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The Endothelial Cells in Islets of Langerhans

Review based on the doctoral thesis
Experimental Studies on the Vasculature of Endogenous and Transplanted Islets of Langerhans

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ABSTRACT

The blood vessels of the pancreatic islets are of crucial importance for oxygen and metabolite supply, and dispersal of secreted hormones. In addition to this, endothelial cells have an important role in the revascularization process after islet transplantation. Studies have reported signs of poor engraftment of transplanted islets, presumably due to impaired revascularization. The aims of this study were to investigate islet endothelial cells and the revascularization process of transplanted islets. The lectin Bandeiraea simplicifolia was found to consistently stain endothelium of both endogenous and transplanted pancreatic islets. By using this marker, we investigated the vascular density of both endogenous and transplanted islets of C57BL/6 mice. One month post-transplantation, a time point when the implants are assumed to be completely revascularized, the graft vascular density was decreased at all investigated implantation sites when compared to endogenous islets. Furthermore, most of the blood vessels were located in the graft connective tissue stroma. Similar results were obtained six months post-transplantation and in cured diabetic animals after one month. In order to evaluate the function of intraportally transplanted islets, we developed a method to retrieve such islets. Enzymatic and mechanic treatment of the liver enabled us to re-isolate the transplanted islets for further in vitro studies. These islets had decreased insulin release, insulin content and glucose oxidation rate when compared to non-transplanted control islets. To understand the role of islet endothelium in the revascularization of transplanted islets we performed angiogenesis microarray studies on islet endothelial cells, from non-cultured, cultured and transplanted islets. We found that the islet endothelium expressed mRNA for both inhibitors and inducers of angiogenesis, and that this expression differed with time. In conclusion, these results provide a useful platform for further studies on the islet endothelium.

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ISLET MORPHOLOGY

The human pancreas contains approximately 1–2 million islets of Langerhans, constituting 1–2% of the gland (1, 2). Their size is usually in the order of 100–400 µm, irrespective of the species studied, representing 2–3000 endocrine cells per islet (3). The islets are surrounded by a thin connective tissue capsule and consist of several different types of cells (2, 4). The islet core contains mainly beta cells, whereas the other endocrine cells, *viz*. alpha-, delta- and pancreatic polypeptide cells are peripherally located. The islets also contain nerves, fibroblasts, macrophages, dendritic cells and endothelial cells (EC), which are interspersed among the endocrine cells. The function of the endocrine cells is to produce the hormones, insulin and glucagon, which regulate especially carbohydrate metabolism.

ISLET VASCULATURE

The pancreatic vasculature constitutes a complex network, which is adapted to the special needs of the exocrine and endocrine parts of the gland, respectively. The islet blood vessels are of major importance for supplying the endocrine organ with nutrients and oxygen, as well as to transport the secreted hormones into the blood stream. The islet vasculature is connected both in series and in parallel to the exocrine blood vessels, and differs morphologically and functionally from that in the exocrine parenchyma (5). The islet microvascular organization is dependent on the size of the islets (6). Thus, smaller islets receive blood from one arteriole, and drain through small venules traversing the exocrine parenchyma. It has been noted that some of the islet efferent capillaries connect with acinar and/or ductular microvessels, thereby forming a so called insulo-acinar portal system (7). The larger islets, on the other hand, have 1–3 arterioles entering the islet, and the islet capillaries drain mainly into postcapillary venules at the edge of the islets, which then empty into intralobular veins. The endocrine capillaries are wider and their EC possess 10 times as many fenestrae as those in exocrine microvessels (8). These fenestrae are likely to be induced by vascular endothelial growth factor-A (VEGF-A) production in the islets (9, 10), and are probably important for the high permeability of the islets capillaries. So far, no direct role of the islet vasculature in the pathogenesis of diabetes mellitus has been proven, except for the role of EC in recruitment of immune competent cells in insulitis (11, 12). Furthermore, changes in the blood perfusion (13), permeability (14) and islet capillary blood pressure have been described (15). However, it is at present unknown to what extent these changes are secondary to damage to or impaired function of the beta cells *per se*.

