Properties of Endothelium and its Importance in Endogenous and Transplanted Islets of Langerhans

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Dissertation presented at Uppsala University to be publicly examined in B22, BMC, Husargatan 3, Uppsala, Saturday, December 5, 2009 at 10:15 for the degree of Doctor of Philosophy in Medicine. The examination will be conducted in English.

Abstract

Transplantation of insulin producing cells is currently the only cure for type 1 diabetes. However, even though the Edmonton protocol markedly increased the success rate of pancreatic islet transplantation, the long term insulin independence is still very poor. An adequate engraftment is critical for islet graft survival and function.

In the present thesis, isolated islet endothelial cells were found to have a low proliferatory and migratory capacity towards vascular endothelial growth factor (VEGF), but this could be reversed by using neutralizing antibodies to the angiostatic factors thrombospondin-1, endostatin or alpha1-antitrypsin.

In the adult islet endothelial cell, VEGF may act as a permeability inducer more than an inducer of angiogenesis. p38 MAP kinase activity has been shown to serve as a switch between these properties of VEGF. Inhibition of p38 MAP kinase by daily injections of SB203580 in the early posttransplantation phase lead to a redistribution of the islet graft blood vessels from the stroma into the endocrine tissue and this was accompanied by a higher oxygen tension.

Besides transports of oxygen and nutrients, beta-cells may require signals from the endothelial cells for their growth and differentiation. It was demonstrated that islet endothelial cells secrete factors, including laminin, that have positive effects on beta-cell insulin release and insulin content.

Our results suggest that improved revascularization of transplanted islets may be achieved by either inhibition of angiostatic factors, or by blocking p38 MAP kinase activity, in the implanted tissue. Islet endothelial cells have a supportive paracrine role for beta-cells that might be hampered by the normally poor revascularization.

Keywords: islet transplantation, endothelial cells, engraftment, beta cells

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ISSN 1651-6206
ISBN 978-91-554-7643-4
urn:nbn:se:uu:diva-109713 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-109713)
List of Papers

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


Contents

Introduction ................................................................................................... 11
  Diabetes Mellitus ...................................................................................... 11
  Pancreatic islets ..................................................................................... 12
  Islet transplantation ................................................................................ 12
  Islet engraftment and revascularization ................................................ 13
  Endothelial cells ................................................................................... 14

Aim ............................................................................................................... 16

Methods ........................................................................................................ 17
  Animals (I-III) .......................................................................................... 17
  Islet isolation and culture (I and III) ......................................................... 17
  Islet isolation and culture (II) ................................................................ 17
  Endothelium isolation (I and III) ............................................................. 18
    Islet endothelium ................................................................................. 18
    Liver endothelium ............................................................................. 18
  Cell migration assay (I) ......................................................................... 19
  Cell proliferation assay (I) .................................................................... 19
  Islet transplantation (II) ........................................................................ 20
  pO2 and blood flow measurements in transplanted islets (II) ............... 20
  Measurements of pO2 in native islets (II) ............................................ 21
  Light microscopic evaluation (II) ........................................................... 21
  Insulin release and total insulin content (III) ........................................ 22
  Insulin measurement (III) .................................................................... 22
  Glucose oxidation (III) .......................................................................... 22
  (Pro)insulin biosynthesis (III) .............................................................. 22
  Insulin degradation (III) ....................................................................... 23
  Immunoprecipitation (III) ................................................................. 23
  Gene expression analysis (III) ............................................................. 23
  Statistical analysis (I-III) ..................................................................... 24

Study design ................................................................................................ 25
  Paper I .................................................................................................... 25
  Paper II .................................................................................................. 25
  Paper III ................................................................................................. 26
Abbreviations

$\alpha_1$-AT  alpha1-antitrypsin
BS-1    Bandeiraea simplicifolia
BSA     bovine serum albumin
FCS      fetal calf serum
ECGS     endothelial cell growth supplement
ELISA    enzyme linked immunosorbent assay
Gck      glucokinase
GLUT-2   glucose transporter 2
HBSS     Hank’s balanced salt solution
IAPP     islet amyloid polypeptide
Ins1     insulin gene
i.p.     intraperitoneal
LM       laminin
mGpdh    mitochondrial glycerol-3-phosphate dehydrogenase
Pcx      pyruvate carboxylase
Pdx1     pancreatic and duodenal homebox gene 1
PEDF     pigment epithelium-derived factor
TBP      TATA-box binding protein
TPU      tissue perfusion units
tsp-1     thrombospondin-1
VEGF     vascular endothelial growth factor
Introduction

Diabetes Mellitus

Diabetes mellitus is usually separated into two major groups; type 1 and type 2 diabetes. Type 2 diabetes is the most common form of diabetes and is often associated with a peripheral insulin resistance and beta-cell failure or demise. When the islets can no longer match the demand, type 2 diabetes develops. It is most commonly seen in older people and is frequently accompanied by obesity. However, as more and more young people are becoming obese, the disease is becoming more common in younger age groups.

Type 1 diabetes is characterized by an autoimmune destruction of the insulin producing beta-cells in the pancreas. Macrophages and T-cells cells invade the islets in an inflammatory reaction called insulitis, leading to a loss of most beta-cells with rise of blood glucose levels as a result. The beta-cell mass is usually reduced by 70–80 % at the time of diagnosis [29]. Type 1 diabetes accounts for about 10 % of all cases of diabetes and even though the increase in incidence of type 2 diabetes has received most attention, there is also a rapid rise of type 1 diabetes. The incidence of type 1 diabetes is increasing by ~4 % per year in both high and low incidence populations. It is predicted that between 2005 and 2020, the prevalence of cases in European children under the age of 15 will increase by 70 % [56]. This confirms, and in fact even exceeds, the predictions for 2010 by earlier estimations [52]. Although the treatment and prognosis for type 1 diabetes has progressed dramatically during the last century, the disease remains a major cause of morbidity and mortality [1]. It is both difficult and expensive to treat and is associated with a number of long-term complications, including kidney failure, blindness, nerve damage, and cardiovascular disease. By measuring glycated haemoglobin (HbA1c) one can assess long-term glucose control. Healthy individuals normally have an HbA1c-value of about 4-6 % (international standard) and it is recommended to have a value under 7 % (or 6 % by the Swedish mono-S method) for most patients. Recent results show that for every 1% increase of HbA1c, cardiovascular disease mortality increased by about 50 % in type 1 diabetes [28]. On the other hand, intensive insulin therapy to lower HbA1c will increase the risk of severe hypoglycaemia.
Pancreatic islets

The endocrine portion of the pancreas takes the form of many small clusters of cells called islets of Langerhans [33]. The adult pancreas contains about one million islets and they usually range in diameter from 30 to 300 μm. Although the islets comprise only 1 to 2 percent of the mass of the pancreas, they receive about 10 to 15 percent of the pancreatic blood flow [23, 36].

