The Microvasculature of Endogenous and Transplanted Pancreatic Islets

Blood Perfusion, Oxygenation and Islet Endocrine Function

RICHARD OLSSON
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Abstract

Type 1 diabetes mellitus affects millions of people worldwide. Islet transplantation is a minimal invasive surgical procedure that restores euglycemia and halts the progression of diabetic complications. However, despite transplantation of islets from multiple donors most patients reverse to hyperglycemia within five years. New strategies to improve long-term outcome of islet transplantation are indispensable. This thesis studied differences in the microvasculature between endogenous and transplanted pancreatic islets, and investigated means to improve islet graft revascularization and function. Islet graft microvessels were similar to endogenous islets responsive to adenosine, angiotensin II and nitric oxide (NO). Recipient hyperglycemia induced a higher basal islet graft blood flow, which also was less dependent on NO than in normoglycemic recipients. Transplantation of freshly isolated instead of cultured islets improved graft revascularization, oxygenation and function. Pretreatment of islets with vascular endothelial growth factor decreased their expression of matrix metalloproteinase-9 (MMP-9) and impaired graft revascularization. Moreover, MMP-9 pretreatment per se improved graft revascularization. In vivo, 20-25% of all endogenous rat islets was low oxygenated (pO2 <10 mmHg). Changes in the islet mass, by means of whole-pancreas transplantation, doubled the fraction of low oxygenated islets in the endogenous pancreas of transplanted animals, whereas this fraction almost completely disappeared after a 60% partial pancreatectomy. Interestingly, oxygenation was related to metabolism, since well oxygenated islets in vivo had 50% higher leucine-dependent protein biosynthesis, which includes (pro)insulin biosynthesis. In intraportally transplanted islets, the low oxygenated fraction of islets was markedly increased one day post-transplantation, and the oxygenation remained low following revascularization. In summary, these data suggest that a better revascularization of transplanted islets can improve graft function. Furthermore, the oxygenation and metabolism of endogenous islets is tightly regulated. This regulation seems to be disturbed following transplantation, which may contribute to long-term islet graft failure.

Keywords: diabetes mellitus, pancreatic islets, islet transplantation, vascular engraftment, islet microcirculation, oxygenation, blood flow, protein biosynthesis

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To the future
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


IV. Olsson, R., Carlsson, P-O. A low oxygenated subpopulation of pancreatic islets constitutes a functional reserve of endocrine cells. (manuscript)

V. Olsson, R., Pettersson, U., Carlsson, P-O. Increased numbers of low oxygenated pancreatic islets after intraportal transplantation. (manuscript)

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<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AT II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>BS-1</td>
<td>Bandeiraea simplicifolia</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FGF-2 / bFGF</td>
<td>Fibroblast growth factor 2 / basic fibroblast growth factor</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<tr>
<td>IAPP</td>
<td>Islet amyloid polypeptide</td>
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<tr>
<td>IBMIR</td>
<td>Instant blood mediated inflammatory reaction</td>
</tr>
<tr>
<td>ICC</td>
<td>Islet-like cell clusters</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>L-NNA</td>
<td>N\textsuperscript{G}-nitro-l-arginine</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>pO\textsubscript{2}</td>
<td>Oxygen tension</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TNF\alpha</td>
<td>Tumor necrosis factor (\alpha)</td>
</tr>
<tr>
<td>TPU</td>
<td>Tissue perfusion units</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial factor</td>
</tr>
<tr>
<td>Vol</td>
<td>volume</td>
</tr>
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<td>Wt</td>
<td>weight</td>
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</table>
Introduction

The pancreas is a gland composed of both endocrine and exocrine cells. In 1869 the German pathological anatomist Paul Langerhans was the first to anatomically describe the endocrine pancreas as cell-clusters in pancreatic tissue sections (134). The main hormone of the pancreatic islets, insulin, was then discovered in 1921 by Banting and Best (114). Insulin is a peptide hormone, which some tissues are dependent on for uptake and storage of glucose. Since type 1 diabetes results in complete loss of insulin-producing cells, restoration of insulin production by transplantation of such cells is a possible cure. However, the long-term results of islet transplantations are still poor.

The endogenous islets have a well-developed vascular network, which is completely disrupted during isolation prior to transplantation. This means that the islet vasculature must be rebuilt following transplantation. Experimental data suggest that the vascular network of endogenous islets is insufficiently restored after transplantation, and this has been suggested to contribute to chronic ischemia and graft failure. In order to develop new strategies to improve the outcome of islet transplantation, this thesis studied the microvasculature of endogenous and transplanted pancreatic islets with special reference to its transport capacities of oxygen.

Diabetes mellitus

Diabetes mellitus is characterized by disturbed glucose homeostasis. There are two major forms of the disease with differences in etiology and treatment. Type 1 is an autoimmune disease destroying the insulin-producing β-cells of the pancreatic islets, whereas type 2 is due to β-cell failure or demise and insulin resistance to varying degrees. The onset of type 1 diabetes is typically before adulthood and both genetic and environmental factors have been described as triggers of the autoimmune attack on the β-cells (60, 61, 79, 244). On the contrary, type 2 diabetes is predominantly seen in the elderly and its etiology is mainly genetic predisposition, as well as obesity and the metabolic syndrome (22, 231).

In 2000, the prevalence of diabetes worldwide was estimated to 171 million and it is estimated to increase to 366 million in 30 years (243). Since most data sources do not distinguish between type 1 and type 2 diabetes in
adults, the figures for subtypes of diabetes are uncertain. However, type 2 is most common and the global prevalence of type 1 diabetes is estimated to 18 million \((137)\). Although both forms of diabetes are increasing the main increase is in type 2. The cause of increase is unknown for type 1, whereas in type 2 the main reason is probably the increasing obesity.

Due to differences in pathogenesis the treatment strategies also differ between type 1 and type 2 diabetes. In type 1 diabetes insulin delivery must be restored. Presently, this is most commonly performed by four daily injections of exogenous insulin. There are also insulin pumps available for continuous delivery of insulin; however, these routes of insulin delivery can not restore the healthy body’s fine tuning of the blood glucose concentration. It is well known that poor blood glucose control clearly increases the risk of developing late complications, e.g. nephropathy, retinopathy, and neuropathy \((1)\). Replacement of insulin-producing β-cells is today the only treatment that can restore euglycemia and ameliorate the progression of diabetic complications \((207)\). This type of treatment is presently only available as transplantation of the whole pancreas, or by transplantation of isolated pancreatic islets. Transplantation may not be a treatment of choice for type 2 diabetes. The main characteristic of this disease is insulin resistance and not complete lack of insulin. In the treatment of type 2 diabetes insulin resistance may be counteracted by weight loss and physical exercise. Moreover, if hyperglycemia persists, there are drugs available both to decrease insulin resistance and increase insulin secretion.

**Pancreatic islets**

In mammals, the pancreatic islets constitute 1-2% of the whole pancreatic volume and they are scattered throughout the entire exocrine parenchyma \((19)\). In the human pancreas this corresponds to 1-2 million islets \((34, 128)\), whereas in rats there are 3000-4000 islets in the pancreas \((140)\). The size of pancreatic islets is fairly constant among different species, i.e. 25-300 μm in diameter. In rodents, each islet is composed of 2000-4000 cells, which either are insulin-producing β-cells (70-80%), glucagon-producing α-cells (15-20%), somatostatin-producing δ-cells (5%), or pancreatic polypeptide-producing PP-cells (15-20%) \((128)\). Notably, the islets of the pancreatic head are rich in PP-cells and poor in α-cells, whereas it is the opposite in the body and tail.

The endocrine pancreas regulates blood glucose homeostasis. The main function of β-cells is to release insulin, which lower blood glucose concentrations by suppressing gluconeogenesis, as well as promoting glucose uptake and storage. Glucose uptake is insulin dependent in skeletal muscle and adipose tissue, whereas other cells have insulin independent glucose transporters \((251)\). Furthermore, insulin stimulates glucose storage
by conversion to glycogen in liver and muscle cells. In contrast, the α-cells release glucagon, which increases blood glucose by degradation of liver glycogen (glycogenolysis), and thereby protects the body from hypoglycemia. The somatostatin, derived from the δ-cells, is a general inhibitor of secretion from both the endocrine and exocrine pancreas. Lastly, pancreatic polypeptide, released by PP-cells, seems to inhibit exocrine secretion (11, 52).

In rodents there is a distinct architecture within the pancreatic islets with only β-cells in the center surrounded by an assortment of non-β-cells (α-, δ- and PP-cells). Interestingly, in horses this pattern has been reported to be inverted with a central core of α-cells (75), whereas in humans no distinct cell architecture is discerned (25, 30).

Microcirculation of the pancreas

During organogenesis the pancreas is formed from the ventral and dorsal buds of the gut endoderm (132), which result in separate vascularization. The pancreatic head is supplied by the superior mesenteric artery, whereas the body and tail are supplied by the celiac artery (19). The pancreas is composed of several lobuli, into which the interlobular arteries and veins run parallel to the major ducts. Thereafter, intralobular arteries pass straight through the center of a lobulus leaving branches to islets, acini, and ductuli. In the rat, the intralobular veins run parallel to the arteries (17), whereas in other species the intralobular veins have been observed to run separate (181, 182). All pancreatic veins empty their blood, rich in secreted islet hormones, into the portal vein.

The exocrine pancreas has an extensive lymphatic drainage, whereas the islets are thought to be devoid of lymph vessels (178, 187, 203). The pancreatic lymphatic system is not prominent during normal conditions. The capacity for drainage of fluids is, however, evident during pathological conditions, such as pancreatitis (15). The lymph vessels are mainly situated in the connective tissue and follow the blood vessels. Both endocrine and exocrine secretions have been reported at low concentrations in the thoracic duct lymph, but this is considered as quantitatively unimportant (187).

Pancreatic islet vascular anatomy and blood flow

The microvasculature of the pancreatic islets has been extensively studied with several different techniques, e.g. dye injections, vascular corrosion casts, in vivo microscopy and non-radioactive microspheres (19). In these studies, the microcirculation of the pancreatic islets has been showed to be independent of the exocrine pancreas (27). The islets are supplied with arterial blood from arterioles, which after penetrating the islet capsule form a
dense glomerular-like capillary network, leaving no endocrine cell more than one cell away from arterial blood (18). The islet capillaries have 10 times more fenestrations than the capillaries found in the exocrine parenchyma (89). These fenestrations, as well as the high vascular density, are dependent on vascular endothelial growth factor (VEGF) produced by the islet cells (116, 133).

In experimental animals, mainly rodents, the islet angioarchitecture differs between larger and smaller islets (19). Small islets (<160 µm) are supplied by one arteriole and empty into several small efferent venules. In the exocrine parenchyma, these venules are either connected to exocrine capillary plexa, thereby forming an insulo-acinar portal system, or empty into larger veins. On the contrary, large islets (>160 µm) receive blood from one to three arterioles and the efferent collecting venules seem to drain exclusively into larger veins without forming any insulo-acinar portal system. Studies of the microvascular angioarchitecture of human islets are few, however, the insular-acinar portal system seems to be well-developed (174, 242).

Seeing that the different islet cell types have distinct locations in the rodent pancreas (cf. above), the direction of the blood flow has been proposed to have significant impact on the ability of cells to intercommunicate within the islet. This has resulted in three competing models of islet microcirculation (27). The first model is based on scanning electron microscopical studies of corrosion casts, which suggest that non-β-cells are perfused before β-cells (173, 175, 182). The second model, is supported by investigations using corrosion casts (17) or anterograde and retrograde pancreas perfusions with anti-insulin/anti-somatostatin gamma-globulin (208). These studies suggest that β-cells are perfused before non-β-cells in both rodents (17, 208) and humans (221). A third model, describes a gated portal pattern from the afferent to the efferent pole of the islet based on intravital microscopical studies (26, 146, 160).

The disseminated nature of the endocrine pancreas complicates measurements of islet blood flow. Continuous recording of the blood perfusion of the whole endocrine pancreas is not technically possible; however, non-radioactive microspheres have been extensively used to study islet blood flow at any given time-point in experimental animals (105). The islets have been reported to receive 5-15% of the whole pancreatic blood flow in rats (10, 34, 97, 140), 2-5% in mice (32) and 15-20% in rabbits (139), although they only constitute 1-2% of the total pancreatic volume. In rats, this corresponds to an islet blood flow of 5-20 ml x min⁻¹ x g islet tissue⁻¹, or approximately 20 nl x min⁻¹ x islet⁻¹ (140). This is a rather high basal organ blood flow, which corresponds to that of the renal cortex (118, 246).

The relationship between pancreatic islet size and blood flow has been carefully investigated in rats (Table 1). In one-year-old Holzman rats the
range of islet diameter was 10-fold (30-300 µm), whereas the islet volume ranged 1000-fold (0.014-14 nl). The largest 20% of pancreatic islets constitute 72% of the islet mass and receive 64% of islet blood flow. On the contrary, the smallest 20% of islets receive 3% of islet blood flow, although they only constitute 1.5% of islet mass. Thus, large islets have a blood flow below average, whereas small islets have a blood flow above average. However, since large islets constitute ~75% of the rat endocrine pancreas and receive the majority of islet blood flow they may be most important for glucose homeostasis.

Table 1. Distribution of blood flow in subpopulations of islets grouped according to their size. Each group of islet size represents 20% of all islets. Modified from reference 140.

<table>
<thead>
<tr>
<th>Islet diameter (µm)</th>
<th>25-57</th>
<th>57-77</th>
<th>77-97</th>
<th>97-140</th>
<th>140-300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single islet volume (nl)</td>
<td>0.01-0.1</td>
<td>0.1-0.25</td>
<td>0.25-0.5</td>
<td>0.5-1.5</td>
<td>1.5-15</td>
</tr>
<tr>
<td>Percentage of total islet volume (%)</td>
<td>1.5</td>
<td>3</td>
<td>6</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td>Percentage of total islet blood flow (%)</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>21</td>
<td>64</td>
</tr>
</tbody>
</table>

The distribution of microspheres suggests that the blood perfusion of different islets is highly variable. When islet blood flow is measured by a microsphere technique, a small fraction of islets may contain more than 6 microspheres, whereas ~90% of islets are devoid of microspheres (107). Repeated microsphere injections show that up to 65% of islets may contain microspheres, but a fraction still remains devoid of microspheres (44). Furthermore, primarily the islets containing microspheres in the first injection contain microspheres after repeated microsphere injections, which suggest that the heterogeneity in blood perfusion is fairly constant over time for different islets.