ENDOTHELIAL CELLS

EC are lining all blood and lymph vessels and they have several unique functions, *e.g*. contributing to local blood flow regulation, coagulation and thrombolytic

processes, serving as a mechanical and immunological barrier between tissues and blood as well as participation in angiogenesis (16). The EC are adapted to the functional needs of the surrounding tissues, and thereby constitute a heterogeneous group of cells. EC differ functionally between large and small blood vessels, as well as between different organs and species (16, 17). Due to this heterogeneity it is difficult to find markers which are specific for all EC. At present several techniques are used to detect EC in vitro or in histological slides, e.g. antibodies (18), lectins (19) and acetylated low density lipoprotein (Dil-Ac-LDL) (20). However, none of these markers consistently labels EC from all parts of the body in every application. In line with this, specific markers for the islet endothelium have been difficult to find, especially for the use in formalin-fixed and paraffin-embedded material. Islet EC are highly adapted to the functional needs of the islets and previous studies have described a technique for isolation and culture of islet endothelium in rats (18) and humans (21). Such in vitro studies have confirmed that there are biochemical and functional differences between endothelium from exocrine and endocrine blood vessels.

DIABETES MELLITUS

Diabetes mellitus is a syndrome characterized by metabolic aberrations due to an absolute or relative lack of insulin. It is commonly divided into type 1 and type 2 diabetes, respectively, both of which are likely to be disorders with a heterogeneous etiology. Type 1 diabetes mellitus (insulin-dependent diabetes mellitus) is characterized by a loss of the insulin-producing beta cells, due to their destruction in an autoimmune process (22). The reasons for the specific targeting of the beta cells are at present unknown. However, the number of diabetes patients is rapidly increasing and is estimated to be 150–220 millions world wide in the year 2010, most of will be type 2 diabetes (23). Type 2 diabetes is due to a combination of an impaired beta cell function and peripheral insulin resistance (24). It is more frequently seen in older people, and is often associated with obesity. However, this form of diabetes is becoming increasingly frequent, and also younger age groups are affected today (25). The number of islets in the pancreas can be normal, although it is usually decreased, albeit never to the same degree as seen in type 1 diabetes (24).

TRANSPLANTATION TO TREAT DIABETES

At present, the only known cure for type 1 diabetes is transplantation of insulin-producing cells (26, 27). Type 2 diabetes patients can often achieve reversal of symptoms if increasing their physical activity in combination with weight reduction, even though glucose intolerance often remains. From now on the discussion will focus on type 1 diabetes, where replacement of beta cells has become the treatment of choice for a small group of selected patients. Beta cells can be substituted either by implantation of a whole pancreas, or as isolated pancreatic islets. The former is a

major surgical procedure which carries significant risks for the patient (28). Arguments in favor of choosing isolated islets for clinical transplantations include the technical simplicity, reduced risks for the patients and the lower medical costs associated with the procedure (26, 27). However, the results of islet transplantations have been poor until the recent application of the so called *Edmonton Protocol*. This encompasses changes in patient selection and choice of immunosuppressive drugs, and has led to a markedly improved outcome of clinical islet transplantation (29, 30). However, also when applying this protocol, transplantation of a large number of islets (>9.000 islet equivalents/kg body weight) is necessary to achieve insulin-independence. Due to the limited availability of islet tissue, this severely restricts the number of patients that may be treated. Methods to reduce the number of islets needed to cure a diabetic individual are therefore warranted.

ENGRAFTMENT OF TRANSPLANTED ISLETS OF LANGERHANS

Engraftment is the adaptation of the transplanted islets to the new environment in the implantation organ. An adequate engraftment process is of major importance for the islet graft survival and function, and constitutes a possible target for interventions to improve the outcome of islet transplantations. There are several processes involved in the engraftment, *e.g*. revascularization, reinnervation and reorganization of stromal and endocrine cell interactions. A rapid revascularization is crucial for islet endocrine function after transplantation, and this has been shown to occur within 7–14 days (31–34). However, the extent of the revascularization has not been thoroughly studied in detail. Recent experiments on islets transplanted to the renal, splenic or hepatic subcapsular space have suggested that the angiogenic process is insufficient to achieve optimal oxygenization of the transplanted islets (35, 36). However, no measurements comparing the vascular density in endogenous islets versus islets transplanted into these implantation sites were performed. Clinically, most islet transplantations are performed intraportally (27, 30), and such intrahepatic islets are difficult to visualize or retrieve, which makes post-transplantation studies difficult to perform. Therefore, the development of techniques for retrieval of intraportally implanted islets would be crucial for functional studies.