The islets are located along various points of the pancreatic arterial tree, with large islets being located closer to the major arterioles and small islets being more deeply embedded in the parenchyma of the pancreas [53]. A fine capsule of reticular fibers surrounds each islet, separating it from the adjacent exocrine pancreatic tissue. The islets are richly innervated by parasympathetic, sympathetic and sensory nerves [46, 59].

Each islet contains approximately 2000 cells, and there are four major cell types in the islets of Langerhans – alpha, beta, delta and polypeptide (PP) cells. In rodents the core of the islet contains mainly beta-cells, whereas the other endocrine cells are preferentially located to the mantle. The human islet cellular architecture is more heterogeneous [6, 8]. The precise interplay of the different islet cell populations results in the fine-tuned release of counterbalanced hormones, which regulate especially carbohydrate metabolism. The beta-cells stand for about 50 to 70 percent of the cells in an islet, depending on the species studied, and produce insulin, an anabolic hormone, which lowers blood glucose concentrations by suppression of gluconeogenesis and promotion of cellular glucose uptake and storage. In addition to insulin, islet amyloid polypeptide (IAPP) is released from the beta-cells [16]. IAPP, also denoted amylin, is a member of the calcitonin superfamily of peptides and has been implicated in the pathogenesis of type 2 diabetes through forming amyloid [7, 73]. The alpha-cells produce glucagon, a catabolic hormone which increases glucose concentrations by promoting glycogen breakdown in the liver, and thereby protect the body against hypoglycemia. Delta-cells produce somatostatin, which is an inhibitor of the release of other hormones, including insulin and glucagon. The product of polypeptide cells seems to inhibit the exocrine secretions of the pancreas.

Islet transplantation

The only present cure for type 1 diabetes is transplantation of insulin producing cells by either whole pancreas transplantation or isolated islet transplantation. Whole pancreas transplantation is often done together with kidney transplantation and is therefore performed mainly in diabetes patients with renal complications. Due to improvements in immunosuppression and surgical techniques, both graft and patient survival rates have improved dramatically over the recent years and are now very high. It is still a technically
complicated procedure though, and there is a risk for complications such as thrombosis and infections [68]. Isolated islet transplantation instead of whole pancreas transplantation holds a relatively small risk for the patient, is techni-
ically simple, and has reduced medical costs associated with the procedure which all speaks in favour for the islet transplantation. However, a large number of islets is required to achieve exogenous insulin independence and due to the shortage of donors, this severely restricts the number of patients who can be cured. Moreover, even though the Edmonton protocol markedly increased the success rate of islet transplantation [65], the long term survival of islets is still poor with a mere 10 % insulin independence after 5 years [62].

Many implantation sites such as the liver, spleen, kidney, pleural cavity and thymus have experimentally been tested, but the liver has clinically gained most popularity due to ease of access and lack of major complications. Another advantage is that the liver is the main target for insulin up-
take. However, the intrahepatic environment is not optimal as it for instance has a low oxygen tension which probably causes an early loss of islets as they depend on diffusion of nutrients and oxygen to survive before the re-
vascularization is established.

Islet engraftment and revascularization

Engraftment is the adaptation of the transplanted tissue to its new implanta-
tion site. There are several processes involved, e.g. revascularization, rein-
nervation and reorganization of the stromal and endocrine cells. During iso-
lation of islets, the vascular and nervous connections are disrupted and un-
like the whole organ transplantation where blood flow is immediately res-
tored by vascular anastomosis, the islets must depend on diffusion of
utrients and oxygen to survive until the revascularization is restored. More-
over, during the culture period that usually precedes transplantation, remnant endothelial cells die or dedifferentiate [48, 51, 55]. A partial restoration of
the islet vascular system is completed within 7-14 days after transplantation
[45, 63], but studies on nude mice transplanted with rodent and human islets showed a low oxygenation of the transplanted islets one month post-
transplantation [10] consistent with an insufficient revascularization [34, 43]. Lack ing blood flow also hamper the drainage of hormones released from the endocrine cells, which could lead to a predisposition to form islet amyloid [58, 71, 72].
In order to regain a proper islet function, new blood vessels have to form and rebuild the capillary network in the islets. Formation of new blood vessels in the adult has long been considered to occur almost exclusively from angiogenesis where pre-existing blood vessels form sprouts or ingrowth of transcapillary tissue pillars into existing blood vessels. However, studies have shown that also vasculogenesis, where blood vessels originate from angioblasts, occurs in the vascularization of transplanted islets [12]. In recent years it has become evident that not only recipient endothelium forms the new blood vessels, but also donor endothelium contributes, at least when transplanting freshly isolated islets. Studies have shown that ~30-40% of the endothelial cells incorporated into the new islet vascular system originate from the donor [5, 37, 48].

![Micrograph of a human islet transplanted to the liver of an immune deficient mouse, one month posttransplantation. Blood vessels are stained with the lectin Bandeiraea simplicifolia (red).](image)

**Figure 1.** Micrograph of a human islet transplanted to the liver of an immune deficient mouse, one month posttransplantation. Blood vessels are stained with the lectin Bandeiraea simplicifolia (red).

**Endothelial cells**

Endothelial cells line all blood and lymph vessels forming a monolayer between the blood and interstitial fluid, but endothelial cells are more than just a passive barrier. They are involved in regulation of vascular tone, fluid and solute exchange, haemostasis, coagulation, inflammatory responses, angiogenesis and vasculogenesis [60].

Endothelial cells are extraordinarily diverse in their morphology, function and gene-expression profile. Morphologically, they differ in size, shape, thickness, number of microvilli, and position of the nucleus. The distinction in characteristics is reflected upon their differences in functional demands. Even the same organ can have different phenotypes of endothelium. The kidney for example has fenestrated endothelium in the peritubular region, discontinuous in the glomeruli, and continuous in other regions. The fene-
strated endothelium is also found in endocrine tissues as pancreatic islets which allows for an easy passage of small molecules.

The islet capillaries are about twice as wide and have ten times more fenestrations than capillaries in the exocrine part of the pancreas [20]. The fenestrations are likely induced by local production of vascular endothelial growth factor (VEGF) in the islets [32].

It has been observed that after transplantation, the blood vessels found in cellular transplants acquire the phenotype of the transplanted tissue, irrespective of the implantation organ. Ingrowing capillaries in neural transplants develop a blood-brain barrier [2], whereas blood vessels formed in transplanted islets become wide with a fenestrated endothelium [19].

Endothelial cells can be isolated and cultured *in vitro*, but a problem with culturing endothelial cells is the fact that cell culture progressively de-differentiates endothelial cells from their relatively inactive *in vivo* state with 0.1 % replications per day to an activated phenotype with 1 % to 10 % replications per day, and with loss of specialized functions associated with diverse vessels and organ systems [11]. Techniques to specifically isolate and culture islet endothelial cells have during the years been developed [38, 42, 67]. Islet endothelial cells retain their endothelial cell specific expression of endostatin, pigment epithelium-derived factor (PEDF) and VEGF after culture and ultrastructurally maintain endothelial cell morphology [40, 42]. They also express highly specific proteins for islet endothelium such as e.g. nephrin [74] and alpha1-antitrypsin (α1-AT) [38].
Aim

The overall aim of this research was to investigate the importance and function of endothelial cells in endogenous and transplanted islets of Langerhans.

