Implantation-Site Dependent Differences in Engraftment and Function of Transplanted Pancreatic Islets

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Abstract

Transplanting pancreatic islets into the liver through the portal vein is currently the most common procedure in clinical islet transplantations for treating patients with brittle type 1 diabetes. However, most islet grafts fail within a 5-year period necessitating retransplantation. The vascular connections are disrupted at islet isolation and implanted islets depend on diffusion of oxygen and nutrients in the immediate posttransplantation period. Rapid and efficient revascularization is of utmost importance for the survival and long-term function of transplanted islets.

In this thesis, the influence of the implantation microenvironment for islet engraftment and function was studied. Islets were transplanted into the liver, the renal subcapsular site or the pancreas. Islets implanted into the liver contained fewer glucagon-positive cells than islets implanted to the kidney and endogenous islets. Intraportally transplanted islets responded with insulin and glucagon release to secretagogues, but only when stimulated through the hepatic artery. Thus, the intrahepatic grafts were selectively revascularized from the hepatic artery. The vascular density in human islets transplanted into the liver of athymic mice was markedly lower when compared to human islets grafted to the kidney. Islets implanted into their physiological environment, the pancreas, were markedly better revascularized. Insulin content, glucose-stimulated insulin release, (pro)insulin biosynthesis and glucose oxidation rate were markedly decreased in transplanted islets retrieved from the liver, both when compared to endogenous and transplanted islets retrieved from the pancreas. Only minor changes in metabolic functions were observed in islets implanted into the pancreas when compared to endogenous islets.

The present findings demonstrate that the microenvironment has a major impact on the engraftment of transplanted islets. Elucidating the beneficial factors that promote engraftment would improve the survival and long-term function of transplanted islets. Ultimately, islet transplantation may be provided to an increased number of patients with type 1 diabetes.

Keywords: Type 1 diabetes, pancreatic islets, human islets, islet transplantation, vascular engraftment, revascularization, implantation-site, microenvironment, vascular density, insulin release, (pro)insulin biosynthesis, glucose oxidation

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To my family

有志者，事竟成

Where there is a will,
there is a way.
List of papers

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

I  Lau J, Jansson L, Carlsson PO. Islets transplanted intraportally into the liver are stimulated to insulin and glucagon release exclusively through the hepatic artery. Am J Transplant 6:967-75, 2006¹.

II Lau J, Carlsson PO. Less revascularization of human islets when experimentally transplanted into the liver than at the renal subcapsular site. Manuscript.


* Shared contribution as first author

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Abbreviations

ANOVA Analysis of variance  
BSA Bovine serum albumin  
BS-1 Bandeiraea simplicifolia 1  
ELISA Enzyme linked immunosorbent assay  
Gck Glucokinase  
GLUT-2 Glucose transporter 2  
HBSS Hank’s balanced salt solution  
IBMIR Instant blood-mediated inflammatory reaction  
KRBH Krebs-Ringer bicarbonate HEPES buffer  
LDH-A Lactate dehydrogenase A  
mGPDH Mitochondrial glycerol-3-phosphate dehydrogenase  
PAK Pancreas after kidney  
PDX-1 Pancreatic and duodenal homeobox gene 1  
RIA Radioimmunoassay  
SPK Simultaneous pancreas kidney  
TCA Tricarboxylic acid  
UCP-2 Uncoupling protein 2  
vol volume  
wtt weight  
YC-3.0 Yellow chameleon protein 3.0
Introduction

Type 1 diabetes is at present treated by daily insulin injections several times daily, but a total normalization of blood glucose concentrations is difficult to achieve. Due to the increased variability in blood glucose concentrations with an associated increase in mean values over the day, these patients have a high risk of developing late complications, e.g. nephropathy, retinopathy and neuropathy [1]. In view of this, islet transplantation is a tempting strategy to treat type 1 diabetes since, if successful, it could restore the fine tuning of blood glucose. However, at present clinical islet transplantation is limited to a selected group of diabetic patients who severely suffer from their disease with transplantation being the last opportunity to achieve a corrected metabolism. Major reasons for not using transplantation as a more common treatment are the need for life long immunosuppression and the lack of donors. At least two donors are usually needed to perform a successful islet transplantation, which makes organ shortage even more of a problem. In order to optimally use the limited organ supply, there is a need to understand the physiology of the transplanted islets and their interactions with the microenvironment of the implantation organ in order to limit posttransplant cell death and dysfunction.
Background

Islet physiology

The islets of Langerhans constitute approximately 1-2% of the mammalian pancreas. In humans this corresponds to 1-2 million islets [2], whereas the rat pancreas contains 4000-5000 islets [3, 4]. The size of an islet is 25-300 μm and they are fairly evenly dispersed throughout the pancreas [5]. There are four main different types of islet endocrine cells: α-cells, β-cells, δ-cells and PP-cells, which secrete glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. Recently it has been suggested that islets also contain a fifth cell type, namely the ghrelin-secreting ε-cells [6]. β-cells are the most common cell type, constituting 60-75% of the endocrine cells. In rodents they are preferentially located in the islet core, whereas the α-cells, δ-cells and PP-cells are found in the periphery of the islet. In humans, the distribution of the different endocrine cells seems to be more random [7, 8].

The endocrine part of the pancreas has 5-10 times higher blood flow than the exocrine pancreas [9]. Approximately 10% of an islet consists of blood vessels, almost exclusively fenestrated capillaries [10]. The formation and maintenance of the dense glomerular-like islet angioarchitecture, as well as the formation of fenestrae, depend on vascular endothelial growth factor (VEGF) [11, 12]. The precise regulation of islet blood perfusion in relation to hormone release, together with modulating influences by the autonomic nervous system, enable the islets to meticulously control the carbohydrate metabolism.