The blood flow of endogenous islets is normally tightly regulated at the arteriolar level by nervous, endocrine and metabolic mechanisms (33, 105). This suggests that an appropriate blood perfusion is essential for islet metabolism and insulin secretion. Indeed, a dose-dependent and time-dependent preferential increase in islet blood flow is induced by glucose (98, 99). However, administration of glucose only seems to increase the blood perfusion of those islets (cf. above; ~10%) that have the highest basal blood perfusion (44, 107). The most important mediator of this islet blood flow increase is the vagus nerve, connected to blood glucose receptors in the brain, oral cavity, and duodenum (38, 100). Moreover, similar glucose receptors are present in the liver, however, their islet blood flow regulation is mediated by sympathetic nerves (39). Islet blood flow is also regulated by locally produced substances. Two important vasodilators are adenosine (46) and nitric oxide (NO) (225), whereas angiotensin II (AT II) (35) and
endothelin (unpublished observation) are potent vasoconstrictors in the endocrine pancreas.

Oxygenation of endogenous islets

The tissue oxygen tension in surface pancreatic islets is approximately 40 mmHg (37), which is comparable to that of venous blood and higher than in the exocrine pancreas (~30 mmHg), renal cortex (~15 mmHg), liver (<10 mmHg) and spleen (~30 mmHg) (42, 118). This may reflect high needs of oxygen for islet metabolism. In vitro, dispersed islet cells exposed to oxygen tension levels less than 12 mmHg have decreased glucose-stimulated insulin secretion (66). Moreover, pO2 was proposed to be the limiting factor for insulin secretion since decreased insulin secretion was observed before any decrease in cell viability (66). In an insulinoma cell line, βTC3 cells, it has been observed that oxygen tension levels below 25 mmHg gradually shift these cells from aerobic to anaerobic metabolism with a concomitantly increased lactate production, whereas insulin secretion is unaffected at pO2 above 7 mmHg (188, 189). Interestingly, in vivo the tight regulation of islet blood flow preserves islet oxygenation during a glucose challenge (43). Decreasing blood flow up to 50% by blockage of NO synthesis did not decrease islet oxygen tension, but decreased insulin secretion. This clearly shows that insulin secretion in vivo can be regulated by other mechanisms than pO2.

In response to low oxygen levels hypoxia inducible factor (HIF)-1α is the most essential transcription factor produced in mammalian cells (196). Interestingly, cultured human and rat islets exposed to 1% O2 (~7.5 mmHg) co-express HIF-1α and Caspase-3 resulting in hypoxia induced apoptosis (172). However, the oxygen threshold for these effects in islet cells is hard to discern, since there are well-known oxygen tension gradients within cultured islets. The spherical form of the islets results in oxygen tension gradients due to oxygen consumption and diffusion properties, respectively. This implies that the pO2 recorded in the culture medium is higher than within the islets. In vivo, the dense vascular network of the islets prevents any oxygen tension gradients. Studies on islet cell susceptibility to low pO2 levels in vivo are sparse and this issue need to be addressed further.

Clinical transplantation of insulin-producing cells

At present, replacement of insulin-producing cells is achieved by either transplantation of the whole pancreas or transplantation of isolated pancreatic islets. For many years, pancreas transplantation has re-established long-term normoglycemia in the majority of the transplanted patients (83). However, as for all pancreas surgery this procedure is associated with
significant risk of complications (83). Therefore, islet transplantation has been developed to constitute a minimal invasive approach for islet replacement. Pancreas transplantation is performed by major open abdominal surgery. In contrast, islet transplantation is performed on a conscious patient by infusing the islets into the portal vein via a transcutaneous catheter. After pancreas transplantation, without any ensuing complications, 2-3 weeks of recovery at the hospital awaits the recipient, whereas following islet transplantation the recipient is discharged within a few days.

In the 90s the clinical outcome of islet transplantations was poor, since insulin independence one year post-transplantation was less than 10% (23). In 2000, this was dramatically improved by the introduction of the Edmonton Protocol (204, 214). The Edmonton group improved the insulin independence one year post-transplantation to 80% by using a steroid-free, low-dose tacrolimus and sirolimus immunosuppressive regimen. Furthermore, they used freshly isolated islets and an increased amount of transplanted islets (>9000 IE/kg) per recipient. This was the first time insulin independence one year after islet transplantation was reported to be similar to that of pancreas transplantation (83, 205, 207). A drawback with the Edmonton Protocol is, however, that it demands multiple donors to retrieve enough isolated islets to cure one patient, whereas in transplantation of the whole gland one donor is enough.

Recently, the long-term graft function, using the Edmonton Protocol, was published. Unfortunately, a faster decline in function for these islet grafts compared to whole pancreas transplants was reported (83, 202). Five years after islet transplantation, only approximately 10% of the patients was still insulin independent (206). The great hope that the Edmonton group revealed for islet transplantation six years ago has now vanished. New research efforts must aim to improve long-term islet graft function.

Human islet isolation techniques have improved greatly over the years, however, currently only 20-50% of the pancreatic islets are recovered (131). In solid organ transplantation it has been known that living-donor organs do better than organs from brain dead donors (228). Interestingly, it was recently reported in rats that brain death of the donor impairs islet isolation yields and possible also affects the graft function following implantation (56). The irreversible damage to the central nervous system elicits a pro-inflammatory response with production of cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-1β and IL-6. Future strategies to reduce this inflammatory response in the donor may both improve islet isolation yields and insulin homeostasis following transplantation.

In pancreas transplantation, the vascular supply is restored immediately after anastomosis of the pancreatic blood vessels to the recipient’s vascular system. In contrast, in islet transplantation it seems as if the vascular network is insufficiently restored after transplantation (118). In addition to
immunological barriers an adequate formation of a new vascular network within the transplanted islets may be one of the main obstacles for successful clinical islet transplantation. Furthermore, allogeneic rejection (241), an instant blood mediated inflammatory reaction (IBMIR; 13, 168) and recurrence of disease (95) have been proposed to contribute to islet graft failure in the clinical setting.

Angiogenesis

There are two mechanisms for the formation of new blood vessels, namely vasculogenesis and angiogenesis (96). Vasculogenesis is the de novo formation of new blood vessels from angioblasts, which frequently occurs in the embryo. In the adult, however, the majority of new blood vessels is formed from pre-existing blood vessels, that is angiogenesis, and it may further be subdivided into vascular sprouting and intussusception. Sprouting is defined as the elongation/outgrowth of a pre-existing vessel, whereas intussusception is the formation of new blood vessels by ingrowth of transcapillary tissue pillars into existing blood vessels, thereby increasing the vascular density.

Tumors are known to not exceed 3-4 mm in diameter, without any neovascularization, due to oxygen diffusion limitations (71, 80). In 1971, Folkman et al reported that tumor tissue produced a soluble factor, which was mitogenic for capillary endothelium (73). A few months later Dr. Folkman proposed that anti-angiogenic treatment may contribute to the treatment of malignant diseases (72). This was the beginning of an Era, where extensive research has been performed to map out the biology of angiogenesis, mainly in tumor tissue. A complex system of pro- and anti-angiogenic factors has been revealed (49). Some of these factors have both pro- and anti-angiogenic properties depending on the tissue environment (96).

In hypoxic cells, the HIF system is activated to induce angiogenesis (196). HIF-1 is an αβ-heterodimer, which is a transcription factor. The HIF-1β subunit is constitutively produced, whereas the HIF-1α subunit is induced by hypoxia. In well oxygenated cells HIF-1α is hydroxylated, which promotes proteolysis in the proteosome and inhibition of transcription activity. This means that the HIF-1 heterodimer is only formed during low oxygenation. However, the cellular threshold for activation of the HIF system seems to differ between tissues (196). After formation, the HIF-1 αβ-heterodimer interacts with the hypoxia response elements to induce transcription of pro-angiogenic factors (e.g. VEGF, VEGFR2, nitric oxide synthase; NOS). Furthermore, HIF-1 indirectly induces expression of several factors, both pro- and anti-angiogenic (196).
Angiogenesis is induced when tissues produce pro-angiogenic factors in favor of anti-angiogenic factors (31, 96). In tumors, the acquired capacity to induce angiogenesis for further development in size is called the “angiogenic switch” (74). Firstly, angiogenesis is induced by NO production, which results in vasodilatation and increased blood flow. Thereafter, production of pro-angiogenic factors, such as VEGF, angiopoietin-2 and fibroblast growth factor (FGF)-2 stimulate endothelial cells to proliferate and migrate. The angiogenic vessel is now “sprouting” towards the low oxygenated tissue, which secretes pro-angiogenic factors. The newly formed vessels are unstable, but endothelial cell derived platelet derived growth factor (PDGF) recruits pericytes and smooth muscle cells for vessel stabilization (54). When the angiogenic stimulus is provided for a sufficient period of time, the matured vessel persists and shear stress from blood flow may be an important factor to inhibit vessel regression (67).

Endothelial cells

Traditionally, endothelial cells have been regarded as inert cells covering the interior surface of the vessels, enabling metabolic exchange between blood and tissues. In recent decades, however, research has revealed that endothelial cells are a heterogeneous cell population with differences in cell markers (78). The vast heterogeneity of endothelial cells depends on both genetic predisposition and environmental factors. The endothelial cells are known to interact with the surrounding tissue both during fetal development and in adult life. Thus, endothelial cells have been reported to induce the development of both the pancreas and liver (132, 153). This cross-talk between endothelial cells and the parenchyma seems also to be present in adult animals, since endothelial cells stimulate β-cell proliferation in pregnant rats (111).

The endothelial cell heterogeneity is also evident at the morphological level (150). The endothelium can be classified as continuous, fenestrated or discontinuous. Continuous capillaries have no openings in their wall. Fenestrated capillaries have small openings, called fenestrations, of about 80-100 nm in diameter. The fenestration is covered by a small, non-membranous, permeable diaphragm, which allows rapid passage of macromolecules. The basal membrane of the fenestrated endothelial cell is continuous. In contrast, discontinuous capillaries (sinusoids) have larger lumens, many fenestrations without diaphragm and a discontinuous or absent basal lamina. The microvascular endothelium is thin, elongated and often fenestrated, whereas in large arteries the endothelium is polygonal, non-fenestrated and thicker. Endothelial cells with fenestrations are often found in endocrine glands, where they are thought to facilitate the disposal of hormones into blood. However, it was recently reported that the
fenestrations of the endothelium in pancreatic islets may not be of crucial importance for glucose homeostasis (133). Capillaries with continuous endothelium are found in skeletal muscle, heart, lung and brain, whereas discontinuous endothelium is found in the bone marrow, liver and spleen.

Despite the great phenotypic heterogeneity of endothelial cells they have a lot of common characteristics (53). Besides forming a continuous monolayer inside all blood and lymphatic vessels, endothelial cells contribute to the regulation of blood coagulation (192). The intact endothelium expresses anti-thrombotic factors, whereas the activated endothelium of damaged vessels expresses factors that promote blood coagulation. Endothelial cells also participate in the regulation of the microcirculation. In this context, NO is an important endothelial cell derived vasodilator, whereas endothelin is a vasoconstrictor secreted from endothelial cells.

Pancreatic islets have highly specialized endothelial cells (185). The islet endothelial cells are extremely thin (100 nm) and have 10 times more fenestrations than the endothelial cells in the exocrine pancreas. Notably, both the islet vascular density and the fenestrations are dependent on a high intra-islet VEGF production by the islet endocrine cells (116, 133). Human islet endothelial cells express alpha-1 anti-trypsin, which seems to be an anti-angiogenic feature (148). Moreover, islet endothelium expresses both inhibitors and stimulators of angiogenesis, such as endostatin and VEGF (159).

Engraftment of transplanted pancreatic islets

The islet isolation preceding transplantation results in islets devoid of vascular and nervous connections. Furthermore, the isolation procedure exposes the islets to hypoxia and inflammatory stress (21), both of which probably are aggravated during the infusion of islets into the portal vein (118, 144). IBMIR is immediately elicited after islet exposure to blood, leading to destruction of islets by activation of the coagulation and complement systems (13, 109, 168). The islets are disseminated into the liver parenchyma and trapped in small branches of the portal vein. Initially, they completely depend on diffusion of nutrients and oxygen for their survival, which seems to result in hypoxia induced apoptosis and necrosis of islet cells (37, 62). The IBMIR-induced blood clots surrounding the islets may further impair the diffusion properties of oxygen and nutrients, however, the coagulated blood and activated platelets may also be important to initiate islet graft revascularization (20).

The revascularization of the transplanted islets has been reported to be initiated a few days post-transplantation and to be completed within the first 7-14 post-operative days (6, 165, 209). However, this revascularization
seems to be insufficient, since both the vascular density and tissue oxygen tension of the islet graft are chronically decreased when compared to native islets (40, 157).

Formation of new islet graft blood vessels

Transplanted pancreatic islets probably stimulate angiogenesis by using similar mechanisms as tumor tissue (cf. above). Isolated islets produce several pro-angiogenic factors (Table 2), such as VEGFs, matrix metalloproteinase-9 (MMP-9) and FGFs (12, 81, 86, 229, 238). These factors are also produced following transplantation. However, a delayed angiogenic response has been reported in diabetic compared to normoglycemic recipients (238, 239). Pancreatic islets also express inhibitors of angiogenesis (Table 2). Thrombospondins (TSPs) are extracellular glycoproteins, which are expressed in islets and may act as potent inhibitors of endothelial cell proliferation and migration (229). In this context, TSP-1 is of particular interest, since TSP-1 knock-out mice have been reported to develop pancreatic islet hyperplasia with increased vascular density (59). Recently, it has been reported that β-cell replication is influenced by endothelial cells (111, 179), which may suggest that the islet hyperplasia seen in TSP-1 knock-out mice is secondary to increased angiogenesis. The pancreatic islets also express other angiostatic substances, such as tissue inhibitors of metalloproteinase (TIMPs) and α1-antitrypsin (12, 148). Furthermore, endothelial cells from isolated islets and islet grafts express endostatin, an angiostatic factor derived from collagen XVIII (159). Interestingly, the islet graft endothelial cell expression of endostatin seems to depend on the implantation organ, since endostatin is expressed in intraportally implanted islets and not in renal subcapsular grafts.

Table 2. Stimulators and inhibitors of angiogenesis produced by pancreatic islets.

