries in the connective tissue stroma to the immediate vicinity of the endocrine tissue is also consistent with this. To our knowledge, this preferential distribution of graft blood vessels to the connective tissue stroma has not been previously described.

In a previous study, a similar vascular density as in the present study was found in endogenous rat pancreatic islets (25). Because of the use of entirely different techniques to visualize blood vessels, it is difficult to compare our results on vascular density in transplanted islets to previous studies quantitating graft blood vessels (6,20,21). Merchant et al. (20) did not mention whether endocrine and connective tissue parts were evaluated separately, and Menger et al. (6) and Heuser et al. (21) evaluated only single islets implanted into a skinfold chamber preparation.

The islet grafts investigated in the present study contained insulin-producing \( \beta \)-cells at all implantation sites, as confirmed by immunohistochemistry. Previous studies have also shown that islets in similar numbers normalize the hyperglycemia of alloxan-diabetic recipients (11). Thus, despite the decreased vascular density of transplanted islets, functional activity remains. However, we have previously noted a 75% decrease in insulin content of transplanted islets compared with islets cultured in vitro (10). Although \( \beta \)-cell death in the immediate posttransplantation period may explain some of the difference, low revascularization with concomitant decreased tissue oxygen tension may also contribute at later stages by suppressing insulin production (26). Indeed, we have previously observed a markedly decreased tissue oxygen tension in transplanted rat islets compared with endogenous islets up to 9 months after implantation (9). This low tissue oxygen tension of islet grafts seems to be independent of implantation site (10) and related to a high degree of nonoxidative glucose metabolism (27). Also, islet graft blood flow, as investigated by means of nonradioactive microspheres or laser Doppler flowmetry, has been found to be decreased compared with endogenous islets (8–10,28,29). In this context, it should be noted that a laser Doppler flow probe measures whole-blood perfusion, i.e., all moving blood cells within the illuminated tissue. In view of the preferential location of blood vessels to the connective tissue in composite islet grafts, as described in the present study, it may therefore be envisaged that the
blood flow within the endocrine parts of the grafts is even lower than previous estimates (≈4 ml · min⁻¹ · g⁻¹) (29). Indeed, in experiments using a combination of microspheres and an ultrasonic flow probe, a nutritional islet graft blood perfusion of only ~10% of that seen in endogenous islets (5–7 ml · min⁻¹ · g⁻¹) was observed (8). An islet blood flow more similar to that of endogenous islets was recorded in islets autotransplanted beneath the renal capsule of partially pancreatectomized animals (30,31). However, in the latter experiments, the influence of the partial pancreatectomy has been difficult to assess.

In conclusion, we found a marked decrease in vascular density of transplanted islets compared with that of endogenous islets. In contrast, the connective tissue surrounding the transplanted islets contained a large number of blood vessels. As suggested by previous studies on blood flow, oxygen tension, and metabolism, the low revascularization of transplanted islets may influence islet graft function.

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