Glucose plays a pivotal role in the release of insulin from β-cells. It is efficiently transported into β-cells by GLUT-2 (glucose transporter 2) and becomes converted to glucose-6-phosphate by the rate limiting enzyme glucokinase (Gck). Pyruvate is ultimately formed in the glycolysis. In the mitochondria, the product is then further processed in the tricarboxylic acid (TCA) cycle to finally yield H⁺, which is exported from the mitochondria by proteins of the respiratory chain (I, II, III, IV). Protons are either transported back into the mitochondrial matrix through the ATP-synthase or the uncoupling protein 2 (UCP-2). When H⁺ is channeled through UCP-2, heat is dissipated. The respiratory chain influx of H⁺, on the other hand, facilitates the synthesis of ATP from ADP by the enzyme ATP-synthase. This results in raised ATP/ADP ratios, which lead to influx of Ca²⁺ through closure of ATP-sensitive K+-channels (K_{ATP}-channels) which depolarizes the cell
membrane and opens voltage-dependent Ca\textsuperscript{2+}-channels. The increased cytoplasmic Ca\textsuperscript{2+} results in exocytosis of insulin granules.

Special shuttle systems, the malate-aspartate shuttle and the glycerol-3-phosphate shuttle, are required for the synthesis of ATP. Their function is to import NADH to mitochondria. The β-cells have a very high activity of mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), which is the rate-limiting enzyme of the glycerol-phosphate shuttle [13]. Furthermore, the activity of pyruvate carboxylase (Pcx), which restores intermediates in the TCA cycle, is also very high [14]. Decreased/alterned activity of mGPDH and Pcx is coupled to type 2 diabetes [15].

While no ATP is yielded through the actions of UCP-2, it is considered to be a negative regulator of insulin secretion, and has been suggested to play an important role in the pathogenesis of type 2 diabetes [16, 17].

When anaerobic conditions occur, pyruvate can in many cells have an alternative route to lactate via the enzyme lactate dehydrogenase (LDH-A). However, expression of LDH-A is normally low in β-cells [18].

![Figure 1. Major pathways involved in glucose-stimulated insulin release. GLUT-2, glucose transporter 2; Gck, glucokinase; G-6-P, glucose-6-phosphate; Pyr, pyruvate; LDH-A, lactate dehydrogenase A; Pcx, pyruvate carboxylase; TCA, tricarboxylic acid; mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase; \( K_{ATP} \)-channel, ATP-sensitive K\textsuperscript{+}-channel; VDCC, voltage-dependant Ca\textsuperscript{2+}-channel; \([\text{Ca}\textsuperscript{2+}]_c\), cytoplasmic Ca\textsuperscript{2+}-concentration. Modified from [19].](image-url)
Pancreas transplantation

The first cadaveric pancreas-kidney transplant was performed in 1966 by Kelly and Lillehei at the University of Minnesota [20]. The procedure has been evolving tremendously since then and today 1-year pancreas graft and patient survival rates are 80-90% and as high as 95%, respectively [21]. Although major advances have been made in this field, such as improved surgical techniques, more effective immunosuppression and better antiviral prophylaxis, the transplantation procedure is still technically complex. Thus, the risk for complications, such as arterial thrombosis, is greater than when performing islet transplantations [22, 23]. Pancreas transplantations are often done in combination with kidney transplantation, either simultaneously or afterwards (SPK = Simultaneous Pancreas Kidney, PAK = Pancreas After Kidney), since immunosuppression is already present [22].

Islet transplantation

The first successful clinical islet transplantation took place in 1977 [24]. Only the endocrine part of the pancreas, the islets of Langerhans, was isolated and transplanted. The liver has so far been the predominate implantation site for islets in humans [25], whereas in animals a variety of implantation sites have been successfully tested including the spleen [26], the omentum [27], the testes [28] and the renal subcapsular space [29]. Orthotopic implantation into the pancreas has rarely been considered due to the risk of complications [30].

A rapid revascularization is of utmost importance for the survival of transplanted islets, since the dense blood vessel network in the islets is disconnected after the isolation process. Highly vascularized implantation sites are therefore preferable as they would theoretically provide better conditions for angiogenesis. The use of the liver as an implantation organ of choice is mainly due to its easy access and since it constitutes a major target organ for insulin. Furthermore, infusing islets into the liver through the portal vein is a minor surgical procedure with almost no serious complications.

The success rate of clinical islet transplantation into the liver was low until the year 2000, when Shapiro and coworkers introduced the so called Edmonton protocol. This encompassed the use of freshly isolated islets for transplantation and a steroid-free immunosuppressive regimen [31]. Moreover, islets from at least two donors were used for transplantation to each patient. By these procedures, an 80% insulin-independence was achieved one year after transplantation. However, the islet function seems to progressively decline after the first year with only approximately 10% of
patients remaining free from exogenous insulin five years post-transplantation [32]. To cope with this, repeated islet infusions are needed.

In view of the long-term functional graft failure after intraportal transplantation in humans, which is seen also in rodents [33], interest has been renewed in finding other implantation sites for islet transplantation. Many sites have been tested, some of the more recent ones are the bone marrow, gastric mucosa and epidymal pad fat in animals [34-36]. Recently, it has clinically been reported that transplantation of islets to the muscle may be feasible [37]. Other strategies have aimed to create a well-vascularized artificial microenvironment by using biocompatible devices [38].

Engraftment

Engraftment is the adaptation of the graft to the implantation organ and encompasses revascularization, reinnervation as well as reorganization of the transplant constituents [39]. During the process of islet isolation, all blood vessels and nerve fibers are disconnected from the surrounding exocrine part of the pancreas. Re-establishing these connections is of utmost importance to achieve an optimal graft function and survival. In view of the decline of graft function after transplantation referred to above, a further understanding of the engraftment process is crucial to improve graft function.

Islet revascularization

Freely grafted syngeneic islets in the hamster dorsal skin-fold chamber show the first signs of angiogenesis already two days posttransplantation. With the help of intravital microscopy, formation and protrusion of capillary sprouts were identified. Furthermore, the newly formed capillaries were perfused within four to six days. Revascularization was completed 10 days post-transplantation and the grafted islets contained insulin-positive cells [40]. Other studies have also shown that blood vessels can be seen within islet grafts three to five days after transplantation and the blood flow is reestablished after 7-14 days [41-45]. The time required for revascularization to occur seems to be independent of whether the islets are implanted singly or in aggregates, or the implantation organ chosen [46]. Interestingly, the newly formed blood vessels in transplanted islets develop into the same phenotype as endogenous islets, i.e. diaphragmed fenestrae, transendothelial channels, and a continuous basal lamina irrespective of implantation organ [42, 47].