More specific questions addressed were:

- Can islet-derived factors affect migration and proliferation of endothelial cells?
- Can the p38 MAP kinase inhibitor SB203580 improve revascularisation in transplanted islets?
- Can endothelium-derived cell products affect beta-cell function and gene expression in an in vitro system?
Methods

Animals (I-III)
Inbred male Wistar-Furth rats weighing approximately 300 g were purchased from Scanbur, Sollentuna, Sweden. Male C57Bl/6 mice weighing approximately 30 g were purchased from M&B Research and Breeding Center, Ry, Denmark. The animals had free access to tap water and pelleted food, and were housed in a room with a 12 hour light/dark cycle and humidity of 70 % throughout the study. All experimental procedures were approved by the animal ethics committee for Uppsala University.

Islet isolation and culture (I and III)
Rat pancreatic islets were isolated by using collagenase digestion followed by separation on a density gradient. Under deep anesthesia with sodium pentobarbital (Apoteket, Gothenburg, Sweden; 200 mg/kg body weight i.p.), a laparotomy was performed and the pancreas exposed. After ligation at the ampulla of Vater, 5 ml collagenase (from Clostridium histolyticum; F. Hoffman-La Roche, Basel, Switzerland) was injected into the pancreas via the common bile duct. The animal was killed and the pancreas dissected from surrounding tissues, removed and incubated in a water bath for approximately 16 minutes at 37 °C. The islets were separated by a density gradient (Histopaque-1077; Sigma-Aldrich, St Louis, MO, USA), centrifuged at 900 g for 22 minutes. After washing, islets were handpicked and groups of 150 islets were maintained free-floating in at 37 °C (air/CO₂; 95:5) in culture medium consisting of RPMI 1640 (Sigma-Aldrich) supplemented with L-glutamine (2 mmol/l), benzylpenicillin (100,000 U/l; Hoffman-La Roche), streptomycin (0.1 g/l; Hoffman-La Roche) and 10 % (vol./vol.) fetal calf serum (FCS) for 4-6 days. Culture medium was changed every 48 hours.

Islet isolation and culture (II)
Mouse pancreatic islets were prepared by a collagenase digestion procedure. The mouse was killed by cervical dislocation. The pancreas was removed, cut into pieces and placed in a flask containing a collagenase solution. The
flask was shaken vigorously in a water bath for 20-22 minutes at 37 °C. The digest was washed three times with Hanks’ balanced salt solution (HBSS; The National Bacteriological Laboratory, Stockholm, Sweden) and the islets were subsequently handpicked under a stereo microscope using a braking pipette. Groups of 150 islets were maintained free-floating at 37 °C (air/CO₂; 95:5) in culture medium.

**Endothelium isolation (I and III)**

**Islet endothelium**

20 hand-picked, apparently clean rat islets were transferred onto a collagen matrix (1.8 mg collagen type 1/ml; Nutacon, Leimuiden, the Netherlands) in a 24-well culture dish. The islets were cultured at 37 °C in 1 ml culture medium supplemented with 20 % (vol/vol) FCS and with 100 μg endothelial cell growth supplement (ECGS; Sigma-Aldrich) /ml. Before the expanding cells reached confluence, they were detached with 0.25 % (wt/vol) trypsin-EDTA solution (Sigma-Aldrich) for <5 minutes at room temperature and washed twice in culture medium. The endothelial cells were extracted from the cell suspension using *Bandeiraea (Griffonia) simplicifolia* (BS-1)-coated Dynabeads (Dynal Biotech, Oslo, Norway). The beads with the bound endothelial cells were resuspended in culture medium supplemented with 20 % (vol/vol) FCS and with 100 μg ECGS /ml in a collagen-coated 24-well culture dish.

**Liver endothelium**

Wistar-Furth rats were anesthetized with sodium pentobarbital (200 mg/kg i.p.) and the liver was perfused retrogradely with 25 ml perfusion buffer (142 mmol/l sodium chloride, 6.7 mmol/l potassium chloride, 10 mmol/l HEPES). The liver was then perfused with a collagenase solution (25 mg collagenase dissolved in 8 ml HBSS, dissected out and cut into pieces. The tissue was transferred to vials containing collagenase solution, and shaken vigorously. The emulsion was washed in HBSS and the liver endothelial cells were retracted using BS-1-coated Dynabeads. The beads with the bound endothelial cells were resuspended in culture medium supplemented with 20 % (vol/vol) FCS and with 100 μg ECGS /ml in a collagen-coated 24-well culture dish.
Cell migration assay (I)

Migratory properties of islet and liver endothelium were assessed by using a Boyden chamber with collagen-coated polycarbonate membrane filter of 8.0 μm pore size (Whatman, Springfield Mill, UK). Migration attractants were added to the lower chambers, whereas the cell suspensions were added to the upper chambers. In some cases, VEGF (20 ng/ml; Sigma-Aldrich), neutralizing antibodies to VEGF (1 μg/ml; Lab Vision, Fremont, CA, USA), endostatin (10 μg/ml; Chemicon, Temecula, CA, USA), thrombospondin-1 (tsp-1; 1 μg/ml; Lab Vision), α1-AT, (10 μg/ml; Sigma-Aldrich) or a nonsense antibody were added to the wells with migration attractants. After incubation at 37 ℃ (air/CO2, 95:5) for 4 hours (liver endothelium) or 5 hours (islet endothelium), the cells were fixed and stained with DAPI (Vector Laboratories, Burlingame, CA, USA). The number of migrating cells was counted under a fluorescence microscope.

Figure 2. Schematic drawing of a Boyden chamber. Migration attractants are added to the lower chambers, whereas the cell suspension is added to the upper chambers. A polycarbonate membrane filter separates the two compartments but allows cells to migrate through.

Cell proliferation assay (I)

The proliferatory properties of liver and islet endothelium were investigated using a Bürker chamber. Islet or liver endothelium was seeded to a collagen-coated 24-well culture dish and cultured at 37 ℃ (air/CO2, 95:5) in culture medium. After 24 hours, the cells in some of the wells were directly counted. Culture medium or islet-conditioned culture medium with VEGF (20 ng/ml), neutralizing antibodies to VEGF (1 μg/ml), endostatin (10 μg/ml), tsp-1 (1 μg/ml), α1-AT (10 μg/ml) or a nonsense IgG antibody (10 μg/ml) (R&D
Systems) were added to the endothelial cells in the remaining wells. After 48-hour incubation at 37 °C, also the number of cells in these wells was counted under a light microscope using a Bürker chamber.