When islets are isolated prior to transplantation they are disconnected from the vascular network, and during the subsequent culture period they depend on diffusion of oxygen and nutrients for survival. Studies have suggested that during culture, islet EC disappear or dedifferentiate (37). This means that the islet vasculature has to be rebuilt after transplantation. There are only few studies on islet endothelium (18, 21, 38, 39), and the knowledge on this cell type is therefore scarce, even though recent studies suggest that there is a vascular dysfunction in grafted islets (40). In order to investigate the islet endothelium of transplanted islets, developments of methods to provide access to these cells are mandatory. Therefore, the aims of the studies presented in my thesis were to find a marker for endogenous and transplanted endothelium. Furthermore, this marker should be applicable to forma-

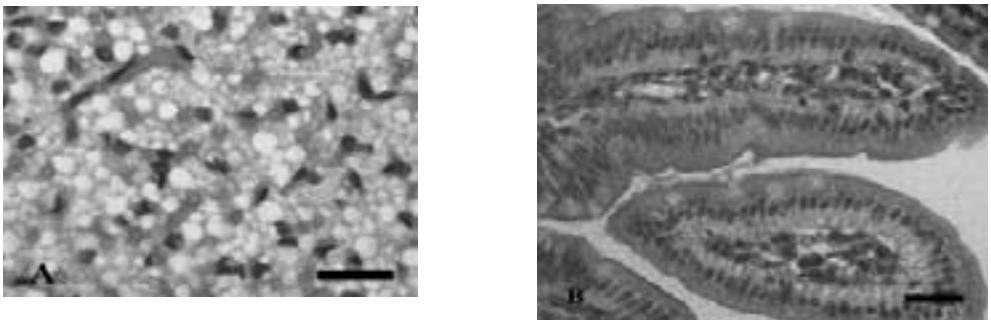

Fig. 1. Staining of endothelium (red) in C57BL/6 mouse brown adipose tissue (**A**) and small intestine (**B**) with Bandeiraea simplicifolia. Scale bars are 25 µm.

lin-fixed, paraffin-embedded specimens and usable for comparisons of the vascular density of endogenous and transplanted islets. In order to make functional studies, we wished to retrieve transplanted islets from the liver and then finally to isolate EC from endogenous and transplanted islets.

When evaluating islet allotransplantation, both the effects of rejection, immune suppressive drugs and possible recurrence of the autoimmune disease must be considered. Many of the drugs used to prevent the immune system from attacking the graft are toxic for the beta cells, and may even by themselves participate in the functional impairment of grafted islets (41). In the present studies, we have consistently used syngeneically transplanted islets to circumvent these confounding factors.

STAINING OF EC IN FORMALIN-FIXED SAMPLES

In these initial studies we evaluated the use of several different markers (Table 1) for use in formalin-fixed, paraffin-embedded specimens of different tissues from mice and rats. We found that Bandeiraea simplicifolia-1 (BS-1), a lectin which binds to α-gal epitopes, stained microvascular EC in all tissues examined (Figures