Since the revascularization process is of crucial importance for the survival of transplanted islets, many research groups have focused on strategies to improve graft vascularization and function. Pre-treating the
isolated islets as well as the implantation site with pro-angiogenic factors
(Endothelial cell growth factor, ECGF or fibroblast growth factor, FGF) has
been shown to accelerate the revascularization [48, 49]. Simple incubation of
islets with matrix metalloproteinase-9 (MMP-9) before transplantation also
seems to promote islet graft revascularization, whereas incubation with
VEGF or FGF-2 does not seem to be effective [50]. When transplanting
islets containing VEGF-overexpressing β-cells, higher insulin content and
increased islet vascular density, which led to improved survival of the islet
grafts, were observed [51, 52]. Besides increasing pro-angiogenic factors,
inhibition of anti-angiogenic factors present within the islets may also
improve revascularization. Transplantation of islets genetically lacking the
angiostatic factor thrombospondin-1 (TSP-1), or of islets treated with siRNA
for TSP-1, was recently shown to improve islet graft vascular density [53].
Improved revascularization of transplanted islets may not only be obtained
by stimulating angiogenesis, since also augmented vasculogenesis by
mobilization of angioblasts in transplanted islets increases their vascular
density [54].

Previous studies have reported that exocrine contamination of islets has a
negative influence on angiogenesis and engraftment after implantation
[55-57]. Despite this, transplantation of freshly isolated islets seems to be
superior to that with islets transplanted after culture [58, 59] due to an
improved degree of revascularization [58]. This is probably due to the
remaining donor endothelial cells that help to initiate angiogenesis and
provide a scaffold for the ingrowth of endothelial cells [60-62].

Microenvironment of transplanted islets

The liver is currently the predominating implantation site for clinical islet
transplantations. However, the liver microenvironment is quite hostile to the
islet grafts. Endocrine cell loss is inevitable due to the low oxygen tension in
this organ [63, 64], the presence of immunologically active Kupffer cells
[65] and in humans and large animals, the instant blood-mediated
inflammatory reaction, IBMIR, which is associated with intravascular
infusion of the islets [66, 67]. The IBMIR leads to activation of the compl-
ment system and subsequent blood clot formation.

The most used experimental implantation organ is the renal subcapsular
site. The reason for this is mainly that the islet graft is easy to find and
retrieve. However, since the islets are transplanted in clusters, diffusion of
oxygen, which is predominating before new vasculature is established, is
insufficient in the core of the grafts, which leads to the formation of
abundant connective tissue rich in blood and lymphatic capillaries
interspersed with the endocrine cells [68, 69]. Clinically, the renal
subcapsular site has rarely been considered, since the volume of the renal
subcapsular space is limited when considering the great number of islets needed to provide a cure. Furthermore, nephropathy is common in patients with type I diabetes.

The spleen is a well-vascularized organ, which would be advantageous for the early oxygenation of transplanted islets [63]. However, the revascularization of implanted islets is poor [68]. Moreover, the spleen is also an organ containing numerous immune cells. This location has clinically been used in a number of cases, but it is unlikely to confer any advantages.

An immunoprivileged site used in experimental studies is the testes. The Sertoli cells play a pivotal role in maintaining the testes as an immunoprivileged environment, but a major drawback with this site is the space limit in humans and that only men can be transplanted.

The presence of an appropriate microenvironment supporting an adequate engraftment of the transplanted islets in the implantation organ is important for long-term survival and regain of function. Different implantation organs are likely to provide different conditions for the transplanted islets, with a variable capacity of the islets for survival, revascularization, proliferation and function. Elucidating the factors beneficial for engraftment of transplanted islets is warranted to determine or design a proper implantation site.
Aims of thesis

General aims

Clinical islet transplantation, i.e. infusing islets into the liver through the portal vein, is a tempting strategy to treat patients with brittle type 1 diabetes. However, most transplanted patients ultimately reverse to the need for exogenous administration of insulin, since the islet graft fails to function with time. Currently, at least two donors are required to achieve insulin independence at one year follow-up, but despite this most grafts fail later on. The continued major organ shortage is an important problem to face in this context. This thesis aimed to investigate the influence of the microenvironment associated with different implantation organs on islet engraftment and function. Understanding the interaction between the implanted islets and their microenvironment may lead to potential interventions to prevent islet loss and dysfunction. If this proves successful, islet transplantation may ultimately be offered to an increased number of diabetic patients in order to avoid the high risk of developing late complications due to the fluctuating blood glucose concentrations.

Specific aims

Paper I

This paper aimed to investigate nutrient-stimulated hormonal release from intraportally transplanted islets when compared to control pancreas as well as islets transplanted beneath the kidney capsule. Moreover, possible changes in endocrine cell composition of islets implanted into the liver or kidney were evaluated.

Paper II

The aim of this study was to evaluate the vascular density in human islets experimentally transplanted into the liver or beneath the renal capsule of athymic mice.
Paper III
The aim of this paper was to examine the pancreas, i.e. the physiological microenvironment for islets, as an implantation site with regard to the degree of revascularization and long-term survival of transplanted islets.

Paper IV
The aim of this paper was to investigate the function and gene expression in vitro of retrieved intrahepatically and intrapancreatically transplanted islets and evaluate whether possible changes are implantation-site dependent.
Study design and methods

A brief description of the methods used is presented here. More detailed information is given in Papers I-IV or in the original references.