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Inhibitors</th>
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<tbody>
<tr>
<td>VEGFs, FGFs, HGF, MMP-9</td>
<td>α1-anti-trypsin, TIMPs, thrombospondins, endostatin</td>
</tr>
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</table>

During culture the majority of islet endothelial cells (180, 190), as well as immune cells (120) and exocrine tissue (152), seems to disappear. Cultured islets are mostly used for transplantation, since the depletion of immune cells (120) and exocrine tissue (90) is considered beneficial for islet engraftment. The islet graft has been considered to be exclusively revascularized by the host (232), but recently it was shown that donor endothelial cells also participate in the revascularization process (24, 143, 180). Furthermore, new islet graft microvessels have been reported to be formed by both angiogenesis (127) and vasculogenesis (57).
The origin of the host vessels that connects to the graft microvessels in islets implanted to different sites is not fully investigated. Experimentally islets are often implanted into the renal subcapsular space, and these multi-islet grafts seem mainly to become revascularized from the renal cortex (127). In the clinical setting, islets are infused into the portal vein were they are trapped in the small branches of the portal vein. Experimental studies indicate that such islets become revascularized by the hepatic artery (6). Recently, whole-liver perfusions were also used to selectively stimulate islet graft insulin secretion with glucose administration via the hepatic artery or the portal vein. Insulin secretion was only stimulated when the hepatic artery was infused, which argues that intraportally transplanted islets are functionally exclusively revascularized from the hepatic artery (135). Interestingly, this finding is similar to the vascularization, which has been reported for liver metastases (195).

The newly formed microvascular network of the islet graft acquires a phenotype similar to that in endogenous islets (87, 149). Thus, the transplanted islet cells influence the creation of a glomerular-like vascular network with fenestrations. This phenomenon has also been reported for transplantation of other tissues, such as development of microvessels with blood-brain barrier induced by neural tissue (2). Notably, in some instances the islet graft blood vessels have been shown to be morphologically aberrant with 20% devoid of an endothelial cell lining 6 weeks post-transplantation (149). The functional property of this finding is unknown, however, it suggests microvascular dysfunction.

The dense vascular network of endogenous islets is not restored following transplantation. In mouse islet grafts implanted into the renal subcapsular space or intraportally into the liver, the capillary density was ~35% of that in endogenous islets, whereas the capillary density in islets implanted into the subcapsular space of the spleen was even lower (155). This low vascular density seems to be chronic, at least up to six months post-transplantation, and independent of the recipient’s glucose homeostasis (157). Furthermore, these studies showed a much higher vascular density in the connective tissue stroma surrounding the implanted islets. This high vascular density of the stroma did not merely reflect a foreign body reaction, since after implantation of inert microspheres a much lower vascular density was discerned. These data may suggest that implanted islets secrete sufficient amounts of pro-angiogenic factors, but for unknown reasons the microvessels are hindered to grow into the islets.

In human islets implanted into immunodeficient mice the graft vascular density is similar to that of mouse islet grafts (47). However, the vascular density of the endogenous islets is lower in humans than in mice, meaning that the vascular density of transplanted human islets is approximately 50% of that in human islets in situ. Notably, in human islet grafts there is a
negative correlation between increasing donor age and graft vascular density. The reason for this finding is to be determined.

In most studies of islet graft revascularization syngeneic or immunodeficient animals have been used. There are few data suggesting that immunosuppressive drugs affect revascularization. In one report, cyclosporine has been shown to impair the islet graft revascularization (245), whereas two other studies concluded that there were no negative effects (164, 233). Moreover, prednisolone has been shown to not affect vascular engraftment (166). Recently, it has also been speculated that sirolimus may negatively affect islet graft revascularization due to inhibition of VEGF production (84). This needs to be determined, since sirolimus is a common immunosuppressive drug in islet transplantation.

Microcirculation of the islet graft

The blood flow of islet grafts has been evaluated by laser-Doppler flowmetry and microsphere techniques, respectively. The laser-Doppler flow value is an arbitrary unit of blood flow that represents the movements of all blood cells in the illuminated tissue, irrespective of vessel type (200). On the contrary, microsphere techniques measure nutritive blood flow and may discriminate between blood flows of different tissue subtypes (99). Thus, laser-Doppler flowmetry values represent the blood perfusion of the whole graft, whereas the microsphere technique may selectively measure endocrine and stromal graft blood flow.

By means of laser-Doppler flowmetry, the blood flow in rodent islet grafts has been reported to be 40-50% of that in the renal cortex irrespective of implantation organ (Table 3), i.e. the subcapsular space of the kidney, spleen or liver (37, 42). The renal cortex has in these studies been chosen as reference organ, since its blood flow is in the range of the blood flow observed in endogenous islets (36). Furthermore, similar blood flow has been observed in human islets implanted into immunodeficient mice (47). Interestingly, there was also a negative correlation of graft blood flow and donor age, corresponding to that reported for vascular density (cf. above). In hyperglycemic syngeneic recipients islet graft blood flow has been reported to be unaltered using laser-Doppler flowmetry (37), and slightly increased when measured with a microsphere technique (36). Microspheres have also been used to study islet graft blood flow in rat islet autografts transplanted beneath the renal capsule. Surprisingly, these studies reported an islet graft blood flow similar to that of endogenous islets (101, 102, 211). However, recent data suggest that the partial pancreatectomy performed in these studies provides unknown stimuli, which improve the vascular density of the grafts (110).

The mechanisms of blood flow regulation in islet grafts have been studied to evaluate whether they acquire functional properties of endogenous islets
or that of the implantation organ. Some studies indicate an altered blood flow regulation of the newly formed graft blood vessels compared to native islets (37, 102, 104). Glucose, the major insulin secretagogue, preferentially increases the blood perfusion of native pancreatic islets, secondary to activation of the vagus nerve (46, 100). In revascularized transplanted adult islets an unaltered (37, 104) or a paradoxically decreased (102) blood perfusion after a glucose challenge have been observed. However, in a study of fetal porcine ICC, acquisition of a glucose-induced islet graft blood flow increase has been observed 16 months post-transplantation (126), which may reflect maturation of blood vessels or reinnervation. Newly transplanted islets probably lack most nerves and it is uncertain if any functional reinnervation of blood vessels occurs. Therefore islet graft blood flow regulation will probably largely depend on locally produced vasoactive mediators. Angiotensin-converting enzyme and NOS are present in endothelial cells of both endogenous and transplanted islets (51, 159, 224). In transplanted islets, there is an up-regulation of the key AT II receptor, i.e. the type 1 receptor (136), and specific inhibition of this receptor results in improved graft blood flow, tissue oxygen tension and first phase of glucose-stimulated insulin release (117).

Table 3. Parameters of the microcirculation in endogenous islets compared to islets transplanted into the renal subcapsular space. Modified from reference 108.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Endogenous Islet</th>
<th>Transplanted Islet</th>
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<tbody>
<tr>
<td>Vascular density</td>
<td>1300 microvessels/mm²</td>
<td>450 microvessels/mm²</td>
</tr>
<tr>
<td>Blood flow</td>
<td>6 ml x min⁻¹ x g⁻¹</td>
<td>3 ml x min⁻¹ x g⁻¹</td>
</tr>
<tr>
<td>Oxygen tension</td>
<td>40 mmHg</td>
<td>5-10 mmHg</td>
</tr>
<tr>
<td>Capillary pressure</td>
<td>4 mmHg</td>
<td>10 mmHg</td>
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</table>

The oxygenation of transplanted islets has been extensively studied in rodents using modified Clark microelectrodes (37, 40, 42). In these studies, a tissue oxygen tension of ~20% of that in endogenous islets has been reported in normoglycemic recipients (Table 3). Notably, in hyperglycemic recipients the oxygen tension of the islet grafts is even lower, but the blood flow is still similar compared to normoglycemic recipients (37, 42). Furthermore, the low oxygenation seems to be chronic, since there is no improvement in oxygenation up to 9 months post-transplantation (40). The oxygen tension of intraportally transplanted islets has not yet been measured due to technical limitations caused by their disseminated nature in the liver. A similar oxygenation as in multi-cellular grafts is indicated by similar vascular densities (cf. above), however, the oxygen diffusion properties may differ.

True hypoxia in islets grafts is supported by anaerobic metabolism and acidosis. In one study, a microdialysis technique was adopted to study
anaerobic metabolism by measuring lactate/pyruvate ratios in rat islet grafts implanted beneath the renal capsule (45). Similarly increased basal lactate/pyruvate ratios were observed in both normo- and hyperglycemic recipients when compared to control (islets isolated from the pancreas). Moreover, a glucose challenge in non-diabetic recipients almost doubled the lactate/pyruvate ratio, which contrasts to the unaffected lactate production for islets exposed to hyperglycemia during culture. In this context, it is interesting to note that β-cells normally express low levels of lactate dehydrogenase (213). Anaerobic metabolism of islet grafts is further supported by acidosis measured by pH-microelectrodes (48). The pH of endogenous islets was identical to arterial blood, whereas the islet graft pH was 0.11-0.15 pH units lower in both non-diabetic and diabetic recipients. Interestingly, a negative correlation between tissue oxygen tension and pH in grafts of diabetic recipients was discerned.

Endogenous pancreatic islets are considered devoid of lymphatic vessels (187) and they have a low capillary pressure of 3-4 mmHg (33). Notably, it has been shown that the islet grafts acquire the hydrostatic pressure of the implantation organ, which in the kidney is ~10 mmHg (Table 3). Any functional consequences of this increase in capillary pressure post-transplantation are presently unknown. However, in some organs, e.g. kidney and retina, an increase in capillary pressure has been reported to affect endothelial cells by increased shear stress, which may have effects on the parenchymal cells (247, 248). Recently, it was also reported that islet grafts in contrast to native islets contain lymphatic vessels (115). Moreover, it was suggested that this may have negative impact on insulin delivery and antigen presentation.

Islet graft function
In clinical islet transplantation, use of the Edmonton Protocol resulted in a one year post-transplantation insulin independence rate of 80%. However, three years after transplantation 50% of the patients was back on insulin and 5 years post-transplantation only 10% was still insulin-free (206). Notably, although >85% of the estimated islet mass is infused to all recipients, their insulin response 10 months post-transplantation is only ~20% of that in healthy people (204). The last six years, the Edmonton Protocol has been reproduced at several transplantation centers worldwide, but there has been no further progress in graft function.

In diabetic rodents, restored normoglycemia has been demonstrated when islets have been implanted into the liver, spleen and kidney. Diabetic mice are cured when 200-300 islets are transplanted, whereas in adult rats approximately 1000 islets are needed to cure the animals. Experimental studies on rats have shown that the renal subcapsular space provides better
long-term graft function than the intraportal site (92). Clinical islet transplantations have been performed to the kidney site (82). However, the majority of human islet transplantations has been performed into the portal vein and presently almost all islets are implanted into this site.

Both in the clinical and experimental setting there are problems to monitor islet mass post-transplantation, especially when the islets are implanted into the portal vein. This means that it has been difficult to differentiate between β-cell death and dysfunction. There is most probably a great death of islets at transplantation, i.e. due to hypoxia (62) and IBMIR (109). Recently, it has also been shown that islets implanted to the liver are dysfunctional (158). Mattsson and co-workers retrieved intraportally transplanted mouse islets by collagenase digestion and reported decreased glucose-induced insulin release of these islets. Moreover, the retrieved islets showed decreased glucose oxidation, suggesting mitochondrial dysfunction. Previously, human and mouse islets retrieved from the renal subcapsular space have been described to have a decreased first phase of glucose-stimulated insulin release, as well as a chronically decreased insulin content, when compared to control islets (215, 216, 226).

Intraportally transplanted islets have been reported to have a defective glucagon response to hypoglycemia both in humans (191) and dogs (85). Surprisingly, the islets respond with glucagon secretion to other stimuli, such as arginine. This poor glucagon response seems to be an implantation site specific phenomenon (85). Moderate exercise has also been observed to result in hyperglycemia in rats with islet transplants in the liver, kidney or peritoneal cavity (186). The rats with intraportally transplanted islets increased their C-peptide and glucagon during exercise, whereas these changes were not seen in the controls or the other transplanted animals. The physiological mechanisms behind these findings are presently unknown.

The mechanisms of vascular engraftment do not seem to be directly affected by hyperglycemia. However, poor glycemic control in the recipient is harmful to mouse islets per se (124), although depending on the mouse genetic background (125). Human islets seem to be more sensitive than mouse islets and a diabetic milieu induces long-term insulin secretion defects in human islets (106). Therefore, strict insulin treatment at the time of islet transplantation is warranted (167)

Strategies to improve graft vascularization and function

In previous studies, local treatments at the implantation sites with pro-angiogenic factors (VEGF or FGF-2) have been reported to be beneficial for revascularization and graft function (88, 119, 220). Furthermore, addition of VEGF to the culture medium during pre-transplantation culture has been observed to reduce islet immunogenicity, improve islet viability and improve
graft function (219). Due to recent advances in molecular biology, overexpression of pro-angiogenic factors may be induced in tissues. Indeed, VEGF overexpression in beta-cells has been reported to augment islet graft revascularization and improve graft function (129, 249). Angiopoietin-1 is another pro-angiogenic factor expressed by islet cells (229), which is an agonist of the endothelial cell specific Tie-2 receptor. Recently, overexpression of angiopoietin-1 was also shown to enhance islet revascularization and to protect from cytokine induced apoptosis (223). Moreover, treatment of the recipients with granulocyte-macrophage colony-stimulating factor to induce vasculogenesis has also been shown to increase islet graft functional mass and revascularization (57). However, since this treatment stimulates the immune system it is not likely to be used in a human allogeneic setting.
Aims

The aims of this thesis were to investigate:

- the blood flow regulation in islet grafts implanted into the subcapsular space of the kidney in both normo- and hyperglycemic recipients (I).
- whether transplantation of pancreatic islets without prior culture influences graft revascularization and function (II).
- whether culture with addition of VEGF or FGF-2 preserves the intraislet vascular network during culture (III)
- whether pretreatment with VEGF, FGF-2 or MMP-9 influences islet graft revascularization and function following transplantation (III).
- the oxygenation of endogenous islets (IV).
- the oxygenation of intraportally transplanted islets (V).
Materials and Methods

Animals (I-V)

Male inbred Wistar-Furth rats weighing 300-350 g (I, II and IV) and male inbred C57BL/6 mice weighing 25-30 g (II, III, and V) were used in the studies. The rats and mice had free access to water and pelleted food and were housed in a room with a 12-hour light/dark cycle and 70% humidity throughout the course of the studies. All experiments were approved by the local animal ethics committee at Uppsala University, Sweden.