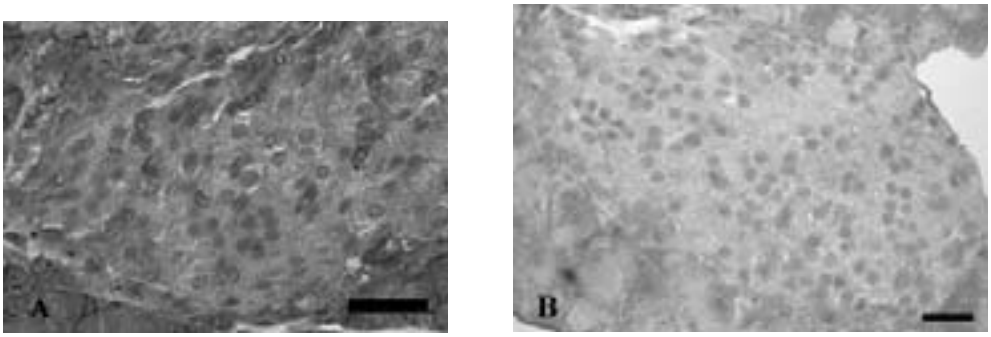

Fig. 2. C57BL/6 mouse pancreatic islets stained with Bandeiraea simplicifolia (**A**) and CD31 (**B**) to detect microvascular endothelial cells (red). Scale bars are 25 µm.

Table 1. Staining of endothelial cells in formalin-fixed, paraffin-embedded tissue samples from rodents.

Lectin / Target antigen	Pancreas			Brown adipose tissue	White adipose tissue			Small intestine		Kidney			Lung		Isolated pancreatic islets			Islet in kidney	
grafts	WF	SD	C57	C57	WF	SD	C57	WF	C57	WF	SD	C57	WF	SD	WF	SD	C57	WF	C57
Bandeiraea simplicifolia	++	+	++	++	++	+	+	++	++	+/-	+/-	+	++	++	+	+	++	++	++
Ulex europaeus	-	-	+/-	+/-	-	-	+/-	-	+/-	-	-	-	-	-	ND	ND	ND	ND	ND
CD34	-	-	+	-	+/-	++	++	++	+	-	-	+	-	-	ND	ND	ND	ND	ND
CD31	-	-	-	+/-	+/-	+	+	++	ND	-	-	-	-	-	ND	ND	ND	ND	ND
CD200	-	-	-	-	-	-	-	-	-	-	-	+	-	-	ND	ND	ND	ND	ND
Ox43	-	-	-	-	-	-	-	-	-	-	-	+/-	-	+	ND	ND	ND	ND	ND
von Willebrand Factor	-	-	-	-	+/-	-	-	-	-	-	-	+/-	-	+	-	-	ND	-	-

–, negative staining; +/–, positive staining in some cells; +, positive staining; ++, strong positive staining; ND, not determined. WF, Wistar-Furth rats; SD, Sprague-Dawley rats and C57, C57BL/6 mice.

1A and B), with the exception of the kidney where only some of the EC, especially in the medulla, were positive (42). Of special interest was the finding that BS-1, in contrast to the other examined markers, consistently stained pancreatic islet endothelium (Figures 2A and B). To evaluate and ensure this, we searched for unstained capillaries, i.e. blood vessels with a diameter <20 µm and preferably containing erythrocytes, without noticing any such structures. The other investigated markers (Table 1) showed no consistency in their staining of EC in sections from either mouse or rat. It should be noted in this context that many of the tested antibodies or lectins referred to above perform well in cryosectioned tissue samples (43–45).

Isolectin B4, an iso-form of BS-1, has previously been used as a marker for microvascular EC in mouse tissues; including the islets of Langerhans (46). This lectin also binds to α-D-galactosyl residues (46), which are present on EC in many species. This may explain the ability of this lectin to stain microvascular EC in rodent tissues.

IDENTIFICATION OF ENDOTHELIUM IN ENDOGENOUS AND TRANSPLANTED ISLETS

Of particular interest was the finding that BS-1 consistently stained endothelium, not only in endogenous pancreatic islets, but also in isolated islets and syngeneically transplanted rat and mouse pancreatic islets (42) irrespective of implantation site (Figures 3A and B). This means that also newly formed EC in recently revascularized islets and the connective tissue stroma can be identified with this lectin. Moreover, these results imply that the staining properties of endothelium in islet grafts are independent of the origin of the newly formed microvessels, i.e. whether they derive from renal, hepatic or splenic blood vessels.