Islet transplantation (II)

After culture, groups of 250 islets were packed in a braking pipette and syngeneically transplanted beneath the capsule of the left kidney in C57Bl/6 mice anesthetized with avertin [2.5 % (v/v) solution of 10 g 97 % (v/v) 2,2,2-tribromoethanol in 10 ml 2-methyl-2-butanol (Kemila, Stockholm, Sweden; 0.02 ml/g body weight i.p.).

pO₂ and blood flow measurements in transplanted islets (II)

One month post transplantation, the mice were anesthetized with avertin (0.02 ml/g body weight i.p), maintained at body temperature and tracheostomized. Catheters were inserted into the right carotid artery and left jugular vein. The former catheter monitored mean arterial blood pressure, whereas the latter catheter was used for continuous infusion of Ringer solution to substitute for loss of body fluid.

The graft-bearing left kidney was immobilized in a plastic cup, embedded in cotton wool soaked in Ringer solution (5 ml kg⁻¹ h⁻¹; Sigma-Aldrich) and covered with mineral oil (Apoteket) to prevent evaporation and keep the tissue moist and at body temperature. The blood perfusion of the islet graft and the adjacent renal cortex was measured by laser-Doppler flowmetry (PF 4001-2 Perimed, Stockholm, Sweden) with a needle probe (411, tip 0.45 mm o.d.; Perimed). The flow probe was positioned perpendicular to the immobilized tissue surface by the use of a micromanipulator, and care was taken not to cause any compression of the tissue. At least five blood flow measurements were performed in the transplanted islets and renal cortex in each animal. The mean of these measurements from each animal was calculated and considered to be one experiment. Since it is difficult to calibrate the instrument in physical units of blood flow, all blood flow values are given as arbitrary tissue perfusion units (TPU).

The partial pressure of oxygen was measured in the islet graft and the adjacent renal parenchyma with modified Clark microelectrodes (Unisense, Århus, Denmark). The electrodes were inserted into the tissues by the use of a micromanipulator under a stereo microscope. At least 10 measurements were performed in both the islet graft and the renal cortex. The mean of all measurements, in each tissue and animal, was calculated and considered to
be one experiment. During the blood flow and pO₂ measurements, blood pressure, body temperature and tissue temperature were continuously recorded with a MacLab Instrument (AD Instruments, Hastings, UK) connected to a computer.

Measurements of pO₂ in native islets (II)

Control animals were anesthetized with avertin (0.02 ml/g body weight i.p) and surgically prepared similar to the transplanted animals. However, in this case the pancreas instead of the kidney was exposed, immobilized, and its islets visualized. Measurements of pO₂ were performed in ≥3 superficial pancreatic islets and in the surrounding exocrine parenchyma of each animal. Multiple measurements were usually performed in the same islet; the mean was calculated to obtain the pO₂ value for one islet. The mean of the islet oxygen tension values in one animal was then considered to be one experiment. The blood perfusion of native islets cannot be determined by laser Doppler flowmetry due to their small size.

Light microscopic evaluation (II)

The graft-bearing left kidneys, or pancreata from control animals, were removed, fixed in formaldehyde and embedded in paraffin. Consecutive sections of the islet grafts and pancreata were prepared and stained with the lectin BS-1 [41] and counterstained with hematoxylin. In each animal, ≥12 tissue sections stained with BS-1 from all parts of the pancreas or islet transplants were randomly chosen and evaluated. The numbers of stained blood vessels in native and transplanted islets were quantified under a light microscope. In the islet grafts, connective tissue surrounded the individual islets in the grafts. The number of blood vessels in the endocrine and connective tissue was therefore counted separately. The fraction of endocrine and connective tissue was determined by a direct point-counting method. For this purpose, the number of intersections overlapping connective tissue stroma and endocrine cells within the islet grafts was counted at a magnification of 600X. Approximately 12 fields (corresponding to ~1500 points) were counted in each islet graft. The area of the investigated native or grafted islets was determined by using a computerized system for morphometry (ImageJ). Vascular density, i.e. the number of stained blood vessels found per measured islet or graft area (mm²), was then calculated.
Insulin release and total insulin content (III)

Groups of 10 rat islets were transferred in triplicates to glass vials containing 250 μl Krebs-Ringer bicarbonate buffer supplemented with HEPES and bovine serum albumin (BSA; MP Biomedicals, Irvine, CA, USA, buffer hereafter referred to as KRBH buffer). The KRBH buffer contained 1.67 mmol/l D-glucose (Sigma-Aldrich) during the first hour of incubation at 37 °C. The medium was removed and stored at -20 °C, and replaced by 250 μl KRBH supplemented with 16.7 mmol/l glucose and incubated for a second hour. The medium was retrieved and stored at -20 °C. To measure the total insulin content, the islets were harvested, pooled in groups of 30, and homogenized by sonication in 200 μl redistilled water. A fraction of the homogenate was mixed with acid-ethanol from which insulin was extracted overnight at 4 °C. Samples were then stored at -20 °C until insulin measurement (see below).

Insulin measurement (III)

After appropriate dilution, insulin concentrations in incubation medium and homogenates were determined by a commercial insulin ELISA (Mercodia, Uppsala, Sweden).

Glucose oxidation (III)

Islet glucose oxidation rates were determined by incubating groups of ten islets in KRBH supplemented with carbon-14 labeled glucose (GE Healthcare Life Sciences, Chalfont St Giles, UK) and non-radioactive glucose to a final glucose concentration of 16.7 mmol/l. The produced $^{14}$CO$_2$ was captured with Hyamin. After incubation for 90 min at 37 °C (O$_2$/CO$_2$; 95:5), the oxidation was terminated by injection of antimycin A into the vials. The $^{14}$CO$_2$ generated by cell metabolism was released by the addition of NaH$_2$PO$_4$ during a 120-minute incubation. The radioactivity in the samples was then measured by liquid scintillation counting.

(Pro)insulin biosynthesis (III)

Groups of 10 rat islets were radioactively labeled by incubating them in duplicates for 2 hours in 100 μl of Krebs–Ringer buffer containing BSA, 16.7 mmol/l glucose and tritiated leucine (American Radiolabeled Chemicals, St Louis, MO, USA) at 37 °C (O$_2$/CO$_2$; 95:5). After incubation the islets were washed in HBSS containing non-radioactive leucine, and then sonicated in
200 μl redistilled water. The islet homogenates were frozen before immunoprecipitation.

**Insulin degradation (III)**

Islet intracellular degradation of insulin was determined using a pulse-chase method. The islet proteins were labeled by maintaining about 150 islets in the presence of tritiated leucine yielding a specific radioactivity of 3.3 GBq/mmol in the medium during a 72 hour incubation. The islets were washed in non-radioactive medium, and duplicate groups of 15 islets were homogenized by sonication in 200 μl of a glycine buffer containing BSA. The remaining islets were divided into 2 aliquots which were incubated for 24 hours without radioactivity. After the chase period duplicates of 15 islets were homogenized by sonicaton in 200 μl glycine-BSA-buffer. 100 μl portions of the incubation medium were also retrieved. The islet homogenates and incubation medium were frozen before immunoprecipitation.