Animals (I-IV)

Inbred male Wistar-Furth rats, weighing approximately 300 g, were purchased from Scanbur (Sollentuna, Sweden). Adult, male C57BL/6 (nu/nu) mice, weighing 25-30 gram, were purchased from M&B (Ry, Denmark). YC-3.0 transgenic mice were kindly donated by Professor R Y Tsien at the University of California, San Diego, CA, USA and subsequently bred at Karolinska Institutet, Stockholm, Sweden. The generation and characterization of these mice have previously been described [70, 71]. Briefly, transgenic YC-3.0 mice express the Yellow chameleon protein 3.0 (YC-3.0) under the regulation of the β-actin and cytomegalovirus promoters [72]. This results in the expression of Enhanced yellow fluorescent protein (EYFP), one part of the hybrid YC-3.0 protein, in most tissues, including all pancreatic islet cells. Glucose homeostasis, islet function, islet mass, islet vascularity and islet blood perfusion in YC-3.0 mice are similar to those of C57BL/6 mice [70]. Tie2-GFP transgenic mice, i.e. animals that express green fluorescent protein (GFP) behind the endothelial cell specific Tie2 promoter, were purchased from The Jackson Laboratories (Bar Harbor, ME, USA) [STOCK Tg(Tie2GFP)287Sato/J] and kept in a local colony at Karolinska Institutet. All animals had free access to pelleted food and tap water throughout the course of the studies. The experiments were approved by the animal ethics committee of Uppsala University.

Chemicals (I-IV)

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless given otherwise.
Islet isolation, culture and transplantation (I-IV)

Pancreatic islets were isolated from rats and mice by collagenase digestion [73], and cultured in groups of 150 islets for 3-4 days at 37°C (O₂/CO₂, 95:5) in 5 ml of culture medium consisting of RPMI 1640 supplemented with L-glutamine, benzylpenicillin (100 U/ml; Roche Diagnostics Scandinavia, Bromma, Sweden), streptomycin (0.1 mg/ml) and 10 % (vol/vol) fetal calf serum. The culture medium was changed every second day.

Human islets were generously provided by the Central Unit of β-Cell Transplant (Brussels, Belgium).

At transplantation, groups of 200 islets were packed in a butterfly needle (25G) and infused via the portal vein into the liver of syngeneic pentobarbital-anesthetized Wistar-Furth rats (Paper I) or avertin-anesthetized C57BL/6 (nu/nu) mice (Papers II and IV). Alternatively, 200 islets were packed in a braking pipette and implanted beneath the left renal capsule of syngeneic pentobarbital-anesthetized Wistar-Furth rats (Paper I) or avertin-anesthetized C57BL/6 (nu/nu) mice (Papers II and III), or packed in a butterfly needle (25G) and injected directly into the splenic part of the pancreas of avertin-anesthetized C57BL/6 (nu/nu) mice (Papers III and IV).

Perfusions of graft-bearing organs (I)

Liver

Ex vivo perfusion of graft-bearing livers was performed one month post-transplantation. The animals were anesthetized with pentobarbital and placed on a heated operating table. The abdominal cavity of each animal was opened. Ligatures were tied around the inferior vena cava immediately below the liver, whereas the portal vein and hepatic artery were cannulated with polyethylene catheters. The catheters were fixed with ligatures and connected to separate infusion pumps. The inferior vena cava was then incised cranially to the liver and the liver perfusion was started. The liver was dissected free, placed in a funnel, and kept at a constant temperature (37°C) and humidity throughout the experiments. The liver preparation was perfused without recirculation at 3.5 ml/min and 1.5 ml/min through the portal vein and hepatic artery, respectively, with a continuously gassed (O₂/CO₂, 95:5) bicarbonate buffer [74] supplemented with 10 mM HEPES and 2 mg/ml each of dextran T70 (Pharmacia, Uppsala, Sweden) and bovine serum albumin (BSA Fraction V; Miles, Slough, UK).

Each graft-bearing liver was allocated to one of three different experimental protocols with regard to stimulation with D-glucose and L-arginine: 1) stimuli provided through both the portal vein and the hepatic artery; 2) stimuli provided only through the hepatic artery; or 3) stimuli...
provided only through the portal vein. The blood vessel through which stimuli were not provided in group 2 (portal vein) and 3 (hepatic artery) was perfused with medium containing low glucose (2.8 mM) during the whole experiment. For the blood vessel(s) through which stimuli were to be provided, the experiments started with a 10-min equilibration period with perfusion medium containing 2.8 mM D-glucose, which was followed by a 20-min stimulation period with high-glucose medium (16.7 mM D-glucose). Thereafter, a 10-min reset period with low-glucose medium (2.8 mM D-glucose) was followed by a 10-min stimulation period with 10 mM L-arginine + 5.5 mM D-glucose added to the perfusion medium. The perfusions were completed with a 20-min period with low-glucose medium (2.8 mM D-glucose). The effluent medium from the preparations was collected on ice at minutes 9-15, 17, 20 25, 30, 35, 40-45, 47, 50, 55, 60 and 70 in tubes containing aprotinin (7.4% of the final sampling volume, 10,000 KIU/ml; Bayer, Leverkusen, Germany).

Separate control experiments were performed to estimate the immediate hepatic uptake of insulin and glucagon. Livers from control, non-transplanted animals were prepared for organ perfusion, as described above. Insulin or glucagon was then added for 1-min pulses to the medium perfusing the hepatic artery in these preparations, while the perfusion medium contained a low (2.8 mM) or high (16.7 mM) glucose concentration or arginine (10 mM).

Pancreas-duodenum
Non-transplanted control rats were anesthetized with pentobarbital. The pancreas and duodenum were removed from the animals as previously described [75, 76]. The pancreas-duodenum preparations were perfused at 1.5 ml/min without recirculation applying the same perfusion protocol with glucose and arginine stimuli and collection of samples as used for the perfusion of graft-bearing livers.

Kidney
One month posttransplantation, animals with an islet graft implanted beneath their left renal capsule were anesthetized with pentobarbital. The graft-bearing kidneys were then removed from the animals as previously described [77]. The kidney preparations were perfused at 3 ml/min without recirculation applying the same perfusion protocol with glucose and arginine stimuli and collection of samples as used for the perfusion of graft-bearing livers and control pancreata.
Hormone measurements in the perfusion effluents (I)

The effluents from the perfusions of pancreata or graft-bearing organs were analyzed for insulin concentrations by a rat insulin ELISA (Mercodia, Uppsala, Sweden). Glucagon measurements in the samples were carried out using a rat glucagon RIA kit (Linco Research, St. Louis, MO, USA).

Insulin and glucagon concentrations in samples obtained from the control experiments for hepatic extraction rates, where insulin and glucagon were infused into the hepatic artery, were analyzed by a human insulin ELISA (Mercodia) and a glucagon RIA kit (Linco Research), respectively.