Recently, expression of α-gal epitopes, *i.e.* those binding to BS-1, was demonstrated in neonatal porcine islet cells (47), whilst no expression was seen in adult

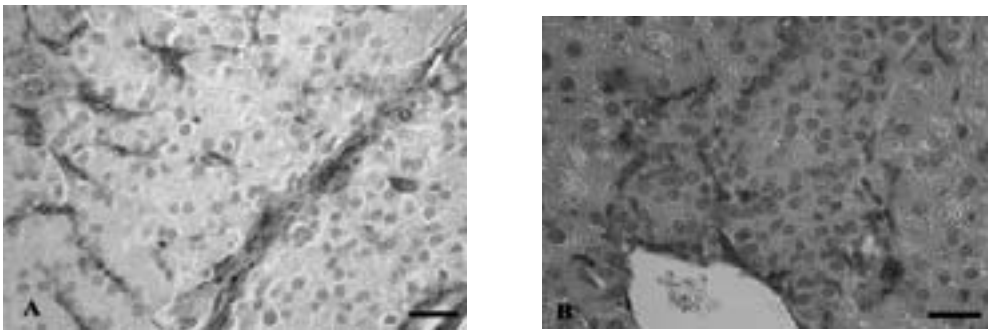

Fig. 3. Staining of endothelial cells (red) in newly revascularized C57BL/6 mouse pancreatic islets syngeneically transplanted to kidney (**A**) and liver (**B**) with Bandeiraea simplicifolia. Scale bars are 25 µm.

endocrine cells (48). Therefore, we further evaluated the staining specificity of BS-1 by simultaneous staining with BS-1 and antibodies for insulin, glucagon or somatostatin in the same pancreatic sections (49). Neither insulin-, glucagon-, nor somatostatin-positive cells in endogenous islets were stained with BS-1. Thus, our results are consistent with the notion that α-gal epitopes are not expressed in adult rodent islet endocrine cells.

CONNECTIVE TISSUE STROMA AND VASCULATURE OF TRANSPLANTED ISLETS

Endogenous islets of Langerhans have a connective tissue capsule, which constitutes a minor part of the islet. Thus, the connective tissue within the grafts, which constitutes approximately 30% of the transplant (49, 50), is likely to derive, at least partially, from a foreign body reaction. Connective tissue was also formed in association with microspheres implanted into the renal subcapsular space, and constituted 57% of these grafts (49). Intraportally transplanted islets were also surrounded by connective tissue. Since most of these islets were entrapped in periportal areas, it was impossible to separate between connective tissue associated with graft or liver.

The vascular density, *i.e*. the number of blood vessels per area, of the transplanted islets was decreased compared to that of endogenous islets, irrespective of the chosen implantation organ (49). Furthermore, islets transplanted into the spleen had an even lower vascular density than islets transplanted beneath the renal capsule or into the liver. The capillary density was markedly higher in the connective tissue stroma of grafts implanted into kidney or spleen than in the endocrine parts of these grafts. The density of stromal capillaries appeared to be higher in the immediate vicinity of the islets at all three implantation sites (49). A markedly lower number of capillaries was found in the connective tissue surrounding microspheres implanted into the renal subcapsular space when compared to connective tissue surrounding implanted islets. Thus, an angiogenic response initiated by the cells within the transplanted islets seems to be of importance to increase the vascular density in the surrounding connective tissue. This preferential distribution of graft blood vessels to the connective tissue stroma rather than among the endocrine cells has not been previously described, and its functional importance awaits further studies. However, when combining the values of vascular density for capillaries in the stromal and endocrine compartments, the value in the total graft is almost as high as in the endogenous islets. It should be noted that a lower oxygen tension in grafted islets, as well as other microvascular disturbances (35), which may be related to the location of graft capillaries, have been previously observed. This suggests that the changed capillary distribution may be of functional importance.