Islet isolation and transplantation (I-V)

Pancreatic islets were isolated by collagenase (Boehringer-Mannheim; Mannheim, Germany) digestion, as previously described (5, 210). In culture, the isolated islets were free-floating in groups of ~150 islets in RPMI 1640 medium (Sigma-Aldrich; Irvine, UK) supplemented with 10% (vol/vol) fetal calf serum (Sigma-Aldrich). The medium was changed every second day. At the time of transplantation, 250 (rat) or 200 (mouse) islets were packed in a braking pipette and implanted beneath the renal capsule on the dorsal side of the left kidney of syngeneic pentobarbital-anesthetized [60 mg/kg, i.p. in normoglycemic and 40 mg/kg i.p. in streptozotocin-diabetic rats (Apoteket, Umeå, Sweden)] rats, and syngeneic avertin-anesthetized [0.02 ml/g i.p. of a 2.5% (vol/vol) solution of 10 g 97% (vol/vol) 2,2,2,-tribromoethanol (Sigma-Aldrich) in 10 ml of 2-methyl-2-butanol (Kemila, Stockholm, Sweden)] normoglycemic or alloxan-diabetic mice. Moreover, for intraportal islet transplantation, islets were packed in a butterfly injection needle (25 gauge) and injected into the portal vein (162) of syngeneic avertin-anesthetized normoglycemic mice.

Islet culture with stimulators of angiogenesis (III)

Pancreatic islets were prepared from C57BL/6 mice by collagenase digestion (5), and cultured free-floating in groups of 150-200 islets. Some islets were cultured for 5-7 days in 5 ml medium composed of RPMI 1640 medium (Sigma-Aldrich) supplemented with 11 mmol/l glucose and 10% (vol/vol)
fetal calf serum, with or without addition of VEGF (Sigma-Aldrich; 20 ng/ml) or FGF-2 (Sigma-Aldrich; 20 ng/ml). Other islets were cultured for 24 hours in 5 ml medium composed of Dulbecco’s modified eagles medium (DMEM; Sigma-Aldrich) supplemented with 11 mmol/l glucose, and 0.5% (vol/vol) fetal calf serum, with or without addition of MMP-9 (Calbiochem, San Diego, CA; 1 µg/ml).

Pancreatico-duodenal transplantations (IV)

This procedure has been described in detail elsewhere (103). Briefly, the donor was anesthetized with an intraperitoneal injection of a mixture of chloral hydrate (175 mg/kg body weight) and pentobarbital sodium (Ekvicitin®, Apoteket; 40 mg/kg), and placed on a heated operating table. The whole pancreas, together with approximately 5 cm of the duodenum, was dissected free from surrounding tissues. Through a catheter in the abdominal aorta the preparation was flushed with 5-7 ml of cold (4 °C) UW-solution (Via-Span; Du Pont Pharmaceuticals, Wilmington, DE) at a pressure of approximately 100 cm H₂O. The graft was then removed from the animal, together with approximately 1 cm of the aorta, which contained the two pancreatic arterial blood vessels, and stored at 4 °C for 1.5-2 h before being implanted into the recipient.

The recipients were also anesthetized with chloral hydrate and pentobarbital sodium and placed on a heated operating table. The left kidney was removed, and the pancreatico-duodenal graft was anastomosed to the renal blood vessels by a non-suturing cuff technique (183). The graft duodenum was sutured end-to-side to a loop of the colon of the recipient by 10 sutures with 7-0 silk. After closure of the abdominal wound, the animals were injected subcutaneously with 10 mg doxycycline (Idocyclin; Leo, Malmö, Sweden) and were observed until fully recovered from anesthesia. Three weeks after transplantation, the whole pancreas transplanted animals were allocated to measurements of pancreatic tissue oxygenation.

Partial pancreatectomy (IV)

Animals were anesthetized with pentobarbital sodium (Apoteket; 60 mg/kg body weight i.p.) and placed on a heated operating table. A transverse incision was made in the left hypochondrium to visualize the pancreas and spleen. The splenic 60% of the pancreas (approximately 520 mg) was freed from surrounding tissues and removed. After surgery, the peritoneum and skin were closed by sutures and the animals allowed recover from the anesthesia. One week later, these animals were allocated to measurements of pancreatic tissue oxygenation.
Endothelial staining (II-III)

The pancreas and the islet grafts were fixed in 10% (vol/vol) formalin, whereas isolated islets were fixed in 4% (vol/vol) paraformaldehyde. All tissues were thereafter embedded in paraffin and sections (5 µm thick) were stained for endothelial cells by the lectin Bandeiraea simplicifolia (154). The sections were incubated with normal goat serum (NGS; DAKO, Glostrup, Denmark), diluted 1:20 with Tris-buffered saline (TBS), for one hour in a moist chamber at room temperature (RT; 20°C). Biotinylated forms of lectin from Bandeiraea simplicifolia (Sigma-Aldrich), diluted 1:100 in TBS, were applied to the sections at 4°C overnight. The sections were then washed and incubated with Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA) for 30 min in a moist chamber at RT. The sections were washed and the chromogen Vector Red (Vector Laboratories) was applied to the sections and left for 30 min to develop in a moist chamber at RT. Thereafter, the slides were washed in TBS, counterstained with hematoxylin, dehydrated and mounted with Mountex (Histolab Products, Gothenburg, Sweden).

Evaluation of vascular density (II-III)

An examiner unaware of the origin of the samples randomly chose histological sections of islets, islet grafts and isolated islets. The number of blood vessels in each tissue was counted in a light microscope at a magnification of 600X. Stromata surrounded the individual islets in the grafts. The number of microvessels in the transplanted islets and stroma was counted separately. The respective fractions of islets and stroma in the islet grafts were determined by a direct point counting technique (240). For this purpose, the number of intersections overlapping the stroma and endocrine cells within the islet grafts was counted (magnification 600X). At least 10 fields (corresponding to ~1,200 intersections) were counted in each islet graft. The areas of the investigated endogenous islets and grafted islets were determined by using a computerized system for morphometry (MOP-Videoplan; Carl Zeiss, Stockholm, Sweden or ImageJ 1.3v; National Institutes of Health, Bethesda, MD). Vascular density, i.e. the number of blood vessels per measured islet or graft area (mm²), was then calculated.

Evaluation of graft volume (II)

Renal subcapsular islet grafts, composed of 250 freshly isolated or 250 cultured rat islets, were retrieved one month post-transplantation and prepared for histological evaluation. The islet grafts were paraffin-embedded, consecutively sectioned (5 µm) and stained with hematoxylin and
To estimate the total graft volumes, the area of every fifth section was measured by using a computerized system for morphometry (MOP-Videoplan). Thereafter, the fraction of endocrine cells in each section was determined by a direct point counting technique (240), and the endocrine graft volume was then calculated.

**Evaluation of islet graft function (II-III)**

Mice were given an intravenous injection of alloxan (75 mg/kg; Sigma-Aldrich) 5 days prior to transplantation and were considered diabetic if they had non-fasting blood glucose concentrations >16.7 mmol/l at this time. The number of transplanted islets (200) was chosen based on our previous studies in this strain (157) and aimed to reach an islet mass insufficient for full reversal of hyperglycemia in most of the diabetic recipients receiving cultured islets. The blood glucose concentrations of the transplanted animals were measured every fifth day up to one month post-transplantation. Animals cured from diabetes were defined as those with non-fasting blood glucose concentrations <11.1 mmol/l.

In paper III, cured diabetic animals were also subjected to an intravenous glucose tolerance test one month post-transplantation. D-glucose (300 mg/ml; Fresenius Kabi, Uppsala, Sweden) was injected into the tail vein of awake animals at a dose of 2.5 g/kg body weight, and blood glucose concentrations were measured in blood obtained from the cut tip of the tail before glucose injection and at 10, 30, 60 and 120 minutes after glucose injection.

In both paper II and III, the graft-bearing kidneys were removed on all cured animals one month post-transplantation to ascertain that they returned to hyperglycemia (>16.7 mmol/l), and that the improved blood glucose concentrations not merely represented regained function of the endogenous pancreas.

**Estimation of pancreatic islet mass (IV)**

The fractional volume of the islets in each pancreas was determined by a point-counting technique (240). The intersections overlapping islets were counted in a light microscope (200X). A total of 10 different fields (corresponding to ~1200 intersections) were examined in each pancreas. Since the densities of the exocrine and endocrine pancreas differ less than 1% (50), the islet mass was estimated by multiplying the pancreatic weight by the islet volume fraction of the whole pancreas.
Measurements of blood flow regulation (I)

Laser-Doppler flowmetry uses coherent and monochromatic light generated by a low-power laser that is directed to the tissue by an optical fiber probe (200). When the photons are scattered by moving blood cells they undergo a Doppler shift, i.e. a change in wavelength. This Doppler shift is recorded by photodetectors and processed to produce a voltage proportional to the blood flow (16). Thus, the number of red blood cells and their average velocity within the illuminated tissue are the major determinants of the blood flow. However, only arbitrary values of blood flow (Tissue perfusion units; TPU) can be obtained, which limits the method to measuring changes of blood flow.

Four weeks after transplantation, the rats were anesthetized by thiobutabarbital [Inactin®, 120 mg/kg i.p., (streptozotocin-diabetic rats 75 mg/kg); Research Biochemicals International, Natick, MA.], placed on a heated operating table (37.5°C), and tracheostomized. Polyethylene catheters were inserted into the ascending aorta (via the right carotid artery), the left femoral artery, and the left femoral vein. The carotid catheter was connected to a pressure transducer (PDCR 75/1; Druck, Groby, UK) to continuously monitor the mean arterial blood pressure throughout the experiment. The femoral vein catheter was used to infuse saline (5 ml x kg⁻¹ x h⁻¹) to substitute for body fluid loss and for administration of adenosine, AT II and N⁰-nitro-l-arginine (L-NNA).

The left kidney was exposed by a left subcostal flank incision and immobilized in a plastic cup. The kidney was embedded in cotton soaked in saline, and covered with mineral oil (Apoteket) to keep the kidney surface moist and at body temperature. The left ureter was catheterized to avoid urinary stasis. The animal was then allowed to rest for at least 15 minutes to minimize the influence of surgical stress on the blood flow measurements.

By means of laser-Doppler flowmetry (Transonic® BLF 21 series, probe diameter 1.2 mm; Ithaca, NY), the blood flow of the islet graft and the adjacent renal cortex was measured. The basal blood flow of the islet graft and the adjacent renal cortex was determined by repeated measurements (n=5) in each animal before administration of any of the test substances, and the mean was considered one experiment. The blood perfusion of the islet graft and adjacent renal cortex was then simultaneously monitored by two laser-Doppler probes to record the changes in blood perfusion upon stimulation with adenosine (0.6 mg x kg⁻¹ x min⁻¹), AT II (0.17 µg x kg⁻¹ x min⁻¹) and L-NNA (25 mg/kg). All substances were dissolved in saline, and given to all animals. Adenosine and AT II were given in random order as an i.v. infusion (0.1 ml/min) for 10 minutes, whereas L-NNA was always given last as a bolus dose of 0.6 ml i.v., due to its sustained effects. A wash-out period of at least 20 minutes, or until the mean arterial blood pressure returned to the basal value, separated each drug administration. Laser-
Doppler flowmetry values were recorded every five minutes. Since it is difficult to calibrate the laser-Doppler flowmeter in physiological units of blood flow, all changes were expressed as relative changes from the blood flow prior to stimulation (0 min, i.e. basal blood flow).

Calculation of vascular conductance (I)

The vascular conductance of the islet graft and renal cortex was calculated by dividing the blood flow measured by laser-Doppler flowmetry with the mean arterial blood pressure at the same point of time. It was then expressed as relative changes from the vascular conductance prior to stimulation (0 min, i.e. basal vascular conductance).

Correlation between blood flow measurements by laser-Doppler flowmetry and microspheres (I)

The laser-Doppler flowmetry values obtained in the present study were correlated to organ blood flow values, as measured by non-radioactive microspheres in the same tissue. For this purpose, the blood perfusion of the left renal cortex was determined by laser-Doppler flowmetry in some animals (n=5), by means of repeated measurements (n=3). The mean of these latter values was then considered to be one experiment. Thereafter, non-radioactive microspheres (diameter 11 µm, NEN-Trac; Du Pont Pharmaceuticals) were injected into the ascending aorta via the carotid catheter, as described in detail elsewhere (97). Blood was collected by free flow via the catheter in the femoral artery for 60 seconds, starting five seconds before microsphere administration. The left renal artery and vein were then occluded by a vascular clip to enable analysis of laser Doppler flowmetry background values (biological zero). Three such values were obtained in each animal and the mean was considered as one experiment.

The animal was killed by cervical dislocation and the graft-bearing left kidney was excised. The blood perfusion of the renal cortex was measured with the non-radioactive microsphere technique. In brief, by means of a scalpel, a part (~250 mg) of the left outer renal cortex (<1 mm from the kidney surface) was dissected from the rest of the kidney, put in 1 ml NaOH (4 mmol/l), and sonicated. At least 200 microspheres were counted under a stereomicroscope in both the blood sample and the renal cortex suspension. This enabled calculation of the outer renal cortical blood flow using the formula: \( Q_{\text{org}} = Q_{\text{ref}} \times N_{\text{org}} / N_{\text{ref}} \), where \( Q_{\text{org}} \) is organ blood flow (ml/min), \( Q_{\text{ref}} \) is withdrawal rate of a blood reference sample (ml/min), \( N_{\text{org}} \) is number of microspheres present in the organ, and \( N_{\text{ref}} \) is number of microspheres in the reference sample.
By means of simple regression analysis, renal cortical blood flow measured by laser-Doppler flowmetry and by the microsphere technique was correlated to obtain a formula to convert laser-Doppler flowmetry values to blood flow values expressed as ml x min⁻¹ x g tissue⁻¹. In the simple regression analysis, each animal contributed with values from the intact kidney and from the kidney with clamped artery and vein (background). The laser-Doppler values of the intact kidney were correlated to the values obtained by microspheres, while the laser-Doppler background (biological zero) was correlated to zero nutritive blood flow.