Morphological studies (I-IV)

In Papers I-III, graft-bearing livers/kidneys/pancreata and control pancreata were fixed in 10 % (vol/vol) formalin and embedded in paraffin. Retrieved islets (Paper IV) were fixed in 4 % (vol/vol) paraformaldehyde and embedded in 5 % (wt/vol) agarose (SeaKem® LE Agarose, Cambrex Bio Science Rockland, Rockland, ME, USA). Thereafter, the agarose-embedded islets were placed in 4 % (vol/vol) formalin and paraffin-embedded.

Embedded tissues were sectioned at 5 μm, mounted on glass slides and stained with lectin or for the antigens mentioned below, followed by counterstaining with hematoxylin:

- For the antigen insulin (Papers I and IV) staining was performed as previously described [68]. Briefly, sections were incubated with hydrogen peroxide to block for endogenous peroxidases, followed by incubation with normal swine serum (Dakopatts, Glostrup, Denmark) to block for non-specific antibody binding. The primary antibody used was guinea pig anti-human insulin serum (ICN, Biomedicals, Aurora, OH, USA) and polyclonal swine anti-rabbit immunoglobulins were used as secondary antibody (Dakopatts). Sections were incubated with peroxidase-anti-peroxidase rabbit polyclonals (Dakopatts) before they were developed with diaminobenzidine tetrahydrochloride, DAB (Dakopatts).

- For the antigen glucagon (Papers I and IV) staining was performed as previously described [68]. The primary antibody used was rabbit anti-porcine glucagon serum (Fitzgerald, Concord, MA, USA). The same protocol as used for insulin described briefly above was used.

- With the lectin Bandeiraea (Griffonia) simplicifolia, BS-1 (Papers II and III), staining was performed as previously described [78] to visualize blood vessel endothelium. Briefly, sections were pre-treated with Neuraminidase Type X to remove blocking sialic acid residues followed by incubation with normal goat serum (Dakopatts) to block for non-specific binding. Biotinylated forms of BS-1 were used. Sections were
incubated with StreptABComplex (Vector, Immunkemi, Sweden) before they were developed with Vectastain red (Vector).

- For the antigens EYFP or GFP, staining procedures are described in detail in Paper III. Briefly, sections were pre-treated with heat-induced antigen retrieval in Target Retrieval solution® pH 6 (Dakopatts). Sections were then incubated with hydrogen peroxide to block for endogenous peroxidases followed by incubation with Dako protein block. The primary antibody used was rabbit anti-GFP IgG (Invitrogen, Carlsbad, CA, USA). The ChemMate EnVision detection kit® (Dakopatts) was then used on the sections according to the manufacturer’s instructions followed by development with DAB (Dakopatts).

Histological evaluations (I-IV)

In Papers I and IV, the stained sections were examined using a point-counting method [79] to determine the percentage of β-cells and α-cells in endogenous or transplanted islets.

In Papers II and III, the vascular density was determined on the stained sections. Briefly, the number of blood vessels within the transplanted islets was counted using a light microscope, and the volumes of the islet grafts were determined by a computerized system for morphometry (Image J, NIH, Bethesda, MD, USA). The islets transplanted in clusters beneath the kidney capsule were interspersed by strands of connective tissue, and the morphometric analysis was in this case combined with a direct point-counting method to determine the fraction of connective tissue in these grafts. The vascular density of the islet transplants, given as number of blood vessels per square millimeter, was then calculated.

Retrieval of transplanted islets (IV)

One month posttransplantation, the intraportally transplanted animals as well as the intrapancreatically transplanted animals were anesthetized with avertin. The islets transplanted into the liver were then retrieved as previously described [80], while the fluorescent islets transplanted into the pancreas were retrieved using a similar collagenase method as used for isolation of endogenous islets. The retrieved islets could easily be distinguished under a fluorescence microscope. Retrieved YC-3.0 islets and control islets were cultured for 1-4 days before studies of glucose-stimulated insulin release, glucose oxidation rate, gene expression and (pro)insulin biosynthesis.
Glucose-stimulated insulin release (IV)

Groups of 10 endogenous or retrieved YC-3.0 islets were investigated for glucose-stimulated insulin release as previously described [81]. Briefly, islets were incubated in glass vials containing KRBH supplemented with 2 mg/ml BSA and 1.67 mM glucose. Incubation conditions were 37 °C (O₂/CO₂, 95:5) in a slow shaking water bath (30 strokes/min) for 1 h. The incubation medium was then collected and the islets were incubated in KRBH supplemented with BSA and 16.7 mM glucose for another 1 h. Afterwards, incubation medium was collected. The insulin released in the incubation media were determined by a mouse insulin ELISA kit (Mercodia).

Glucose oxidation (IV)

Islet glucose oxidation rates in groups of 10 endogenous or retrieved YC-3.0 islets were determined according to a previously described method [81]. Briefly, islets were incubated in glass vials containing KRBH supplemented with D-[U-14C]glucose (Amersham, Amersham, UK) and non-radioactive glucose to give a final concentration of 16.7 mM. Incubation conditions were 37 °C (O₂/CO₂, 95:5) in a slow shaking water bath (30 strokes/min) for 90 min. The glucose oxidation was terminated with Antimycin A and Na₂HPO₄ (pH 6) was added to release the CO₂ formed. To trap the CO₂, Hyamine 10-X (New England Nuclear, Boston, MA, USA) was added and the glass vials were further incubated for 2 h. Ultimate Gold scintillation liquid (Packard Instrument, Meriden, CT, USA) was then added in order to measure the radioactivity by liquid scintillation counting. Glass vials without islets were used as blanks.

(Pro)insulin biosynthesis (IV)

Islet (pro)insulin biosynthesis in groups of 10 endogenous or retrieved YC-3.0 islets were determined according to a previously described method [82]. Briefly, islets were incubated in KRBH supplemented with 2mg/ml BSA, 16.7 mM glucose and 50 μCi/ml L-[4,5-3H]leucine (Amersham) for 2 h, 37 °C (O₂/CO₂, 95:5). The islets were then washed in HBSS containing 10 mM non-radioactive leucine followed by disruption in 200 μl redistilled water in an ultrasonicator.