**Immunoprecipitation (III)**

Portions of 10 μl from the islet homogenates and the 100 μl medium samples from the islet incubations were mixed with 100 μl of a glycine buffer supplemented with BSA and Triton® X-100, and in duplicates supplied with 10 μl of guinea pig anti-human insulin serum or 10 μl normal guinea pig serum. The immune binding was allowed to proceed for 1 hour at room temperature. Subsequently 50 μl rProtein-A-Sepharose™ Fast Flow suspended in glycine-BSA-Triton® X-100 buffer was added and the material was tumbled for 15 minutes at room temperature and then centrifuged for 3 minutes at 4000 x g. The Sepharose™ sediment with the anti-insulin-insulin complex was washed twice with glycine-BSA-Triton® X-100 buffer and finally resuspended in 500 μl acetic acid with BSA. After addition of 4 ml Ultima Gold™ scintillation fluid, the radioactivity was determined by a liquid-scintillation spectrometer using external standardization. Non-specific binding was corrected for by subtracting the radioactivity bound by normal guinea pig serum from that precipitated by immune serum.

**Gene expression analysis (III)**

Isolation of total RNA was performed with RNeasy Micro Kit (Qiagen, Hilden, Germany) and all the samples were treated with RNase-free DNase (Qiagen). Conversion of RNA to cDNA was performed with reverse transcription system (Promega, Madison, WI, USA) using oligo (dT)15 primers.
The LightCycler System (Hoffman-La Roche) and detection with SYBR Green (SYBR Green JumpStart Taq ReadyMix), was used to amplify and analyze generated cDNA. The primers used were glucose transporter 2 (GLUT-2), pyruvate carboxylase (Pcx), glucokinase (Gck), mitochondrial glycerol-3-phosphate dehydrogenase (mGpdh), pancreatic and duodenal homebox gene 1 (Pdx1), insulin gene (ins1) and laminin (LM) chains: α4, α5, β1and β2. TATA box binding protein (TBG) was used as housekeeping gene. PCR amplifications were performed in a total volume of 10 μl, containing 1 μl of cDNA, 1 μl of each primer, 4 μl SYBR Green JumpStart Taq ReadyMix and RNase-free water added to the final volume. The results are presented as threshold cycle values (CT), i.e., the estimated amplification cycle number when the fluorescence exceeds a specific threshold value. The CT values were used to calculate the amount of PCR product in comparison to TBP by subtracting the CT value for TBP from the CT value for the gene studied (ΔCT). Relative mRNA expression was calculated as \(2^{-\Delta CT}\).

Statistical analysis (I-III)

Values are expressed as the mean ± SEM. When only two groups were compared, Student’s t-test was applied. Multiple comparisons between data were performed using analysis of variance (ANOVA) and Bonferroni’s post-hoc test. For all comparisons, P-values <0.05 were considered statistically significant.
Study design

Paper I
In this study we investigated the migration and proliferation properties of isolated liver and islet endothelial cells in response to islet cell products, using Boyden and Bürker chambers.

Islet and liver endothelial cells were isolated by the use of Dynabeads coated with a lectin that binds endothelial cells. Rat islets were isolated and cultured for 4 days to minimize contamination with exocrine tissue and passenger leukocytes. Islet-conditioned medium was then obtained by culturing the isolated islets in fresh culture medium for 48 hours. Migration and proliferation were investigated with islet-conditioned culture medium with or without VEGF, neutralizing antibodies to VEGF, endostatin, tsp-1, or α1-AT.

Paper II
The effect of the p38 MAP kinase inhibitor SB203580 on islet revascularization was investigated in this study. Islets were isolated and kept free-floating for 5 days. After culture, groups of 250 islets were syngeneically implanted beneath the kidney capsule of male C57Bl/6 mice. During the first 14 days following transplantation, the islet-transplanted mice received a daily i.p. injection SB203580 dissolved in saline or saline alone. One month post transplantation the blood perfusion and partial oxygen pressure of the graft and the adjacent renal cortex was measured by laser Doppler flowmetry and modified Clark microelectrodes respectively. Control animals were surgically prepared similar to the transplanted animals. However, in this case measurements of partial oxygen pressure were performed in superficial pancreatic islets, and in the surrounding exocrine parenchyma of the pancreas instead of the kidney. The blood perfusion of native islets cannot be determined by laser-Doppler flowmetry due to their small size.
This study aimed to elucidate the importance of endothelial cell products for islet function. Islet endothelial cells were isolated by the use of Dynabeads coated with a lectin that binds endothelial cells. Endothelium-conditioned culture medium was obtained by incubating the endothelial cells in fresh culture medium for 24 hours. Rat pancreatic islets were isolated and cultured for 5 days and subsequently incubated in endothelium-conditioned culture medium with or without addition of LM-111 or control culture medium for 24 hours. After the incubation the islets were collected and insulin release, insulin content, glucose oxidation rate, (pro)insulin synthesis and intracellular insulin degradation were investigated. mRNA expression of genes encoding GLUT-2, Pcx, Pdx-1, Gck and mGpdh was also studied in the islets. In order to investigate whether laminin is expressed in the endothelial cells, the mRNA expression of genes encoding laminin chains α4, α5, β1 and β2 were measured in endothelial cells and whole islets.

Figure 3. Isolated islets were cultured and the outgrowing cells were purified with magnetic Dynabeads coated with the lectin BS-1. Endothelial-conditioned culture medium was obtained by incubating the purified endothelial cells in fresh culture medium for 24 hours. After the incubation in the endothelium-conditioned medium, islets were collected and insulin release, insulin content, glucose oxidation rate, (pro)insulin synthesis and intracellular insulin degradation were investigated.
Results

Paper I

Endothelial cell migration

Rat liver endothelium had a higher tendency to migration than rat islet endothelial cells towards islet-conditioned culture medium. Liver endothelial cells also displayed an increased migration towards islet-conditioned culture medium when compared to migration towards control medium. This chemotactic effect of the islet-conditioned culture medium was fully prevented by addition of a neutralizing VEGF-antibody. Moreover, addition of VEGF but not a nonsense antibody to culture medium caused migration of liver endothelial cells. The supplementation of neutralizing antibodies directed towards endostatin, tsp-1 or α1-AT to the islet-conditioned culture medium had no additive effect on the liver endothelial cell migration. Although we extended the time for migration from 4 to 5 hours, we could not find a positive chemotactic effect of either control medium or islet-conditioned culture medium on rat islet endothelial cells. However, when neutralizing antibodies directed towards either endostatin, tsp-1 or α1-AT was added, the migratory capacities of islet endothelial cells towards islet-conditioned culture medium were improved. No additive effects were observed when all three antibodies were applied. Neither a VEGF-antibody, nor a nonsense IgG antibody, had any effects on islet endothelial cell migration.