Oxygen tension in surface endogenous islets (II-III)

Non-transplanted rats were anesthetized by thiobutabarbital (Inactin®, 120 mg/kg i.p.; Research Biochemicals International) and non-transplanted mice were anesthetized by avertin [2.5% (vol/vol) solution of 10 g 97% (vol/vol) 2,2,2-tribromoethanol (Sigma-Aldrich) in 10 ml 2-methyl-2-butanol] (Kemila, Stockholm, Sweden). The animals were placed on an operating table, tracheostomized and maintained at body temperature (37°C). Polyethylene catheters were inserted into the left femoral artery and the left femoral vein. The arterial catheter was connected to a Statham P23dB pressure transducer to continuously monitor the mean arterial blood pressure throughout the experiment. The femoral vein catheter was used to infuse saline (5 ml x kg⁻¹ x h⁻¹) to substitute for body fluid loss. The abdomen was opened by a mid-line incision and the pancreas was immobilized over a hollow cylindrical plastic block attached to the operating table. The pancreas was then continuously superfused with mineral oil (Apoteket) at body temperature to prevent desiccation of the tissue. After allowing the mean arterial pressure to stabilize, 0.8 ml sterile-filtered 2% (wt/vol) neutral red (Kebo Grave, Stockholm, Sweden) was injected intravenously to selectively stain the islets within the pancreas. This dye has previously been evaluated and shown not to affect pancreatic oxygenation, whole pancreatic and islet blood flow, or glucose homeostasis (33, 34). The rats were then allowed to rest for 30 min to minimize the influence of surgical stress and neutral red administration on the pO₂ measurements.

The pO₂ was measured by modified Clark microelectrodes (outer tip diameter 2-6 μm; Unisense, Aarhus, Denmark)(34, 37, 145). The microelectrodes use the polarographic method, which is based on the principle that a unique, characteristic current-voltage curve is obtained when solutions of electro-oxidizable or electro-reducible substances are electrolyzed in a cell (69, 91, 123). The voltage-current relationship in a system for characterization of oxygen is represented by a sigmoid curve with a current plateau between -0.5V and -0.9V (122). In this voltage range, the plateau current depends on the pO₂ of the surrounding solution of the
cathode surface. The oxygen will immediately be reduced (consumed) at the cathode surface, resulting in a current which is limited by the rate of oxygen diffusion to the cathode. This rate is limited by permeation factors in the electrolyte solution, the electrode membrane itself and on the medium outside the membrane. This results in a current directly proportional to the pO₂ in the tissue surrounding the microelectrode (123, 141).

The modified Clark microelectrodes are composed of three electrodes in a glass shaft (Figure 1). The sensing cathode is a platinum wire (f), the guard electrode is a silver wire (c), and the reference anode is composed of Ag/AgCl wire (d). The electrolyte (e) consists of 0.5 mol/l KCl buffered with 50 mmol/l K₂CO₃ and 75 mmol/l KHCO₃ (pH 10.2). Functionally, the pO₂ electrode consists of two separate circuits. The platinum cathode and the silver anode form the true oxygen sensing system, whereas the silver cathode and the silver anode form the guard system. Both circuits are supplied with -0.8 V from separate voltage sources and the current of the oxygen sensitive circuit is recorded.

The electrode reaction at the cathode surface (reduction of dissolved oxygen) is as follows:

\[
\begin{align*}
O_2 + 2H_2O + 2e^- & \rightarrow H_2O_2 + 2OH^- \\
H_2O_2 + 2e^- & \rightarrow 2OH^- \\
O_2 + 2H_2O + 4e^- & \rightarrow 4OH 
\end{align*}
\]

The anode reaction (oxidation) is:

\[
4Ag + 4 OH^- - 4e^- \rightarrow 2Ag_2O + 2H_2O
\]

The microelectrodes were calibrated in water saturated with Na₂S₂O₅ or air at 37 °C before and after the experiment. The drift of the microelectrode recordings was <0.5%/h. The microelectrode tip was inserted into the islets and the exocrine pancreas with a micromanipulator under a stereomicroscope. Measurements of pO₂ were performed in 3-6 islets in each animal. Multiple measurements (≥3) were frequently performed in each islet and the mean of these measurements was then calculated to obtain the pO₂ in one islet. The mean of the pO₂ values in one animal was considered one experiment in the subsequent statistical analysis.
Oxygen tension and blood flow in islet grafts (II-III)

Transplanted rats and mice were anesthetized and surgically prepared similarly to the non-transplanted animals (cf. above). However, in this case the abdomen was opened by a left subcostal flank incision and the graft-bearing left kidney was immobilized in a plastic cup attached to the operating table. The kidney and the islet graft were embedded in cotton and mineral oil (Apoteket) to prevent heat loss and desiccation. The animals were then allowed to rest for 30 minutes to minimize the influence of surgical stress on the subsequent measurements.

Repeated measurements \(n \geq 10\) of \(pO_2\) were conducted in the transplanted islets and adjacent renal parenchyma, and the mean was considered one experiment. In conjunction with the \(pO_2\) measurements, blood flow in the islet graft and the adjacent renal cortex was recorded by laser-Doppler flowmetry (PF 4001-2, Perimed, Stockholm, Sweden). The blood flow in the islet graft and the adjacent renal cortex was determined by repeated measurements \(n \geq 3\) in each animal, and the mean was considered one experiment.
Threshold for pimonidazole accumulation in islet cells (IV)

Freshly isolated rat islets were incubated at 95% air/5% CO₂ and 37°C for approximately 6 hours. Thereafter, free islet cells were dispersed from groups of 300 islets from each animal. In brief, islets were washed in Ca²⁺-free Hanks (Sigma-Aldrich) followed by trypsin digestion at 37 °C. The trypsin digestion was terminated by washing with regular culture medium (see above). Droplets of dispersed islet cells were placed on cover glasses (diameter 13 mm) pre-coated with poly-L-lysine (Sigma-Aldrich). The cover glasses were kept in a 10 ml Petri dish and incubated for 30 min at 37 °C to allow the majority of the dispersed islet cells to attach to the cover glasses. Thereafter, 10 ml of culture medium was added to the Petri dish and the dispersed islet cells were cultured overnight.

The next day RPMI 1640 medium (Sigma-Aldrich) was changed for Krebs Ringer bicarbonate buffer (KRBH; 5.6 mmol/l glucose) with 200 µmol/l pimonidazole (Hypoxiaprobe-1; Chemicon International, Temecula, CA). Dispersed islet cells from each animal (n=4) were incubated at 37 °C for 4 hours in air tight chambers containing the different gas mixtures as given in table 4.

Table 4. Gas mixtures with different oxygen concentrations and corresponding pO₂.

<table>
<thead>
<tr>
<th>Gas mixture</th>
<th>pO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50±0.01%O₂/95%N₂/5.0±0.1%CO₂</td>
<td>~4</td>
</tr>
<tr>
<td>1.00±0.02%O₂/94%N₂/5.0±0.1%CO₂</td>
<td>~8</td>
</tr>
<tr>
<td>1.50±0.03%O₂/93.5%N₂/5.0±0.1%CO₂</td>
<td>~12</td>
</tr>
<tr>
<td>2.00±0.04%O₂/93%N₂/5.0±0.1%CO₂</td>
<td>~16</td>
</tr>
</tbody>
</table>

To assure complete equilibrium between the chamber’s gas mixture and the incubation medium, the KRBH was pre-gassed for 10 minutes with the corresponding gas mixture, and the incubation time was doubled compared to standard in vitro experiments. All oxygen gas mixtures were produced by a certified manufacturer (Air Liquid Gas, Stockholm, Sweden) and the oxygen level within the air tight chambers was continuously monitored with an oxygen sensor (Dräger PAC III, Lübeck, Germany). The drift of the oxygen sensor was <0.05% hour. After incubation at different oxygen pressures, the dispersed islet cells were fixed in 4% paraformaldehyde for 5 min, washed in phosphate buffered saline (PBS; pH 7.4) and immunostained for pimonidazole. Due to practical reasons these cover slides were stained for pimonidazole manually. The manual staining protocol differed from the automatized protocol (described below) by permeabilizing cells with 0.2% Triton X for 10 min prior to antigen retrieval and incubation with the anti-pimonidazole monoclonal antibody (1:50) overnight at 4°C. In control
experiments, the manual and automatic staining procedures produced similar results on tissue sections.

Moreover, islet cell viability after incubation at 0.5%, 1.0%, 1.5% or 2.0% O₂ was evaluated by stainings with propidium iodide (Sigma-Aldrich; 20µg/ml) and bisbenzimide (Sigma-Aldrich; 5mg/ml). Captures of stained cells in a fluorescence microscope were processed in a computerized system for morphometry (ImageJ 1.3v). Total numbers of cells (nuclei stained with bisbenzimide) and dead cells (cytoplasm stained with propidium iodide) were counted separately, followed by calculations of the percentage of vital cells.

**Induction of decreased oxygenation in vitro (IV)**

Culture medium was removed after 1, 5 or 10 days of culture. Whereas normal amounts of medium (5 ml) were added to half of the culture dishes with islets, 15 ml medium were added to the remaining. After 24h of additional culture, all culture dishes were incubated, in the presence of 200 µmol/l pimonidazole (Chemicon International), at 95% air/5% CO₂ for 2 hours. The rationale for this experimental set-up was that excess non-stirred culture medium markedly increases the diffusion distance of oxygen from air to the pancreatic islets and, thus, decreases the oxygenation of isolated pancreatic islets with resulting increased frequency of central islet necrosis (4).

**Induction of decreased tissue oxygenation in vivo (IV)**

The animals were anesthetized with thiobutabarbitral sodium (120 mg/kg body weight i.p.; Inactin; Research Biochemicals International), placed on an operating table maintained at body temperature (37°C) and tracheostomized. Polyethylene catheters were inserted into the right femoral artery and vein. The arterial catheter was connected to a blood pressure transducer (PDCR 75; Groby) to monitor blood pressure. The abdominal cavity of the animals was opened by a midline incision and the pancreas was exposed and its surface islets visualized, as previously described (33). Tissue oxygen tension in superficial pancreatic islets was measured with modified Clark microelectrodes (37). Islet oxygen tension and femoral artery blood pressure were then continuously recorded during the application of a graded aortic vascular clamp superior to the coeliac artery. The vascular clamp was positioned to allow a femoral arterial blood pressure of ~20 mmHg and an islet oxygen tension of 5-10 mmHg for 2 hours, meanwhile pimonidazole (Chemicon International) injected intravenously at the start of the vascular clamp was allowed to bind to low oxygenated regions. Blood pressure, islet
oxygen tension and body temperature were continuously recorded during the experiment with a MacLab Instrument (AD instruments, Hastings, UK).

Measurements of oxygenation by means of pimonidazole (IV-V)

Islet oxygenation was studied in cultured islets, the endogenous pancreas of non-transplanted rats and mice (IV-V), partially pancreatectomized rats (IV), whole-pancreas transplanted rats (IV) and in intraportally transplanted mice (V). Pimonidazole (Chemicon International) was either added to the culture medium (200 µmol/l) or injected intravenously into the tail vein of the animals (60 mg/kg body weight). Two hours later, islets were retrieved from culture or the animals were killed and their pancreas/pancreata and liver dissected out. The tissues were fixed in formalin overnight, and prepared for immunohistochemical staining for pimonidazole (see below).

Immunohistochemical staining for pimonidazole (IV-V)

Isolated islets were fixed in 4% paraformaldehyde overnight, dehydrated in ethanol and embedded in paraffin. Pancreata and livers were fixed in 10% (vol/vol) neutral buffered formalin overnight, washed in water, dehydrated in ethanol and embedded in paraffin. Sections, 5 µm thick, were produced and mounted on poly-L-lysine-treated glass slides. In paper IV an automatized immunostaining procedure (Dako Autostainer; Dako) was used, whereas in paper V the immunostaining procedure was manual. The staining protocols only differed in the incubation time of the primary antibody (see below). The slides were deparaffinized, washed and stained with antibody for pimonidazole. Antigens were retrieved from the sections by incubation of the glass slides with 0.01% (wt/vol) pronase (Dako) for 40 min at 40°C. The slides were then washed with TBS, (pH 7.6; Dako) for 2x5 min at 4°C. The slides were incubated with Dako blocking solution (Dako) for 30 min and incubated with primary antibody against pimonidazole (Chemicon International), diluted 1:25 in TBS with 0.2% (wt/vol) Brij 35, for 40 min (Dako autostainer) or diluted 1:50 in TBS with 0.2% (wt/vol) Brij 35, overnight at 4°C (manual staining procedure). Thereafter, the slides were washed in TBS and incubated with a biotinylated secondary goat F(ab')2 anti-mouse antibody (Southern Biotechnology, Birmingham, AL), diluted 1:500 in TBS with 0.2% Brij 35 and 0.035% (vol/vol) Dako blocking solution (Dako) for 30 min. The slides were again washed and incubated with Alkaline Phosphatase Standard ABC kit (Vector laboratories) for 30 min. The sections were washed in TBS and incubated with Vector Red
Alkaline Phosphatase substrate kit (Vector laboratories) for 25 min, followed by counterstaining with hematoxylin. Liver tissue was used as positive control slides (8), whereas animals not given an intravenous injection of pimonidazole were used as negative controls.

Light microscopical evaluation of pimonidazole stainings (IV-V)

The fraction of islets in the endogenous pancreas (IV-V), transplanted whole-pancreas (IV), remaining pancreas after 60% pancreatectomy (IV) and intraportally transplanted islets (V) staining positive for pimonidazole was evaluated under a light microscope (magnification 200X) by an examiner unaware of the origin of the samples. Small transplanted islet sections were difficult to discern in the liver parenchyma. Therefore, only islet sections with a diameter exceeding 20 µm were evaluated, both in the endogenous pancreas and in the liver following transplantation (V).

Islet oxygenation and protein biosynthesis in vivo (IV)

A previous study protocol for 3H-L-Leucine autoradiography (147) was modified to fit this study. Briefly, pimonidazole (60 mg/kg) was injected into the tail vein of awake rats. After 90 min the same rats were given an intravenous injection of 25 µCi 3,4,5-3H-L-Leucine (American Radiolabeled Chemicals, St. Louis, MO; 1 mCi/ml) dissolved in saline. The animals were killed 30 min later and their pancreata and livers were retrieved for histological evaluation of both pimonidazole incorporation and 3H-L-Leucine autoradiography. Tissue sections were first immunostained for pimonidazole (described above). Thereafter, wet slides were dipped in 50% autoradiography film emulsion (Eastman Kodak Co, Rochester, NY), and dried over night in a light-proof chamber. The slides were kept for 1 week at 4 °C before being developed, fixed and counterstained with hematoxylin. In a light microscope (1000X) digital pictures were made of both islets positive and negative for pimonidazole. An examiner unaware of the origin of the samples counted the number of autoradiographic silver grains in pimonidazole-positive and -negative islets, respectively. In each animal >0.02 mm$^2$ of islet tissue was evaluated using a computerized system for morphometry (ImageJ 1.3v). Leucine-dependent protein biosynthesis was then expressed as silver grains per mm$^2$ islet tissue.
Insulin, VEGF and bFGF content in isolated rat islets (II)

Groups of 125 freshly isolated or cultured rat islets (5 days of culture) were placed in 500 µl Hanks and sonicated. Homogenates were then stored at -70°C until analysis. The insulin content of the homogenates was measured with a rat insulin ELISA (Mercodia, Uppsala, Sweden), whereas the VEGF and bFGF contents were analyzed with a mouse VEGF ELISA and human bFGF ELISA, respectively (R&D Systems, Minneapolis, MN). Amino acid homology >90% between species is recommended by the manufacturer for their ELISAs to ascertain specific cross-reactivity. The amino acid homology for rat and mouse VEGF is 98%, and for rat and human bFGF 95.5%.