The influence of the hormone production from intact endogenous islets on the function and revascularization of ectopically implanted islet grafts is largely unknown. To investigate this we used both normoglycemic and hyperglycemic recipients, i.e. animals with or without functioning endogenous islets (50). The

number of islets was chosen to either reverse (500 islets) or not reverse (200 islets) hyperglycemia. The recipients were injected with alloxan prior to transplantation and they all had blood glucose concentrations >14 mM at the time of implantation. Transplantation of 500 islets fully reversed the hyperglycemia (<8 mM), while the animals transplanted with 200 islets remained slightly hyperglycemic (~11.1 mM) throughout the course of the study. The vascular density in transplanted islets was significantly decreased to the same extent, when compared to that of native islets, in both groups of recipients (50). Thus, this study showed that a remaining normal number of native pancreatic islets does not affect the formation of new blood vessels in an islet graft. Furthermore, the possibility to cure diabetic recipients demonstrates that the function of the islets can be adequate, even though the vascular density is decreased. We also found that the vascular density in the transplanted islets did not improve up to six months post-transplantation (50). Thus, no redistribution of graft blood vessels from the connective tissue stroma into the endocrine tissue occurred with time.

RETRIEVED TRANSPLANTED ISLETS

Since islets implanted into the liver are injected directly into the portal vein they distribute in the portal tributaries throughout the hepatic parenchyma. This means that they are difficult to localize, and few techniques are available for their study post-transplantation. To circumvent this problem we developed a method to retrieve transplanted islets from the liver for further functional evaluations (51). On an average 25–50% of those implanted, could be retrieved from each processed mouse liver. The identity of retrieved transplanted islets was confirmed by several different methods. Retrieved islets stained pink/red after intravital staining with neutral red, which allegedly selectively stains pancreatic islets (52, 53). Sections from the re-isolated islets also stained positive with an insulin antibody, and beta and alpha cell secretory granules were observed with transmission electron microscopy.

Insulin release from retrieved islets incubated with 1.67, 16.7 or 16.7 mM glucose + 5 mM theophylline was investigated either immediately after retrieval or after 3–4 days of culture and compared to non-transplanted freshly isolated or cultured islets. Immediately after retrieval, the insulin release was lower compared to non-transplanted control islets during all three incubations. After culture, the insulin response to 16.7 mM glucose and 16.7 mM glucose + theophylline remained markedly impaired in retrieved islets, whereas basal insulin release was similar to that from similarly cultured control islets. Retrieved transplanted islets contained less insulin, both immediately after retrieval and after culture, compared to control islets. However, whereas the insulin content of control islets decreased markedly after culture, the insulin content of retrieved islets did not change (Figure 4).

Glucose oxidation rates of retrieved islets were markedly lower than those of control islets when exposed to 16.7 mM glucose, both immediately after retrieval and after culture. The glucose oxidation rates were not affected by culture in any of

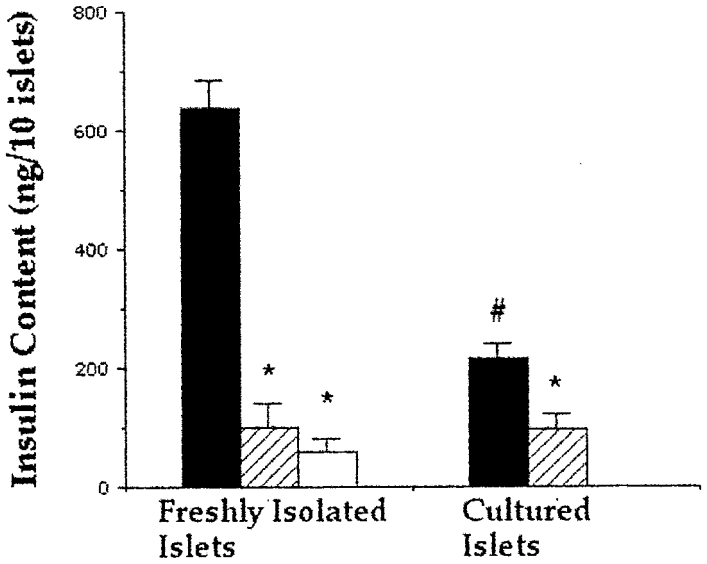

Fig. 4. Insulin content in isolated non-transplanted C57BL/6 mouse islets (closed bars) or intraportally transplanted islets retrieved from normoglycemic (hatched bars) or cured diabetic recipients (open bars). The investigated groups were freshly isolated islets, freshly retrieved islets from normoglycemic or cured diabetic recipients, isolated and cultured islets and cultured islets retrieved from normoglycemic recipients. Values are expressed as means±SEM. * denotes $P<0.05$ when compared to corresponding isolated non-transplanted islets, whereas # denotes $P<0.05$ when compared to corresponding freshly isolated islets. Modified from (51).

the groups. There were no differences in DNA content between freshly retrieved transplanted islets and freshly isolated control islets, thereby arguing against the notion that the decreased glucose-stimulated insulin release, insulin content and glucose oxidation rate of retrieved islets compared to control islets merely reflect a lower amount of islet tissue in the former preparations.