For each sample, aliquots of the homogenate were incubated at room temperature for 1 h with a mixture of glycine buffer with BSA and Triton X-100 and either guinea pig anti-bovine insulin serum (ICN Biochemicals, Cosa Mesa, CA, USA) or guinea pig serum (for non-specific antibody
binding). Next, protein A-Sepharose (Amersham) was added before incubation for another 15 min during slow shaking. Finally, the samples were washed twice with glycine buffer, containing BSA and Triton X-100, before resuspension in acetic acid solution and transfer to scintillation vials. Ultimate Gold scintillation liquid (Packard Instrument) was then added in order to measure the radioactivity by liquid scintillation counting. To obtain a measure of the (pro)insulin biosynthesis, counts representing guinea pig serum was subtracted from the counts representing anti-insulin serum.

For the analysis of total protein biosynthesis, aliquots of the homogenate were mixed with glycine buffer, containing BSA, and trichloroacetic acid. The samples were then centrifuged and the pellets were resuspended in NaOH and transferred to scintillation vials. Finally, Ultimate Gold scintillation liquid (Packard Instrument) was added in order to measure the radioactivity by liquid scintillation counting.

**Islet insulin and DNA content (IV)**

Groups of 10 endogenous or retrieved YC-3.0 islets were homogenized by sonication in 200 μl redistilled water. A fraction of the homogenate was mixed with acid-ethanol (0.18 M HCl in 95% (vol/vol) ethanol) and insulin was extracted at 4°C overnight. Insulin contents in homogenates were determined by a mouse insulin ELISA kit (Mercodia). Moreover, aliquots of the aqueous homogenate were used for DNA measurements by fluorophotometry (PicoGreen® dsDNA Quantitation Kit cat # P-7589, Molecular Probes, Eugene, OR, USA).

**Gene expression analysis (IV)**

Isolation of total RNA was performed with RNeasy Mini Kit (Qiagen, Hilden, Germany) and all the samples were treated with RNase-free DNase (Qiagen). The RNA quality was analyzed with the Agilent Bioanalyzer 2100 using RNA Pico Chips (Agilent Technologies, Waldbronn, Germany). Conversion of RNA to cDNA was performed with reverse transcription system (Promega, SDS, Falkenberg, Sweden) using Oligo (dT)_{15} primers. The LightCycler System (Roche Diagnostics, Mannheim, Germany), and detection with SYBR Green (SYBR® Green JumpStart™ Taq ReadyMix), was used to amplify and analyze generated cDNA. The primers used are listed in Paper IV. The results are presented as threshold cycle values (C_T values), i.e., the estimated amplification cycle number when the fluorescence exceeds a specified threshold value. The C_T values were used to calculate the amount of PCR product in comparison to TATA-box binding protein (TBP) as a "house-keeping gene", by subtracting the C_T value for
TBP from the C\textsubscript{T} value for the gene being studied (ΔC\textsubscript{T}). Relative mRNA expression was calculated as 2\textsuperscript{-ΔCT}.

Statistical analysis (I-IV)

All values are given as means±SEM. All statistical comparisons were made with SigmaStat\textsuperscript{®} (SPSS Science Software, Erfurt, Germany). Multiple comparisons for normally distributed data were performed using ANOVA and the Bonferroni post-hoc test, whereas non-parametric values were compared using non-parametric ANOVA and Dunn’s test. When only two groups were compared, Student’s unpaired two-tailed t-test was applied. For all comparisons, a P-value <0.05 was considered to be statistically significant.
Results and Discussion

Response to secretagogues in islet grafts (I)

In this paper we investigated whether there are changes in the cellular composition of intraportally transplanted syngeneic islets when compared to those in the endogenous pancreas, and if hormone release from the transplanted islets is affected by stimuli administered through either the portal vein or hepatic artery. Previous studies with perfusion of islet graft-bearing livers have shown that there is a biphasic insulin release from intraportally transplanted islets in response to glucose, but no comparisons to endogenous islets or islets implanted to other organs have been made [83-85]. In the present study, we compared the hormonal release from islets implanted beneath the kidney capsule, islets transplanted intraportally to the liver and endogenous islets in the pancreas.

Islets implanted to the kidney or intraportally into the liver had a delayed and decreased first-phase of insulin release in response to glucose (16.7 mM) when compared to endogenous islets. Furthermore, insulin could only be measured in the effluent from perfused graft-bearing livers when stimuli to insulin release (high glucose and arginine) were provided through both the hepatic artery and the portal vein (experimental group 1) or through the hepatic artery alone (experimental group 2). In contrast, no insulin response at all could be detected when stimuli to insulin release were administered only through the portal vein (experimental group 3). Likewise, no glucagon release in response to arginine was detected when stimuli were provided through the portal vein, but was consistently seen when provided through the hepatic artery or both blood vessels. Since intrahepatic islets did not sense stimuli provided through the portal vein, which is the physiological nutrient supply, it is likely that nutrient sensing of the islet α- and β-cells in the islet transplants occurs only through the newly formed blood vessels from the hepatic artery. Thus, our data are consistent with a previous study, which reported that intraportally transplanted islets become revascularized by tributaries mainly from the hepatic artery [45].

Some of the insulin secreted by intraportally transplanted islets is likely to be metabolized by the hepatocytes before leaving the liver. We therefore estimated the immediate hepatic clearance of insulin and glucagon to evaluate the approximate fraction of studied hormones that enter the systemic circulation. We found that the hepatic clearance for insulin and
glucagon was $43.6 \pm 3.8\%$ and $67.5 \pm 5.1\%$, respectively. These estimates are consistent with previous reports [86-90].