Insulin, VEGF and bFGF content in rat islet grafts (I-II)

Islet grafts were dissected free from the surrounding renal parenchyma, placed in 1 ml acid ethanol (0.18 mol/l HCl in 70% vol/vol ethanol) and sonicated to disrupt the islet cells. The samples were left to extract overnight at 4°C, and then stored in a freezer until analysis. The insulin content of the homogenates was measured with a radioimmunological assay (paper I; ICN ImmunoBiologicals, Lisle, IL) or a rat insulin ELISA (paper II; Mercodia). The VEGF and bFGF contents were analyzed with a mouse VEGF ELISA and human bFGF ELISA, respectively (R&D Systems).

Measurements of islet VEGF release (III)

200 islets were cultured in 5 ml DMEM, with or without addition of MMP-9, for 24 hours. Thereafter, the culture medium was stored at -20 °C until analysis of VEGF content by means of a mouse VEGF ELISA (R&D Systems).

Total RNA extraction and cDNA synthesis (III)

Ultraspec™ RNA Isolation System (Biotexx Laboratories, Houston, TX) was used to extract total RNA from islets cultured for 5-7 days with or without addition of VEGF (20 ng/ml). Briefly, approximately 150 islets were homogenized in 200 µl Ultraspec™, 40 µl chloroform was added and the samples were shaken vigorously. The homogenates were centrifuged at 4°C and the aqueous phase containing RNA was transferred to a new tube. The
RNA was precipitated by isopropanol, washed in 70% ethanol, and vacuum-dried to remove all liquid. For cDNA synthesis 0.5 µg total RNA was incubated with random primers (Promega, Madison, WI) at 70°C for 5 min. After adding M-MuLV RNA H’ Reverse Transcriptase (Finnzymes, Espoo, Finland), deoxy-nucleotides (Promega) and RNase inhibitor (Amersham Biosciences, Piscataway, NY) reverse transcription was performed at 37°C for 60 min. The reverse transcriptase was heat inactivated at 95°C for 5 min and the samples stored at -20°C until polymerase chain reaction (PCR) measurements.

Real-time PCR measurements (III)

Primers were designed to amplify specific regions of mouse thrombospondin-1, vasohibin, VEGF and MMP-9 (Table 5). PCR amplifications were carried out in a real-time LightCycler® (Roche Diagnostics, Mannheim, Germany), using the DyNAmed™ Capillary SYBR®-Green qPCR kit (Finnzymes). To obtain relative mRNA expression values, PCRs were performed for β-actin and TATA-binding protein for each of the experimental groups. The amount of PCR product relative to these housekeeping genes was calculated comparing the differences in crossing point (Cp) values according to the formula $2^{-\left(Cp_{\text{sample}} - Cp_{\beta-\text{actin/TATA-binding protein}}\right)}$. All PCR products were verified by agarose gel electrophoresis and SYBR Gold (Molecular Probes, Eugene, OK) staining.

Table 5. Primer pairs used for real-time PCR measurements of stimulators and inhibitors of angiogenesis in cultured pancreatic islets.

<table>
<thead>
<tr>
<th>Primer Pairs</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5’-GCTCTGGCTCCTAGCACC-3’</td>
<td>5’-CCACGATCCACAGAGTACTTG-3’</td>
</tr>
<tr>
<td>TATA-binding protein</td>
<td>TATA-binding protein</td>
<td></td>
</tr>
<tr>
<td>5’-ACCCTCCAAATGACTCTATG-3’</td>
<td>5’-ATGATGACTGACCAATCG-3’</td>
<td></td>
</tr>
<tr>
<td>Thrombospondin-1</td>
<td>Thrombospondin-1</td>
<td></td>
</tr>
<tr>
<td>5’-GGAACGGAAAGACAACACTG-3’</td>
<td>5’-AGTTGAGCCTGCCAGTTG-3’</td>
<td></td>
</tr>
<tr>
<td>Vasohibin</td>
<td>Vasohibin</td>
<td></td>
</tr>
<tr>
<td>5’-AGATCCCCATACAAATGTTG-3’</td>
<td>5’-GAGCCTCTTTTGGTCATTTCC-3’</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>VEGF</td>
<td></td>
</tr>
<tr>
<td>5’-ACTTGCTGAGATGCAAGCC-3’</td>
<td>5’-GTGGTGACATGGTAAATC-3’</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>MMP-9</td>
<td></td>
</tr>
<tr>
<td>5’-CCTACTCTGGCTGCCAATAAA-3’</td>
<td>5’-CTGCTTGCCCCAGGAAAGC-3’</td>
<td></td>
</tr>
</tbody>
</table>
Analysis of blood glucose concentration (I-V)

Glucose reagent strips were used to measure blood glucose concentration in venous blood obtained from the cut tip of the tail (MediSense; Baxter Travenol, Deerfield, IL). Blood glucose values above the detection range were set to 27.4 mmol/l, which is the highest reliable blood glucose concentration evaluated for this device.

Statistical analysis (I-V)

Values are expressed as the means±SEM. Probabilities (P) of chance differences between two experimental groups were calculated using Student’s unpaired or paired two-tailed \( t \)-test. Multiple comparisons between data were performed using analysis of variance (ANOVA) and Fisher's protected least significant difference or Bonferroni’s \textit{post-hoc} test. For all comparisons, P-values <0.05 were considered statistically significant.
Results and Discussion

Validity of blood flow measurements by laser-Doppler flowmetry

Laser-Doppler flowmetry is a simple and well evaluated method for measuring blood flow. It has been proposed that there is a linear correlation between real blood flow and laser-Doppler flowmetry values (201, 218). In view of this, we aimed to estimate our renal laser-Doppler flowmetry values to blood flow estimates expressed as ml x g tissue\(^{-1}\) x min\(^{-1}\) (paper I). For this purpose, we correlated renal cortical blood flow measured by laser-Doppler flowmetry to blood flow measured by a non-radioactive microsphere technique. Indeed, there was a linear correlation between renal cortical blood flow obtained by laser-Doppler flowmetry and by a microsphere technique (P<0.01, r=0.94, n=5 animals).

Thus, in normo- and hyperglycemic animals the renal cortical blood flow obtained by laser-Doppler flowmetry was estimated to be 9 ml x g tissue\(^{-1}\) x min\(^{-1}\). Likewise, the blood flow of the islet graft was estimated to be 4 ml x g tissue\(^{-1}\) x min\(^{-1}\) in normoglycemic recipients and 6 ml x g tissue\(^{-1}\) x min\(^{-1}\) in hyperglycemic recipients. These values are slightly higher than those obtained in previous studies in this animal model, using a non-radioactive microsphere technique (36). The discrepancy between blood flow values measured by laser-Doppler flowmetry or a microsphere technique is likely to be explained by inherent methodological differences. The laser-Doppler flow value represents the movement of all blood cells within the illuminated tissue, irrespective of blood vessel type. In contrast, the microsphere technique measures the blood flow in the smaller arterioles and capillaries, which is more likely to represent the nutritive blood flow responsible for oxygen delivery. Methodological errors affecting the microsphere technique, such as influence of the anatomical organization of the vasculature causing steric restriction and underestimation of blood flows, or uneven distribution of microspheres (28, 176) are, however, avoided with laser-Doppler flowmetry. Another advantage with laser-Doppler flowmetry is that the blood flows can be continuously measured, whereas the microsphere technique only provides a "snapshot" of the blood flow at a given time point.

To achieve reliable islet graft measurements by laser-Doppler flowmetry, the illuminated tissue must not include the renal tissue beneath the islet graft. Since a 250 islet graft is ~1.25 mm deep (37) and the laser beam of the
laser-Doppler flowmeter only penetrates ~0.5 mm in the renal cortex (222), it is most likely that we only measure islet graft blood flow. Moreover, similar laser-Doppler flowmetry values have been reported in a 1000 islet graft as in a 250 islet graft (42).

An important drawback with laser-Doppler flowmetry is, however, the background signal (biological zero), which can be estimated by arrest of the blood flow (227). This biological zero has previously been shown to arise from Brownian motion of macromolecules within the interstitium (121). In paper I, the biological zero was estimated to 3-4 TPU in both the islet graft and the adjacent renal cortex of all animals. It seems therefore unlikely, that differences in the background signal may interfere with our results.

Validity of oxygen measurements by modified Clark microelectrodes

In several previous studies modified Clark microelectrodes have been used to measure the oxygen tension of endogenous and transplanted islets (37, 40, 42, 47). When polarographic microelectrodes are used to measure oxygen tension in tissues, it has been reported that errors in these measurements to a large extent are caused by the size of the electrode tip. Large electrodes (tip diameter >10 µm) damage tissues and compress capillaries, thereby decreasing the tissue oxygen tension (3). Large electrodes also have a high diffusion current, which results in a high oxygen consumption (217). The modified Clark microelectrodes used in the present studies have previously been thoroughly evaluated for in vivo measurements of tissue oxygen tension in the kidney (145), and in endogenous as well as transplanted islets (34). The small tip diameter of these modified Clark microelectrodes (outer diameter 2-6 µm) minimizes tissue damage and compression. Moreover, a major advantage with these electrodes is that all oxygen diffusing towards the sensing cathode is removed from the internal electrolyte reservoir by the guard cathode, placed 200-600 µm behind the sensory cathode. This enables accurate recording of the oxygen tension in the surrounding tissues.

It should be noted that the tissue oxygen tension of endogenous islets could only be recorded in surface islets and that neutral red was used for their visualization. This stain has been shown to not interfere with glucose homeostasis or the oxygen tension of the exocrine pancreas (34). Thus, it is highly unlikely that this dye affects the measurements of oxygen tension in endogenous islets.
Validity of endothelial cell stainings by means of the lectin *Bandeiraea simplicifolia*

Endothelial cells are a heterogeneous population of cells, and their expression of cell type markers differ largely between different organs (78). The lectin *Bandeiraea simplicifolia* (BS-1) binds to D-galactosyl residues on endothelial cells (130). Recently, BS-1 has been shown to be a reliable marker of endothelial cells in both endogenous and transplanted pancreatic islets (156). In the present studies (II-III), we used the lectin *Bandeiraea simplicifolia* (BS-1) for specific stainings of endothelial cells in cultured, endogenous and transplanted pancreatic islets. This enabled us to perform reproducible calculations of the vascular density in histological tissue sections.

Validity of 2-nitroimidazoles as markers of low oxygenation

The bioreductive metabolism of 2-nitroimidazoles provides a way of labeling low oxygenated cells *in vivo* (93). The 2-nitroimidazoles chemically belong to nitro-aromatic compounds, which seldom occur naturally. However, most organisms including mammals can metabolize 2-nitroreductases, using nitroreductase enzymes to reduce the nitro-group. Oxygen inhibits binding by competing for the electrons in the first reductive step (Figure 2). Normally, oxygen readily reverses the first step of reaction, regenerating the parent compound. The nitro-radical anion, produced in the first one-electron reduction step, oxidizes back to the parent nitroimidazole so efficiently in normoxic cells that there is no substrate for the second reduction (151, 236). However, at low oxygen tension levels (pO₂<10 mmHg) the bioreductive metabolism of 2-nitroimidazoles proceeds as a series of one-electron reductions to the nitroso (2 e-), hydroxylamine (4e-) and amine (6e-) derivatives, eventually generating reactive adducts that covalently bind to macromolecules, such as glutathione in the cells (161, 237). The 2-nitroimidazole-derived side chains can thereafter be recognized by antibodies (197), using techniques such as immunohistochemistry and ELISA, to assess tissues/cells low in oxygen tension.
Bioreductive metabolism and accumulation of 2-nitroimidazole adducts in low oxygenated tissue. 2-nitroimidazoles such as pimonidazole (1) are reductively activated by nitroreductases, forming a nitro radical anion (2). Normally oxygen reverses the formation of the nitro radical anion after the first electron transfer, regenerating the parent compound. Full reduction therefore only occurs in cells with low oxygenation (i.e. pO$_2$<10 mmHg). Under these conditions, the nitro radical anion of pimonidazole is further reduced to a hydroxylamine intermediate (3). This intermediate binds to –SH-containing molecules, such as glutathione and other intracellular proteins (4), and these adducts accumulate in the cell. Modified from reference 161.

In mammals, there are several nitroreductase enzymes present within the cytoplasm, mitochondria and microsomes. These enzymes include aldehyde oxidase, DT-diaphorase and xanthine oxidase in the cytoplasm, NADPH-cytochrome reductase, and cytochrome P-450 in the microsomes and the mitochondrial enzymes dihydrolipoamide dehydrogenase, and succinate dehydrogenase (93). The 2-nitroimidazoles do not accumulate in dead or necrotic cells, since this process requires functional nitroreductase activity (199). In contrast to other nitroreductases, DT-diaphorase is capable to reduce 2-nitroimidazoles in 2-electron steps in an oxygen-insensitive process. Thus, it has been suggested that accumulation of 2-nitroimidazoles in some tissues may be oxygen-insensitive. However, a 1000-fold overexpression of DT-diaphorase produced a very small increase in the rate of 2-nitroimidazole binding in monkey kidney cells (112). Furthermore, this study overexpressed cytochrome P-450 reductase 80-fold, which produced a 5-7-fold increase in the rate of 2-nitroimidazole binding. Despite this finding, the 2-nitroimidazole binding rate is still considered relatively insensitive to cytochrome P450 reductase concentration (93), which suggests...
that variations in P450 reductase concentration in tissues may be much less important than oxygenation status in determining the rate of reduction.