Another possible explanation for the poor function of the retrieved islets is that the re-isolation procedure in itself damages the islet cells. However, we used the same amount of collagenase as for isolation of islets from the pancreas (54, 55). In separate experiments we also decreased this concentration, or even omitted it completely, and nevertheless obtained similar results. However, the number of retrieved islets was markedly reduced. The mechanical dispersion of the liver used for retrieval is similar to that used during normal islet isolations and is therefore unlikely to inflict any further functional impairment, as suggested by the similar survival of the retrieved islets. An issue of importance is to what extent the observed impaired function is dependent on the implantation organ, that is the liver, or if it mainly reflects the transplantation and engraftment trauma in itself. The present functional impairment seen in intraportally implanted islets is much more pronounced than that seen after transplantation under the kidney capsule, suggesting that the implantation organ is indeed of major importance. In confirmation of this

notion, experimental studies in rodents have also indicated a lower long-term rate of function of intraportally transplanted islets (56). It might also be a difference if the islets are implanted as single islets or as a cluster of islets.

ISOLATION OF ENDOTHELIAL CELLS FROM ENDOGENOUS OR TRANSPLANTED ISLETS

When islets are isolated prior to transplantation, the efferent blood vessels are disconnected and the islet cells depend on diffusion of oxygen and nutrients. What effects this has on the islet EC are unknown, we wished to investigate this further. We were able to isolate EC from intact and dispersed freshly isolated islets; islets pre-cultured for 7 days as well as transplanted islets retrieved from both the kidney and liver. By the use of BS-1 coated Dynabeads we could separate EC from contaminating cells and achieve a purity >90%. The identity of the EC was confirmed by uptake of acetylated light density lipoproteins, and staining with the lectin BS-1, as well as antibodies against angiotensin-converting enzyme and endothelial nitric oxide synthase.

Isolated endothelial cells have been studied with microrray technology to evaluate the presence of angiogenesis stimulators and inhibitors (57). We were consistently able to obtain expression responses in all investigated groups, *i.e*. EC from dispersed islets, EC outgrown from freshly isolated islets or 7 days pre-cultured islets, as well as EC outgrown from retrieved transplanted islets from both the kidney and liver, when applying the angiogenesis microarrays. A rather surprising finding is that the endothelium per se fails to produce factors able to stimulate angiogenesis, but rather produce inhibitors of this process, such as endostatin (58) and pigment epithelium-derived factor (PEDF). PEDF normally decreases EC proliferation (59), and induces apoptosis in EC in sprouting blood vessels, but spares those in quiescent microvessels (60). Indeed, previous studies have shown that the pancreas develops substantial stromal vascularity and epithelial cell hyperplasia in the absence of PEDF (61). These findings are of interest in view of our previous reports suggesting the presence of a vascular dysfunction in transplanted islets, manifested by a decreased vascular density, low oxygen pressure and low blood flow (40). It is tempting to speculate that expression of an angiogenesis inhibitor, such as PEDF, may participate in this response. Ongoing experiments aim to verify the accuracy of these experimental analyses.

CONCLUSIONS

The lectin BS-1 stains endothelium in both endogenous and transplanted rodent islets of Langerhans in formalin-fixed, paraffin-embedded sections. The vascular density of syngeneically transplanted islets of Langerhans is decreased when compared to endogenous islets irrespective of implantation site. Neither hyperglycemia nor prolonged transplantation time increased vascular density. Transplanted islets

retrieved from the liver have decreased insulin release, insulin content and glucose oxidation compared to isolated non-transplanted islets. EC can be isolated from freshly isolated, cultured and transplanted pancreatic islets and they differ in their expression of angiogenically active substances.

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