Endothelial cell proliferation

Islet-conditioned culture medium had a stimulatory effect on rat liver endothelium proliferation compared to control medium. As for the liver endothelial cell migration, this effect of the islet-conditioned culture medium was fully preventable by addition of a neutralizing VEGF-antibody. Moreover, addition of VEGF, but not a nonsense antibody, to culture medium caused increased proliferation of the liver endothelial cells. The supplementation of neutralizing antibodies directed towards endostatin, tsp-1 or α1-AT to the islet-conditioned culture medium had no additive effect on the liver endothelial cell proliferation. Islet endothelial cell number increased very slowly during culture. A slight stimulatory effect of islet-conditioned culture medium on islet endothelial cell numbers was observed when compared to con-
control medium. This effect could be abolished by addition of a VEGF-antibody to the islet-conditioned culture medium, and was mimicked by addition of VEGF but not a nonsense antibody to control culture medium. However, a further increase in islet endothelial cell proliferation was obtained after addition of neutralizing antibodies directed towards either endostatin, tsp-1 or α₁-AT to the islet-conditioned culture medium. As for the effects on cellular migration, no additive effects were observed when more than one of the antibodies was applied.

Paper II
Vascular density
The vascular density of the transplanted islets was only ~20 % of that in native islets, but the connective tissue surrounding each individual transplanted islet was, on the other hand, highly vascularized. There was a rather large variation in the total number of blood vessels between different grafts. However when evaluated for each individual animal, such variations could be disregarded and this analysis showed that treatment with SB203580 in the early post transplantation phase led to a redistribution of the islet graft blood vessels from the stroma into the endocrine tissue.

Oxygen tension and blood flow
There was a higher oxygen tension in the transplanted islets following p38 MAP kinase inhibition compared to control islets, although all transplanted islets had a markedly lower oxygen tension than native islets and the total number of vessels in the grafts were unaffected by SB203580. The blood perfusion of the islet grafts was also similar in control and SB203580-treated animals and was only 40-50 % of that in the adjacent renal cortex.

Paper III
Purity and characteristics of endothelial cell preparations
CD31-staining was used to characterize the endothelial cells purified by BS1-coated Dynabeads. Evaluation by manual counting showed that the cultured sorted cells contained 89±6 % of cells positive for the endothelial cell marker CD31. The purified isolated cell preparations were further validated as endothelial cells by positive staining for a second CD31 antibody, vascular endothelial cadherin (VE-cadherin) and by induced uptake of Dil-Ac-LDL. Human dermal microvascular endothelial cells were used as positive control. Moreover, the vast majority of the isolated cells and rat brain micro-
vascular endothelial cells were negative for the smooth muscle cell marker alpha smooth muscle actin (α-SMA) while mesenchymal stem cells (MSC) were positive for α-SMA but negative for all the endothelial cell markers.

Insulin release and content
The basal insulin release at 1.67 mmol/l glucose did not differ between islets that had been exposed to endothelium-conditioned culture medium or control medium, whereas the response in insulin release to 16.7 mmol/l glucose was augmented. Addition of a function-inhibiting antibody towards the β1-chain of LM neutralized the positive effects of endothelium-conditioned culture medium on glucose-stimulated insulin release. Moreover, there was an increase in insulin release from islets incubated in culture medium with an addition of LM-111 when compared to islets incubated in culture medium alone. The total islet insulin content was increased in islets that had been exposed to endothelium-conditioned culture medium. Neutralization of the LM β1-chain in the endothelium-conditioned culture medium prevented its positive effects on islet insulin content. Moreover, there was an increase in insulin content from islets incubated in culture medium with addition of LM-111 when compared to culture medium alone.

Glucose oxidation rate
When exposed to 16.7 mmol/l glucose, the islets cultured in endothelium-conditioned culture medium had a 60 % increase in glucose oxidation rate compared with islets cultured in control culture medium.

Islet (pro)insulin biosynthesis and total protein biosynthesis
The (pro)insulin biosynthesis did not differ between the islets that had been cultured in control culture medium compared with endothelium-conditioned culture medium. Similar results were observed irrespectively of whether the (pro)insulin biosynthesis per se was measured or was expressed as a ratio of total protein biosynthesis.

Insulin degradation
Islet intracellular degradation of insulin was determined using a pulse-chase method including a 72 hour pulse with [3H]leucine and a subsequent 24 hour chase in control culture medium or endothelium-conditioned culture medium. After the chase period the content of radioactively labelled insulin was measured and calculated as a percentage of the radioactive insulin in the islets retrieved immediately after the pulse incubation. Lacking insulin was regarded as degraded intracellularly, since no insulin degradation occurs in
the incubation media at the present conditions. The islets incubated in control culture medium during the chase period degraded 57% of the intracellular insulin, whereas the islets incubated in endothelium-conditioned culture medium only degraded 41% of the intracellular insulin.

Gene expression
There was no difference in mRNA expression of genes encoding GLUT-2, Pcx or Pdx1 in islets that had been cultured in endothelium-conditioned culture medium compared with control culture medium. However, islets cultured in endothelium-conditioned culture medium had increased mRNA expression of Gck when compared with islets cultured in control culture medium. Likewise, the mRNA expression for the gene for mGpdh was increased in islets cultured in endothelium-conditioned culture medium compared with control culture medium. In order to investigate whether LM is indeed expressed in the endothelial cells, the mRNA expression of genes encoding LM chains α4, α5, β1 and β2 were measured in endothelial cells and whole islets. All tested LM chains displayed markedly higher mRNA expression in endothelial cells compared with whole islets. There was no difference in mRNA expression of the insulin gene Ins1 in islets cultured with control culture medium with addition of LM-111 compared with islets cultured in control culture medium alone.
Discussion

Transplantation of pancreatic islets is currently the only treatment for curing patients with type 1 diabetes mellitus. At present, however, islets from at least two donor pancreata are usually needed to cure a diabetic individual. The reasons for the need of this huge number of islets is unclear, but likely reflects both cell death in the immediate post transplantation period [3, 14, 15], as well as dysfunction of surviving islets [35, 44, 57]. Pancreatic islets normally have a dense vasculature, which is of importance to provide oxygen and nutrients, allow for an accurate glucose sensing and disperse hormones to the systemic circulation. Recent evidence also indicates an important role for the islet endothelium in sustaining beta-cell functions and growth [27, 47]. Poor revascularization of the transplanted islets is therefore one potentially contributing factor to islet graft dysfunction, by causing low delivery of oxygen to the islet cells with resulting lactate formation in the islet tissue [24], but perhaps also by causing lack of paracrine support for the beta-cells. Hence, strategies to speed up and improve islet revascularization are needed.