We used the commercially available 2-nitroimidazole, pimonidazole (Hypoxiaprobe-1; Chemicon International), for immunohistochemical detection of pimonidazole-accumulation (IV-V). Pimonidazole is non-toxic and it is rapidly taken up by all tissues, including brain (93). The piperidine side-chain of pimonidazole is positively charged leading to a 3-fold higher intracellular than extracellular concentration at physiological pH (63). Oxygen dependent bioreductive metabolism of pimonidazole has been reported in several tissues of both humans (7, 235) and laboratory animals (58, 77, 250). Most studies have been performed on tumor tissue, since low tumor oxygenation is associated with resistance to radiotherapy (184). In tumors pimonidazole has been shown to accumulate at pO\textsubscript{2} less than 10 mmHg (198). Most studies on normal tissues such as the pancreas only report an oxygen dependent accumulation of pimonidazole without investigating the threshold for pimonidazole accumulation (161). Hyperglycemia may induce intracellular levels of NADPH, which theoretically can induce bioreductive metabolism of 2-nitroimidazoles (142). However, in the liver the reductive metabolism of pimonidazole has been shown to be independent of the NADPH levels (9). Since this problem has not been addressed in islets, we chose to perform all experiments at normoglycemia.

We determined the threshold for pimonidazole-accumulation in dispersed islet cells to exclude problems with oxygen tension gradients within islets. The cell viability of the dispersed islet cells was similar (~95%) at all oxygen tension levels (0.5-21\% \text{O}_2) tested. In dispersed islet cells accumulation of pimonidazole was induced at pO\textsubscript{2} less than 10 mmHg. When investigated in whole islets during culture, preferentially larger islets displayed a pronounced gradient of pimonidazole- staining towards the islet core with unstained islet periphery. In some cases, larger islets investigated \textit{in vitro} also had central necrosis surrounded by pimonidazole-staining. The necrotic islet core did not stain, because accumulation of 2-nitroimidazoles requires functional nitroreductase activity, which is absent in dead cells (199). The capacity of islet cells to incorporate pimonidazole after induced hypoxia \textit{in vitro} and \textit{in vivo} was also corroborated in control experiments.

Validity of measurements of islet protein biosynthesis

We modified a previous study protocol for measurements of protein biosynthesis by means of \textsuperscript{3}H-L-Leucine autoradiography (147). This enabled us to assess islet protein biosynthesis \textit{in vivo}, including (pro)insulin. The \textsuperscript{3}H-L-Leucine in plasma was observed to be cleared by 70\% within 10 minutes, which suggests that \textsuperscript{3}H-L-Leucine is rapidly taken up by the tissues. The
pancreata were retrieved 30 minutes after injection $^3$H-L-Leucine, fixed in formalin and prepared for autoradiographic measurements. Thus, the majority of $^3$H-L-Leucine detected by autoradiography in the islets was incorporated intracellularly. Moreover, the incorporation of $^3$H-L-Leucine did not merely reflect differences in blood flow, since injections of either 25 µCi or 50 µCi $^3$H-L-Leucine gave similar values for protein biosynthesis in both pimonidazole-positive and -negative islets.

Blood flow regulation in islet grafts (I)

In paper I, we investigated the blood flow regulation in renal subcapsular islet grafts of both normo- and hyperglycemic recipients. One month post-transplantation, by means of laser-Doppler flowmetry, the islet graft blood flow response to intravenous administrations of adenosine, AT II and L-NNA was monitored.

The vasoactive substance adenosine is locally produced during augmented metabolism with ATP-depletion (29). In most tissues adenosine increases blood flow, but in some cases such as the renal cortex both decreased (65, 193) and increased (68) blood flow values have been reported. In endogenous islets, adenosine does not contribute to basal islet blood flow, but it is an important mediator of glucose-induced islet blood flow increase (46). The initial islet blood flow increase at 3 min after a glucose challenge is regulated by the vagus nerve, whereas after 10 and 20 min both the vagus nerve and adenosine contribute to the increased islet blood flow. Moreover, the adenosine mediated vasodilatation seems to be independent of NO and is mediated by A1 receptors (46). During infusion of adenosine the islet graft blood flow was unaffected in both normoglycemic and hyperglycemic animals. However, adenosine reduced the mean arterial blood pressure by ~45% in both groups. This means that the vascular conductance of the islet grafts was markedly increased by adenosine similar to that observed in endogenous islets (46).

An intrinsic angiotensin system has been described in the native pancreas (41, 138). AT II regulates pancreatic blood flow (35), and has also been observed to decrease islet (pro)insulin biosynthesis as well as insulin secretion (136). We observed that AT II decreased blood flow and vascular conductance in both the islet graft and renal cortex of diabetic animals. In non-diabetic animals, neither the islet graft blood flow nor the renal cortex blood flow was significantly decreased (P<0.10). However, the vascular conductance of both the islet graft and renal cortex was decreased in these animals. Most interestingly, in a previous study on rats, a similar concentration of AT II decreased both whole pancreatic blood flow and vascular conductance, whereas islet blood flow and vascular conductance were unaffected (35). Thus, it seems as if the blood vessels in the islet graft
are more sensitive to AT II than those of the endogenous islet. This is also supported by recent data. In an elegant study, Lau and co-workers have shown that the key receptor for AT II, the AT₁-receptor, is upregulated in islets implanted into the subcapsular space of the kidney (136). Furthermore, systemic treatment with losartan, an AT₁-receptor antagonist, improved both blood flow and oxygenation in the islet grafts, which resulted in improved first phase glucose-stimulated insulin release (117).

We also report that both the blood flow and vascular conductance of the renal cortex were further decreased by AT II in diabetic compared to non-diabetic animals. This is in line with the previously reported enhanced AT II induced vasoconstriction in the kidney, which is commonly seen in diabetic subjects (70, 177). In contrast, the islet graft microvessels did not seem to acquire hypersensitivity to AT II, since the fractional decrease in islet graft blood flow in hyperglycemic animals was similar to that in normoglycemic animals during infusion with AT II. Moreover, this finding may suggest that the influence of AT II on islet graft blood flow, at least in the hyperglycemic state, is independent of the implantation organ.

NO is produced locally by endothelial cells and it increases blood flow in the native islets (225) as well as in the kidneys (234). There are also experimental data that suggest that the vagal increase in islet blood flow is mediated by NO (171). Considering the volatile properties of NO, we chose to study the effects of NO indirectly. For this purpose, an irreversible inhibitor of both inducible and constitutive NOS (L-NNA) was administered to the animals (76). Thus, we did not investigate the direct effects of NO, but rather those induced by the absence of NO in the tissues. After administration of L-NNA, we observed a decrease in blood flow and vascular conductance in both the islet graft and renal cortex of normoglycemic animals, which was of the same order of magnitude as that previously reported in native islets (225). However, the corresponding values in diabetic animals were not affected to the same degree. Moreover, the mean arterial blood pressure differed between normo- and hyperglycemic animals after injection with L-NNA. The normoglycemic recipients increased their mean arterial blood pressure to 150 mmHg, whereas hyperglycemic animals only increased their mean arterial blood pressure to 130 mmHg. This difference in mean arterial blood pressure was not statistically significant (p<0.10), but the combined systemic and the local effects (i.e. in the renal cortex and islet graft) of L-NNA in our study suggest that diabetic animals are not as dependent on NO as non-diabetic animals for their blood flow and blood pressure regulation. This is consistent with previous studies, where a dysfunction of the NO-dependent vasodilatation has been observed in diabetic subjects (194, 230). Most interestingly, in view of the present results, this also seems to affect the islet graft circulation in diabetic animals, which is less dependent on NO to maintain its basal blood flow than islet grafts of non-diabetic animals. Since the dysfunction of
NO-dependent vasodilatation is a diabetic phenomenon, it seems likely that the diminished effect of NO inhibition in the islet grafts of diabetic recipients is independent of the implantation organ.

Influence of donor islet properties on revascularization and islet graft function (II-III)

The islet isolation process disrupts all islet vascular connections and during islet culture preceding transplantation the donor islet endothelial cells dedifferentiate or die (149, 180, 190). Thus, a decade ago, transplanted islets were considered to be revascularized exclusively from the host (232). However, recently it was shown that donor endothelium is important in islet graft revascularization (24, 143, 180). Firstly, Linn and co-workers observed that 33% of the endothelial cells incorporated in the islet graft microvessels were of donor origin (143). Shortly thereafter, Brissova et al reproduced these data and, most elegantly, also showed that these donor derived microvessels were perfused, and not merely represented remnant donor cells (24).

In view of these findings, we used syngeneic rodent models to investigate whether freshly isolated islets become better revascularized than cultured islets after transplantation (paper II). The rationale of this investigation was that the increased numbers of donor endothelial cells in freshly isolated islets might facilitate revascularization and improve function. Noteworthy, the most successful clinical islet transplantation protocol, the Edmonton Protocol, uses freshly isolated islets instead of cultured islets for transplantation. Moreover, we hypothesized that islet culture with stimulators of angiogenesis (VEGF, FGF-2) may preserve or expand islet endothelial cells. If successful, these endothelial cells could thereafter contribute to the revascularization process and maybe improve function following transplantation (paper III).

Indeed, transplantation of freshly isolated islets improved vascular density and tissue oxygen tension as evaluated one month post-transplantation. The tissue oxygen tension in islet grafts composed of freshly isolated islets was twice that in islets transplanted after culture and this was associated with improved function in a minimal mass model. All animals transplanted with freshly isolated islets were cured, whereas only 33% in the control group returned to normoglycemia. Notably, already four days post-transplantation there was a tendency to an increased oxygen tension in grafts composed of freshly isolated islets (P=0.09). However, the blood perfusion of rat islet grafts composed of freshly isolated and cultured islets differed neither four days nor one month post-transplantation. The likely explanation to this is that the blood flow measured by laser-Doppler flowmetry represents not
only the nutritive blood flow to the endocrine cells, but total blood perfusion, i.e. all moving blood cells within the illuminated tissue. A large number of the intra-graft capillaries was stroma capillaries (155), which therefore are likely to contribute to total graft blood perfusion substantially. In contrast, it could be expected that the blood flow in capillaries in the endocrine parts mainly contributes to the delivery of oxygen to the endocrine cells (66). Indeed, one-month-old grafts composed of freshly isolated islets had a 70% higher islet vascular density than corresponding grafts of cultured rat islets. Moreover, the oxygen tension measurements suggested that the higher number of blood vessels in the endocrine parts of one-month-old grafts of freshly isolated rat islets not merely were explained by remnant donor endothelial cells, but also by functional blood-perfused capillaries. In contrast to our results, a previous histological study in rat (163) has suggested that transplanted cultured islets may become revascularized more slowly than freshly isolated islets, but that all islets become fully revascularized within a week. Mendola and co-workers did, however, not mention whether endocrine and connective tissue parts were evaluated separately (163).

We measured the concentrations of the potent angiogenic factors VEGF and FGF-2 in freshly isolated and cultured rat islets, as well as in four-day-old and one-month-old rat islet grafts, in an attempt to explain our findings. We observed, however, similar VEGF content in islet grafts composed of freshly isolated or cultured rat islets when investigated during active angiogenesis four days after transplantation. Thus, differences in VEGF production seem unlikely to explain the better revascularization of islets transplanted immediately after isolation. This is also indirectly supported by results from a previous study, where in vivo blockage of VEGF receptor 2 did not impair the revascularization of the transplanted islets (212). On the contrary, induction of VEGF hyperexpression in islets prior to transplantation through gene transfer has in several studies been shown to improve revascularization and graft function (129, 249). This proposes that isolated islets normally are incapable of producing enough VEGF for their revascularization and survival post-transplantation.

Likewise, the production of FGF-2 in four-day-old rat islet grafts was similar in grafts composed of freshly isolated and cultured islets. One month-old rat islet grafts had a higher production of FGF-2 than four-day-old islet grafts. There was also a tendency (P=0.07) to higher FGF-2 content in the one-month-old-grafts composed of freshly isolated rat islets than that of the cultured islets. This may have contributed to the better revascularization of transplanted freshly isolated islets, since newly formed microvessels are known to be unstable (96) and FGF-2 has been reported to improve blood vessel stability (55).

Despite reports of increased cell viability (219) and vessel stability (55) after stimulation with VEGF or FGF-2, islets cultured with addition of
VEGF (20ng/ml) or FGF-2 (20 ng/ml) for 5-7 days lost their vascular structures similar to controls. One month post-transplantation the vascular density, blood flow and tissue oxygen tension were all decreased in VEGF pretreated islets grafts, whereas FGF-2 pretreated islets were revascularized similar to controls. In order to find an explanation for the impaired revascularization of VEGF pretreated islets, we measured the expression of some stimulators and inhibitors of angiogenesis in isolated islets exposed to VEGF and compared to controls. Interestingly, the expression of MMP-9 was reduced by 50% in islets pretreated with VEGF for 5-7 days. MMP-9 is a pro-angiogenic protease, which is produced by cells such as macrophages and endothelial cells. The main feature of MMP-9 in revascularization is tissue re-modeling, but it has also been shown that MMP-9 turns normal islets angiogenic by releasing matrix bound VEGF (14).

Thus, we also pretreated islets with MMP-9 (1µg/ml) for 24 hours prior to transplantation. One month post-transplantation, the vascular density was improved by 75%, whereas the blood flow and tissue oxygen tension were increased by approximately 100%, suggesting improved vascular engraftment. However, in a minimal islet mass model VEGF or MMP-9 pretreated islets cured similar number of animals as control islets. Animals receiving MMP-9 pretreated islets even lowered their blood glucose at a slower rate than controls in the minimal islet mass model and responded slightly slower than controls to a glucose challenge. In acute and chronic pancreatitis MMP-9 has been suggested to be diabetogenic, since it degrades insulin (64). This property of MMP-9 might have delayed the islet graft function, thereby overexposing these islets to glucose toxicity in the minimal islet mass model. Moreover, since MMP-9 is a protease, it is likely that it may be harmful to islets per se. In this case, MMP-9 treatment may have resulted in a reduced viable islet mass at transplantation. When considering the low vascularization of grafts composed of VEGF pretreated islets, these grafts did better than expected in the minimal islet mass model. This may be explained by previously reported anti-apoptotic effects of VEGF (219). Nevertheless, the present data strongly imply that islets do not benefit from VEGF treatment prior to transplantation.

The formation of a highly vascularized stroma in islet grafts is probably due to growth factors secreted by the transplanted islets, whereas the reason(s) for the defect revascularization of the endocrine tissue per se remain(s) to be determined. However, some clues were provided in the study (paper II), where transplanted freshly isolated islets were more efficiently revascularized than transplanted cultured islets. This argues that remnant donor endothelial cells are indeed important in the revascularization process. It could be argued that the remaining microvessels in freshly isolated islets may serve as channels for the migration of new vessels, which may induce the process of revascularization. Remaining endothelial cells may also attract and become incorporated within the newly formed microvessels (24, 143.
Interestingly, when investigated in paper III the vascular density was similar in the one-month-old islet transplants to that in the cultured islets prior to transplantation, which proposes that these remnant vessel structures are important as scaffolds or pre-formed channels for new capillaries to grow into.