The cellular composition of the transplanted islets was changed when compared to that of endogenous islets. Islets transplanted beneath the kidney capsule had a decreased fraction of $\beta$-cells compared to control islets. One likely explanation for this is that the $\beta$-cell rich core of rodent islets is more prone to hypoxic injury in the immediate posttransplantation period than more peripheral islet cells due to oxygen tension diffusion gradients [91, 92]. In contrast, the $\beta$-cell number in intraportally transplanted islets showed only a tendency to a decrease. However, the fraction of $\alpha$-cells was markedly decreased in the intraportally transplanted islets, both when compared to endogenous islets and islets implanted beneath the kidney capsule. Our data support previous reports of a defective glucagon response from intraportally transplanted islets [93]. The decrease in $\alpha$-cell numbers may be due to factors associated with the hepatic environment per se, or be a result of trauma imposed by the intraportal transplantation procedure.

We have observed that islets implanted into the liver respond to secretagogues via the newly formed vessels, i.e. tributaries of the hepatic artery. However, clinical islet transplantations have reported that most patients return to hyperglycemia within a five years period. The reason(s) for the islet graft failure over time is unknown. One possible explanation is deposits of islet amyloid polypeptide (IAPP) amyloid in transplanted islets. Studies have shown that human islets experimentally transplanted into the subcapsular site, the spleen and the liver of athymic mice develop IAPP amyloid deposits already within two to three weeks posttransplantation [94-96]. IAPP is cosecreted with insulin and aggregates of IAPP are believed to exert toxic effects on $\beta$-cells leading to cell death and subsequent reduction of $\beta$-cell mass. Moreover, rapid and adequate revascularization is of utmost importance for the survival and regain of function in transplanted islets since they are avascular after isolation. For this purpose, we wanted to investigate the vascular density in human islets experimentally transplanted into the liver of athymic mice and this was done in Paper II.

**Vascular density in transplanted human islets (II)**

It has previously been reported that the vascular density in human islets transplanted beneath the renal capsule of athymic mice was markedly decreased when compared to endogenous human islets. Furthermore, a low oxygen tension correlated to the decreased vascular density [97].

In view of these findings, we wanted to estimate the vascular density in human islets implanted into the liver, which is currently the dominating implantation organ for clinical islet transplantation. For comparison, human
islets were, as previously, also transplanted beneath the renal capsule of other athymic mice.

Human islets transplanted intraportally into the liver or beneath the renal capsule showed normal morphology with numerous insulin-positive cells and no infiltration of inflammatory immune cells one month post-transplantation.

It is noteworthy that the vascular density in human islets implanted into the liver was strikingly lower when compared to human islets implanted beneath the renal capsule. The intrahepatic islets were surrounded by a narrow rim of stroma which was richly vascularized. Similarly, a lot of blood vessels were found in the connective tissue interspersing islets grafted in the kidney. However, few blood vessels were discerned within the endocrine tissue at both implantation sites.

There is a great heterogeneity in endothelial cells; every organ has a specific endothelial phenotype in order to adapt to different physiological functions [98]. The sinusoidal endothelium in the liver is highly fenestrated in order to facilitate efficient uptake of nutrients for storage and metabolism. Furthermore, it is phagocytotic to cleanse the blood from toxic substances. Likewise, fenestrated endothelium is found in islets in order to provide easy access of the secreted hormones in the circulation. Endothelial cells of different origins have a tendency to respond differently to angiogenic stimuli [98-100]. As an example, only endothelial cells from the hepatic artery, but not those of the portal vein, seem to revascularize islets intraportally transplanted into the liver ([45], Paper I). Islet endothelial cells seem particularly resistant to proliferate in response to angiogenic stimuli when compared to other endothelial cells [99].

Markedly decreased vascular density has been reported in islets implanted into heterotopic sites such as the liver, spleen or kidney when compared to endogenous islets [68]. For the ingrowth of blood vessels into the transplanted islets to occur, specific angiogenic stimuli are needed from the microenvironment in order to form the appropriate endothelial phenotype. We therefore hypothesized that islets implanted into their physiological environment, i.e. the pancreas, would become more appropriately revascularized.

**Influence of microenvironment on angiogenesis in islet grafts (III)**

In this study we transplanted syngeneic islets into the pancreas or beneath the renal capsule in mice. The rationale for this investigation was that islets implanted into their normal microenvironment might improve
revascularization. We chose to use the renal subcapsular site for comparison, since the revascularization process in that site is already carefully mapped.

In order to distinguish between transplanted and endogenous islets in the pancreas, we used islets from the transgenic Yellow chameleon protein 3.0 (YC-3.0) mouse [70]. Enhanced yellow fluorescent protein (EYFP), which is expressed in all somatic cells, was used as a reporter for the transgene. Following immunohistochemical staining with a GFP-antibody, known to also detect EYFP, transplanted islets could easily be identified.

Indeed, the vascular density in islets implanted into the pancreas was remarkably higher when compared to islets grafted in the kidney. Interestingly, the capillary number in islets grafted in the pancreas was only slightly lower than the dense capillary network in endogenous islets. The low vascular density observed in islets grafted beneath the renal capsule was in line with previous studies [50, 68, 97].

Next we wanted to elucidate if the high graft vascular density in islets implanted into the pancreas reflected expansion of remnant donor endothelial cells or increased ingrowth of blood vessels from the host. For this purpose, we used islets from the transgenic Tie2-GFP mouse, with specific expression of GFP only in the endothelial cells, as donor islets. By using these islets for transplantation, the contribution of donor endothelium in the revascularization process was much more simple to evaluate than in islets of the YC-3.0 mouse where all somatic cells in the islets express fluorescence.

Donor endothelial cells have previously been reported to participate in the revascularization process when freshly isolated islets were transplanted [60-62]. In our study, we cultured the islets before transplantation, which has been shown to dedifferentiate or degenerate the endogenous islet vasculature [62, 101]. Tie2-GFP islets grafted in the pancreas or beneath the renal capsule also revealed that the new vascular structures formed in the islet grafts at both sites contained very few GFP-positive cells. These data indicate that the acquired vascular network in our transplanted islets was mainly of recipient origin, and that the improved revascularization of intrapancreatically transplanted islets was due to increased ingrowth of blood vessels, not a redifferentiation or regeneration of intra-islet endothelial cells.

Culture of islets before transplantation enables modifications of islets in order to increase the survival. For instance, islet surface heparinization prevents the IBMIR occurring when islets are intraportally transplanted into the liver of pigs [102]. Moreover, addition of pro-angiogenic factors or modulation of angiogenic inhibitors important for stimulation of angiogenesis is possible during culture.