VEGF and revascularization

The liver has so far been the most commonly used transplantation site for islets due to its accessibility and since the liver is the main target for insulin uptake. The first ingrowing blood vessels can be seen within a few days after transplantation and blood flow is established after 7-14 days. In general, angiogenesis is known to be driven by hypoxia in cells lacking sufficient blood supply and this leads to the formation of a number of different growth factors which stimulate angiogenesis, such as hepatocyte growth factor and VEGF. VEGF is expressed in islets both in vitro and after transplantation and is a potent mitogen and survival factor for the endothelial cells [70]. In our study (I), islet-conditioned culture medium caused liver endothelial cells to migrate and proliferate, suggesting that indeed some islet secretory product has the capacity to stimulate attraction of recipient endothelium following islet transplantation. This is also consistent with our earlier observations in vivo that transplanted islets induce a dense vascular network in their immediate vicinity, which fails to develop if plastic microbeads of similar size as islets instead are implanted [43]. We hypothesized that the main islet se-
cretory product that stimulated liver endothelial cells to migrate and proliferate was VEGF. By administering VEGF-neutralizing antibodies to the islet conditioned medium, the induced liver endothelial cell proliferation and migration was blocked. Moreover, addition of VEGF to the culture medium mimicked the boosting proliferatory and migratory effect obtained with islet-conditioned culture medium.

A large number of blood vessels can be found in the connective tissue surrounding implanted islets, but few blood vessels are present within the transplanted endocrine tissue [43], suggesting incapability of endothelial cells within islets to proliferate and migrate. Migration and proliferation of endothelial cells within islets may be important to connect remnant intra-islet blood vessels to recipient blood vessels, and to support ingrowth of capillaries towards the beta-cell rich islet core. In our assay, rat liver endothelium had a higher tendency to migrate towards islet-conditioned culture medium than rat islet endothelial cells (I). The islet endothelial cells normally had a very low capacity for both migration and proliferation towards islet secretory products including VEGF. Increased concentrations of VEGF are secreted from beta-cells in response to hypoxia and transplantation, but this is not enough to prevent islets from becoming poorly revascularized. Only a marked over-expression of VEGF in islets [31, 75] has been shown to stimulate angiogenesis and improve intra-islet revascularization. In fact, occurring concentrations of VEGF does not seem to affect islet revascularization at all [64]. This could be explained by VEGFs ability to induce p38 MAP kinase by binding to its receptors. p38 MAP kinase serves as a signaling molecule to separate between angiogenesis- and vascular permeability-inducing properties of VEGF [22]. In our study using SB203580 to inhibit p38 MAP kinase activity (II), we could see a redistribution of islet graft blood vessels from the stroma into the endocrine tissue. The vascular density of the transplanted islets was only ~20 % of that in native islets but the connective tissue surrounding each individual transplanted islet was, on the other hand, highly vascularized. This means that p38 MAP kinase activity in islet graft endothelial cells normally interferes with the migration of these cells into the endocrine tissue towards VEGF secreted by beta-cells. The redistribution of blood vessels into the endocrine tissue was accompanied by an increased oxygenation of the islet cells, although all transplanted islets had a markedly lower oxygen tension than native islets and the total number of vessels in the grafts were unaffected by SB203580. The blood perfusion of the islet grafts was similar in control and SB203580 treated animals. The rich VEGF-induced fenestrations of the adult islet endothelial cells are known to facilitate rapid passage of proteins such as secreted insulin. Treatment with SB203580 did, however, in our experiments not induce diabetes during the treatment period. This is in line with previous observations when VEGF was deleted in either most pancreatic beta-cells or the entire pancreatic epithelium of mice; in both scenarios this did not induce overt diabetes, but only mild glucose intolerance [21, 32]. These results implicate that
VEGF can stimulate endothelial cells to proliferate and migrate towards the transplanted islets, but fail to stimulate ingrowth and/or the capability for remnant intra-islet blood vessels to grow and connect to recipient blood vessels. This mirrors the effects of VEGF to preferentially induce vascular permeability instead of promoting migration and proliferation and may contribute to the low revascularization of transplanted islets. Inhibition of the p38 pathway may be a new therapeutic approach to improve clinical islet transplantation results, given that it proves safe and have the same beneficial effects on the outcome of islet transplantation also in larger animals.

Angiostatic factors

Instead of promoting occurring proangiogenic factors, an even more effective way to promote angiogenesis could be to silence the angiostatic factors. Especially since some of these angiostatic factors exert their way through inducing apoptosis in proliferation endothelium [26]. Furthermore, finding ways to pharmacologically inhibit angiostatic factors during a short period after transplantation are more likely much more feasible in the clinical setting, than trying to increase the expression of proangiogenic factors in the transplanted tissue. The angiostatic factor α1-AT is produced by the endothelial cells themselves and is important for the low basal proliferation rate seen in islet endothelium [38]. On the other hand, presence of this factor in the endothelium also seems to be of importance to prevent the development of type 1 diabetes, at least in a non-obese diabetic mouse model [66]. We therefore decided to test not only to inhibit α1-AT, but also two other potent angiostatic factors, i.e. endostatin and tsp-1, which has been described to be produced by islets including the islet endothelial cells themselves [9, 13, 69]. When neutralizing antibodies directed towards either endostatin, tsp-1 or α1-AT was added, the migratory capacities of islet endothelial cells towards islet-conditioned culture medium were improved (I). Notably, addition of neutralizing antibodies to endostatin or tsp-1 showed to be as potent as inhibition of α1-AT to improve islet endothelial migration and proliferation in our assays. Mice deficient of tsp-1 have been described to not only have hyperplastic but also markedly hypervascular islets [13], which indicates that tsp-1 is important to prevent islet vascular expansion in vivo. In our recent experiments, depletion of tsp-1 in islets for transplantation also seems to improve islet graft revascularization and function [50]. Most interestingly, we could find no additive effects to even further increase islet endothelial cell migration and proliferation by blocking all three investigated angiostatic factors. This suggests that intervention towards only one of these factors is needed to fully activate the angiogenic properties of islet endothelium.
Figure 4. Angiogenesis is tightly regulated by a number of angiogenic and angiosstatic factors. Under normal conditions, angiostatic factors are predominant and angiogenesis does not occur.

Endothelium and beta-cell function

The pancreatic islets in the adult are among the most vascularized of all organs in the body with a unique dense glomerular-like angioarchitecture. This means that in the islet, each beta-cell is surrounded by at least one islet endothelial cell, and therefore these cells by necessity are exposed to each others products [4]. Endothelial cells produce a number of growth factors, cytokines and other substances that may affect not only the endothelial cells themselves, but also surrounding cells [60].

We exposed rat pancreatic islets to islet endothelial cell products and found that insulin release was increased with more than 40 % in response to 16.7 mmol/l glucose and that the insulin content was increased with 35 % compared to control. At least part of the improved insulin release after exposure to endothelial cell product was explained by increased mitochondrial activity as assessed by glucose oxidation. This was also reflected in an upregulation of the mitochondrial gene mGpdh.