Furthermore, it should be noted that macrophages residing within the pancreatic islets are known to disappear during culture (190). Monocytes and tissular macrophages seem strongly involved in adult angiogenesis due to their local secretion of metalloproteinases, which causes the formation of capillary lumens through local tunneling in the parenchyma (169, 170). Indeed, since both freshly isolated and MMP-9 pretreated islets became better revascularized than control islets, this may suggest that macrophages are important also in islet graft revascularization.

Oxygenation and protein biosynthesis in endogenous islets (IV)

The blood perfusion of the endocrine pancreas is high and similar to that of the renal cortex (118). However, the blood perfusion of different islets seems to be extremely variable, since when blood flow is measured by a microsphere technique only ~10 % of the islets contain microspheres (107). The average islet blood flow has been estimated to 20 nl/min in the rat (97, 107), and in these experiments one microsphere represents ~375 nl/min. Thus, a reasonable interpretation is that the islets devoid of microspheres (~90%) have a blood perfusion below the detection limit, i.e. the blood flow each microsphere represents.

In paper IV, we hypothesized that the highly variable islet blood perfusion observed in the rodent pancreas may result in a heterogeneous islet oxygenation and reflect differences in islet function. Since only a few surface islets may be assessed by means of modified Clark microelectrodes, we had to adopt a new technique for measurements of the oxygenation of pancreatic islets irrespective of their size and location in the pancreatic gland. For this purpose we used the bioreductive metabolism of pimonidazole as a marker of low oxygenation (pO2<10 mmHg) in the endocrine pancreas.

Islet pimonidazole accumulation was studied in normal animals, in whole-pancreas transplanted animals and in 60% pancreatectomized animals. Most interestingly, in normal rats 20-25% of the pancreatic islets accumulated pimonidazole. This fraction of low oxygenated islets was similar in the pancreatic caput and cauda, respectively. Notably, in contrast to the investigated isolated islets (cf. above) pimonidazole-positive islets in vivo always displayed homogenous staining. This staining pattern argues
against any limitations in oxygen diffusion from the islet capillaries to distant islet cells. The homogenously pimonidazole-stained islets rather indicate vasoconstriction at the islet arteriolar level, resulting in a low blood perfusion of the whole islet. The islet blood perfusion is normally regulated at the islet arteriolar level (33) independently from the exocrine pancreas by a complex interplay between hormonal, neural and locally produced factors (105).

After whole-pancreas transplantation the fraction of pimonidazole-positive islets was doubled in the native pancreas of the transplanted animals, whereas the pancreas transplant had similar fraction of pimonidazole-positive islets as observed in control animals. Thus, increased islet mass due to decreased metabolic demands seems to recruit more islets to the low oxygenated subpopulation. Moreover, since the transplanted pancreas is denervated, it is most likely that this phenomenon is due to nervous down-regulation of the oxygen supply to the endogenous islets. One week after decreasing the islet mass to ~40% by partial pancreatectomy almost no islets stained positive for pimonidazole. This indicates that all islets were well-perfused and that all accessible islet function was needed to maintain normoglycemia in these animals. The mechanism for the recruitment of pimonidazole-positive islets is most likely to reflect local mediators or local nervous mechanisms and not the extrinsic nervous mechanism involved in the closing down of excess islets, since the transplanted denervated pancreas had similar fraction of pimonidazole-positive islets as normal animals.

Moreover, we hypothesized that the pimonidazole-positive islets would differ from other islets in their metabolic activity including protein biosynthesis. For this purpose, we modified a previously reported protocol for 3H-L-Leucine autoradiography to measure leucine-dependent protein biosynthesis, of which (pro)insulin biosynthesis normally accounts for approximately 25% in endogenous islets (147). Indeed, these experiments showed that the leucine-dependent protein biosynthesis was 50% higher in pimonidazole-negative compared to pimonidazole-positive islets. Since the pimonidazole-positive islets probably have a low blood perfusion, concerns were raised that their lower leucine-dependent protein biosynthesis merely resembled differences in blood flow. However, measurements of leucine-dependent protein biosynthesis seemed to be independent of blood flow, since variations in the 3H-leucine dose did not affect the recorded protein biosynthesis of neither pimonidazole-positive nor –negative islets.

The observed low degree of oxygenation is probably well sufficient to maintain basal functions and viability in islet cells, which we also confirmed in vitro. However, in vivo a low metabolic activity in such cells is probably necessary, since these islets otherwise would be restricted by their oxygen supply. This is unlikely, since endogenous islets have a tightly regulated blood flow (27, 105), where locally produced metabolic mediators such as
adenosine probably would dilate afferent arterioles in any case of anaerobic metabolism (46).

**Oxygenation of intraportally transplanted islets (V)**

After intraportal islet transplantation the islet vascular density is not restored (155). In previous studies of islet graft revascularization it has not been technically possible to measure tissue oxygen tension in intraportally transplanted islets, but only in liver subcapsular grafts (42). In paper V, we used pimonidazole to assess the oxygenation of intraportally transplanted syngeneic mouse islets. Consistent with our observation in rats there was a subpopulation of endogenous islets (approximately 30%) that stained positive for pimonidazole. One day after intraportal transplantation, when islets completely depended on oxygen diffusion for their survival, the fraction of islets with low oxygenated cells was doubled compared to that recorded in the pancreas of control animals. Despite that transplanted islets generally are considered to be fully revascularized within 14 days (165, 209) the percentage of low oxygenated islets remained similarly increased one month post-transplantation, i.e. approximately 70% of the transplanted islets had an oxygen tension less than 10 mmHg. These data indicate that intraportally transplanted islets normally may suffer from ischemia both before and after revascularization, and that formed blood vessels transport insufficient quantities of oxygen similarly to what previously has been reported for islets implanted into the renal subcapsular site (37, 40, 42).

We used normoglycemic recipients to avoid problems with proposed glucose dependent accumulation of pimonidazole (142). However, it is unlikely that the use of normoglycemic recipients confound the present findings in intraportally transplanted islets, since denervated transplanted islets are not capable of down-regulating their oxygenation (cf. whole-pancreas transplant). The low oxygenation of intraportally transplanted islet should therefore be caused by either high oxygen consumption or low oxygen delivery. The latter is the most likely case, since intraportally islets are known to have a decreased vascular network (155). Whereas low oxygenated endogenous islets regularly displayed a homogeneous staining pattern for pimonidazole, suggesting low blood perfusion of the entire islets, transplanted islets often displayed a more heterogeneous staining pattern. Especially one day post-transplantation, but also one month post-transplantation, islets with a pimonidazole-positive core and unstained periphery were common similarly to what was seen in isolated islets. Most likely, this reflects dysfunctional islet graft blood vessels causing the formation of oxygen consumption gradients from the surrounding tissue.

The functional activity of differently oxygenated intraportally transplanted islets has as yet not been determined. However, intraportally
transplanted islets retrieved for experimental investigations one month post-transplantation seem in general to have markedly decreased insulin content, nutrient stimulated insulin release and glucose oxidation rate, the latter suggesting mitochondrial dysfunction (158). This opens the possibility that treatments to improve oxygenation of intraportally transplanted islets may not only be beneficial in the immediate post-transplantation phase (94), but, as for renal subcapsular islet grafts, be important also at later time points after transplantation (113).
Conclusions

Paper I
- Islet graft blood vessels are responsive to stimulation by adenosine, angiotensin II, and nitric oxide.
- Recipient hyperglycemia results in a basal islet graft blood flow, which is less dependent on nitric oxide.

Paper II
- Immediate transplantation of isolated islets, without preceding culture, improves vascular engraftment and function.
- The improved vascular engraftment of freshly isolated islets is likely mainly due to angiogenic properties of remnant donor microvessels, endothelial cells, and/or macrophages.

Paper III
- Treatment with VEGF or FGF-2 during culture does not preserve intraislet vascular density.
- FGF-2 treatment during culture has no effect on islet graft revascularization.
- Treatment with VEGF during culture impairs islet graft revascularization. This may be due to a decreased production of MMP-9 in VEGF exposed islets, which makes these islets less angiogenic and results in a decreased vascular density, blood flow and oxygen tension compared to control grafts.
- Incubation of islets with MMP-9 prior to transplantation improves islets graft revascularization.
Paper IV

- The oxygenation of endogenous islets is greatly heterogeneous.
- Approximately 25 % of the islets in a normoglycemic rat constitutes a low oxygenated (pO$_2$<10 mmHg) subpopulation of islets.
- The size of this low oxygenated subpopulation is dependent on the islet mass and metabolic demand.
- The low oxygenated subpopulation of pancreatic islets has a decreased protein synthesis compared to islets with a higher oxygen tension.
- The low oxygenated subpopulation of endogenous islets may constitute a functional reserve of islet cells.

Paper V

- The oxygenation of intraportally transplanted pancreatic islets is heterogeneous.
- One day post-transplantation the low oxygenated subpopulation of transplanted pancreatic islets is doubled compared to endogenous islets.
- After revascularization of intraportally transplanted islets, i.e. one month post-transplantation, the fraction of low oxygenated transplanted islets remains high and is similar to that recorded one day post-transplantation.
- These findings suggest that intraportally transplanted islets have deficient oxygen supply.
Bakgrund


Transplantation av bukspottkörteln är en komplicerad operation med tämligen stor risk för kirurgiska komplikationer, medan transplantation av Langerhanska öar är ett mini-invasivt ingrepp där cellöarna sprutas in i levern.

Ett år efter transplantation av cellöar är 80% av patienterna botade, men fyra år senare är det bara 10% som inte återigen har fått börja med insulinbehandling. Det finns troligen flera orsaker till att de Langerhanska cellöarna dör efter transplantation, varav en kan vara bristande blodförsörjning. Inför transplantation isoleras cellöarna från resterande delar av bukspottkörteln, vilket medför att de helt saknar blodtillförsel när de sprutas in i levern. För cellöarnas tillförsel av syre och näringsämnen samt för distribution av insulin till kroppens övriga celler krävs att det återupprättas ett fungerande kärlsystem i cellöarna. Detta tycks dock inte ske och kronisk syrebrist skulle kunna bidra till att cellöarna dör några år efter transplantation.

De i avhandling ingående delarbetena studerar blodförsörjningen till transplanterade Langerhanska cellöar jämfört med de normala förhållandena i bukspottkörteln, med särskild tyngdvikt på syresättning och på metoder att förbättra densamma i transplanterade öar.

Avhandlingens delarbeten

I det första delarbetet studeras blodflödesregleringen i cellöar transplanterade under njurkapseln på normala respektive diabetiska råttor. Vi undersökte effekterna av blodflödesreglerande substanser med känd effekt hos cellöar i bukspottkörteln för att bedöma ”mognadsgrad” i transplanterats nybildade blodkärl. I likhet med endogena öar reglerades transplantatets blodflöde av adenosin (ökar blodflödet), angiotensin II (minskar blodflödet) och kväveoxid (ökar blodflödet). Känsligheten för angiotensin II var möjligvis större i transplantaten jämfört med tidigare rapporterade data för cellöar i bukspottkörteln. Hos diabetiska mottagare var transplantatets basala blodflöde något högre och dessutom mindre beroende av kväveoxid.

I tredje delarbetet försökte vi bevara alternativt expandera kärlstructurerna i odlade cellöar för att se om det skulle förbättra kärlnybildningen efter transplantation. För detta ändamål odlade vi cellöarna under 5-7 dagar med tillsats av tillväxtfaktorer med känd effekt på blodkärl (vascular endothelial growth factor; VEGF, fibroblast growth factor-2; FGF-2). Tyvärr bevarade denna behandling inte kärlstructurerna under cellodling. När vi sedan transplanterade öarna visade det sig att VEGF-stimulering under odling medförde en försämrad kärlnybildning, medan stimulering med FGF-2 inte påverkade kärlnybildningen. Studier av genuttrycket hos VEGF-stimulerade cellöar visade sänkt produktion av matrix metalloproteinase-9 (MMP-9). Denna substans är normalt aktiv vid kärlnybilning och vi behandlade därför cellöar med MMP-9 före transplantation. Förbehandling med MMP-9 resulterade i en förbättrad kärldensitet, högre blodflöde och högre syresättning i transplantaten. Detta antyder att MMP-9 har en central roll i kärlnybildningen i transplanterade cellöar.

Den vanligaste metoden för att mäta syrgastension i vävnad baseras på microelektoder. Cellöarna är små (25-300 µm i diameter) och utspridda i hela bukspottkörteln, vilket innebär att mikroelektroder endast kan mäta syresättningen i ett fåtal ytliga och stora öar. I fjärde delarbetet ämnade vi mäta syresättningen i alla cellöar oberoende deras lokalisation. Vi valde att använda oss av pimonidazole som är en markör för låg syresättning. Pimonidazole ansamlas i celler med låg syresättning (pO₂<10 mmHg), vilket sedan kan visualiseras i histologiska vävnadssnitt. Vi observerade att 20-25% av cellöarna i en normal råtta är lågt syresatta (pO₂<10 mmHg). Fraktionen av lågt syresatta cellöar ökade när vi gav rättorna ytterligare en bukspottkörtel (helpkreatransplantation) och minskade när vi tog bort drygt hälften av rättornas egen bukspottkörtel (pankreatektomi). Därför var den låga syresättningen associerad med en låg proteinproduktion i cellöarna. Dessa data antyder att det finns en lågt syresatt funktionell reserv av cellöar i bukspottkörteln.

Vid klinisk transplantation sprutas cellöar in i levern. Det har spekulerats i att cellöarna kan vara lågt syresatta även efter fullbordad kärlnybildning, vilken avslutas inom 14 dagar efter transplantation. På grund av metodologiska begränsningar har man inte tidigare kunnat mäta syresättningen i cellöar transplanterade till lever. I femte delarbetet använde vi oss av pimonidazole (se ovan) för att uppskatta syresättningen av cellöar transplanterade till levern. Den lågt syresatta fraktionen cellöar (pO₂<10 mmHg) i musens bukspottkörtel var ~30%. En månad efter transplantation var den lågt syresatta fraktionen av transplanterade cellöar fördubblade jämfört med motsvarande fraktion i bukspottkörteln. Således har cellöar som är transplanterade till levern nedsatt syresättning även efter fullbordad kärlnybildning.
Slutsats
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