Concerns have been raised when transplanting islets into the pancreas, since there is a high risk of complications due to leakage of enzymes from the exocrine cells causing tissue damage and inflammation. Interestingly, none of the intrapancreatically transplanted animals in our study developed
acute pancreatitis posttransplantation. Neither did Stagner and coworkers find any evidence for pancreatitis after intrapancreatic islet transplantations in canines and in rats [103]. Nevertheless, intrapancreatic islet transplantation is not likely to become a clinical reality due the associated risks of exocrine pancreatic damage. However, studies on intrapancreatic transplantations can hopefully help us to evaluate the factors in the pancreatic microenvironment with positive effects on engraftment. That should in turn enable us to modify a heterotopic implantation site in order to obtain efficient engraftment also at that site.

No inflammatory cells were seen within the islets implanted into the pancreas either at two days, one month or six months posttransplantation. However, inflammatory cells in limited numbers could in some cases be observed in the periphery of the intrapancreatic islet grafts two days post-transplantation.

In view of the findings in Paper III, we wanted to investigate whether the high vascular density in islets grafted in the pancreas was correlated to improved function.

Influence of microenvironment on functional properties of islet grafts (IV)

The technique to retrieve intraportally transplanted islets for investigation was previously established in our group [80]. We showed that islets retrieved from the liver one month posttransplantation had perturbed endocrine function when compared to isolated endogenous islets. We wanted to further investigate functional properties of retrieved islets from the liver to elucidate whether this dysfunction of intraportally transplanted islets is due to the liver microenvironment or merely relates to the transplantation per se. Therefore, we choose to compare intraportally transplanted islets with islets implanted into their physiological microenvironment, the pancreas, as well as endogenous islets (hereafter referred to control). In addition, islets implanted into the pancreas become better revascularized (Paper III).

Our results from Paper IV confirmed previous findings [80] of a decreased insulin release, insulin content and glucose oxidation rate in intraportally transplanted islets when compared to non-transplanted control islets. However, islets transplanted into the pancreas had an insulin content and glucose oxidation rate similar to that of control. Furthermore, only a modest alteration in glucose stimulated insulin release, i.e. increased basal insulin release and a decreased stimulation index compared to control, could be seen in islets implanted into the pancreas. Islets transplanted into the liver showed markedly decreased (pro)insulin biosynthesis when compared to both control and islets implanted into the pancreas.
Thereafter we wanted to investigate possible mechanisms which could explain the observed functional impairment of islets transplanted into the liver. We selected to study some genes that are essential for β-cell differentiation and function. Downregulation of PDX-1, GLUT-2 and Pcx could be seen in islets retrieved from both the liver and pancreas. In addition to this, islets implanted into the liver had decreased mRNA levels of glucokinase and mGPDH when compared to control islets, whereas no such downregulation was seen in islets implanted to the pancreas. Interestingly, a gene that is normally suppressed in β-cells, namely LDH-A, was markedly upregulated in islets implanted into the pancreas, but with only a tendency to upregulation in the liver, when compared to control. The observed increase in basal insulin release at low glucose concentrations in islets implanted into the pancreas may be explained by this finding, since lactate may stimulate β-cells to release insulin, a notion previously discussed in the literature [104, 105]. All observed gene expression changes in the intrahepatic islets could cause impairments in glucose-induced ATP-synthesis and insulin secretion, thereby providing a molecular basis for the β-cell dysfunction preferentially seen in intraportally transplanted islets.

The observed dysfunction of islets transplanted into the liver seemed to be at least partially site-specific, since changes seen in islets implanted to their physiological microenvironment, the pancreas, were less pronounced. Whether the functional difference reflects various degrees of revascularization between intrapancreatically and intrahepatically transplanted islets, or an influence of the implantation per se, remains to be determined. In this context, it would have been of interest to investigate subcapsular islets as well. However, we were not able to retrieve and investigate single islets from this site, since the grafted islets adhered tightly to one another and to the connective tissue stroma.

The findings in this thesis demonstrate that the microenvironment of the implantation organ has a major influence on the transplanted islets. Islets implanted into the liver become poorly revascularized and the low vascular density correlates to dysfunction of grafted islets. In contrast, islets implanted into the pancreas only show minor changes in metabolic functions when compared to endogenous islets. Furthermore, the islets grafted in the pancreas are well-vascularized. Thus, using the pancreas as implantation site for islet transplantation is a good experimental model for studying engraftment and function of transplanted islets. Elucidating the beneficial factors from the local microenvironment that promote revascularization could in the end lead to increased survival and long-term function of transplanted islets.
Conclusions

Paper I

- Islets transplanted intraportally into the liver were stimulated to insulin and glucagon release only when stimuli were supplied through the hepatic artery.
- A delayed and decreased first-phase of insulin release in response to glucose was observed in islets implanted into the liver and kidney when compared to endogenous islets.
- Intraportally transplanted islets contained fewer glucagon-positive cells than endogenous islets and islets transplanted beneath the renal capsule.
- The fraction of insulin-positive cells was decreased in islets transplanted beneath the renal capsule, but not in islets transplanted intraportally into the liver, when compared to endogenous islets.

Paper II

- The vascular density in human islets transplanted into the liver of athymic mice was markedly decreased when compared to human islets implanted to the subcapsular space of the kidney.

Paper III

- Islets transplanted into the pancreas displayed a much higher vascular density when compared to islets transplanted beneath the renal capsule.
- The observed high graft vascular density in islets implanted into the pancreas reflected increased ingrowth of blood vessels from the recipient.
- Islets implanted into the pancreas survived for at least six months post-transplantation.
Paper IV

- Site-specific properties of the liver markedly impaired the metabolic functions of intraportally transplanted islets, i.e. decreased insulin content, glucose-stimulated insulin release, (pro)insulin biosynthesis and glucose oxidation rate. Intraportally transplanted islets also displayed pronounced gene expression changes.

- Islets transplanted into their normal microenvironment, i.e. the pancreas, displayed gene expression changes, but only minor changes in metabolic functions compared to endogenous islets.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)