We could see no increase in (pro)insulin biosynthesis despite the fact that both insulin release and total insulin content was increased in the islets exposed to endothelial cell products. This suggests that any raised expression of the insulin gene [47] was not translated to protein levels. The culture medium was analyzed to make sure that the increased insulin content in the islets did not result from an effect of inhibited insulin release to the medium during the culture in endothelium-conditioned culture medium. Instead, we found a decrease in intracellular insulin degradation by 57 %. In an attempt to find the factors responsible for the positive effects of the endothelial cell products on beta-cell function, a number of different neutralizing antibodies or receptor antagonists were tested to see if they could remove these positive effects. The grounds for the selection of the interventions were based on
previous observations by us and others. Tsp-1 is a natural inhibitor of angiogenesis expressed in islet endothelium, which we in tsp-1 deficient mice recently have observed is important for beta-cell differentiation [49]. Endothelin-1 is a potent vasoconstrictor derived from endothelial cells, which is known to stimulate insulin release [17]. HGF was recently shown by us to be expressed by islet endothelial cells and to stimulate beta-cell proliferation [27]. It has previously been shown that beta-cells do not form their own basement membrane, but rely on endothelial cells to form a vascular basement membrane containing laminins next to the beta-cell [47]. LM-111 has been demonstrated to promote differentiation of pancreatic precursor cells into beta-cells in vitro [25] and to promote glucose-stimulated insulin secretion in vitro [18, 30, 54]. Prostaglandins exert a number of effects on islet beta-cells, although mostly negative [39, 61].

The only factor investigated that could neutralize the positive effects of endothelial cell products on islet insulin content and glucose-stimulated insulin release, was an antibody towards the β1-chain of LM. A pronounced increase in insulin content and glucose-stimulated insulin release compared to control islets could be seen when the positive effect of LM was further investigated by adding LM-111 to islets in culture. The results from the RT-PCR studies showed that islet endothelial cells had markedly higher mRNA-levels of LM chains α5 and β1 than whole islets. The results demonstrate that endothelial cells can be important not only for transport functions by providing nutrients and oxygen to parenchymal cells, but also for paracrine functional support. At least part of the beneficial effect of islet endothelial cells on beta-cells can therefore be attributed to LMs.
Conclusions

- Islet-derived factors such as VEGF cause increased migration and proliferation of liver endothelial cells. However, the migration and proliferation of islet endothelial cells are hampered by angiostatic factors.

- Treatment with the p38 MAP kinase inhibitor SB203580 redistributes islet graft blood vessels from the stroma into the endocrine tissue and increases oxygenation of the islet cells.

- Islet endothelium-derived factors, including laminins, have a positive effect on islet function.
Sammanfattning på svenska

Bakgrund


Delarbete I


Leverendotelet visade en ökning av både proliferation och migration mot faktorerna utöverade från öar. Dessa effekter försvann om antikropp mot VEGF tillsattes. Till skillnad från leverendotelet så kunde inte ö-producerad VEGF få endotelcellerna isolerade från öar att migrera och det hade mycket
liten effekt på ö-endotelsproliferationen. Det gick dock att aktivera både proliferation och migration av ö-endotelet genom att tillsätta antikroppar mot angiostatiska faktorer som endostatin, thrombospondin-1 eller alfa1-antitrypsin.

Sammanfattningsvis visar studien att VEGF producerat av öar kan attrahera blodkärl mot öar transplanterade i levern, men att blodkärlsbildningen inuti öarna hindras av angiostatiska faktorer som bildas av öarna och dess endotel. Ett sätt att få en bättre blodförsörjning i öarna skulle kunna vara att blockera angiostatiska faktorer tidigt efter transplantationen.

Delarbete II

VEGF är en tillväxtfaktor för nybildning av blodkärl men inducerar också blodkärlspermeabilitet. p38 MAP kinas har visats kunna fungera som en växel mellan dessa funktioner av VEGF. Vår hypotes var att blodkärlsnybildningen i transplanterade öar hindrades av VEGFs förmåga att inducerera permeabilitet istället för migration och proliferation. Ett stort antal blodkärl kan ses i bindväven runt om de transplanterade öarna, men få blodkärl växer in. I det andra delarbetet undersökte vi om p38 MAP kinas-inhibitorn SB203580 kunde förbättra VEGF-inducerad kärlbildning och samtidigt förhindra VEGF-inducerad vaskulär permeabilitet.

Möss transplanterades med 250 öar under njurkapseln och gavs SB203580 dagligen under de första 14 dagarna efter transplantationen. Efter 1 månad tittade vi på hur syrgastrycket, genomblödningen och kärldensiteten i transplantaten hade förändrats i de behandlade djuren jämfört med kontroll.

Behandling med SB203580 visade sig omfördela blodkärlen från den omkringliggande bindväven till de transplanterade öarna och detta följses av en ökad syresättning av ö-cellerna. Blodperfusionen i öarna var dock densamma för kontrollmössen och för mössen behandlade med inhibitorn.

Våra resultat visar att VEGFs förmåga att företrädelsevis inducera permeabilitet i blodkärl kan delvis bidra till den dåliga blodkärlsbildningen i transplanterade öar.

Delarbete III


Ö-endotelceller från rätta renades fram och med hjälp av dessa produce-rades endotelcells-konditionerat medium, innehållande faktorer utsändrade
från ö-endotelet. Funktionen och genuttrycket hos isolerade öar från råtta som odlats i detta medium undersöktes därefter.

Den glukos-stimulerade insulinfrisättningen och insulininnehållet i öarna ökade markant efter odling med endotelcells-konditionerat medium. Detta kunde åtminstone delvis förklaras av förbättrad mitokondriefunktion som utvärderades genom glukosoxidation och en uppreglering av mitokondriegenen mGpdh. Trots att både insulinfrisättningen och insulininnehållet i ökade, kunde vi inte se en ökning av insulinbiosyntesen. Istället såg vi att den intracellulära nedbrytningen av insulin minskade. För att ta reda på vilken faktor det var i vårt endotelcellsmedium som gav dessa effekter på öarna, testade vi ett antal antikroppar mot faktorer som bildas av endotelceller. En antikropp mot laminin var den enda som kunde neutralisera de positiva effekterna av endotelet i vår studie. Tillsats av laminin till kontrollmedium visade sig också ge ökad insulinfrisättning och innehåll.

Studien visar att endotelceller kan påverka betacellsfunktionen och att detta åtminstone delvis beror på laminin.
Acknowledgements

Even though my name is the only one on the front page, the making of this thesis was not a one-woman job. I wish to express my sincere gratitude to the many people who have contributed in different ways. In particular, I would like to thank:

My supervisor, Professor Per-Ola Carlsson, for introducing me to this field of research, for your guidance, support and never ending enthusiasm!

My co-supervisor, Professor Leif Jansson, for sharing his deep knowledge in the field of islet transplantation.

Former Head of Department, Professor Arne Andersson, for valuable feedback and advice.

Professors and senior scientists, Stellan Sandler, Håkan Borg, Nils Welsh, Michael Welsh, Carina Carlsson, Gunilla Westermark, Ulf Eriksson, Parri Wentzel, Mia Philipsson, Johanna Henriksnäs, Monica Sandberg and An-dreaa Barbu for critical questions and creative suggestions.

The former and present members of my group; Göran Mattsson for staining advice and letting me take over his computer, Magnus Johansson for all protocols and for introducing me to how things work at the lab, Joey Lau for help with PCR, Johan Olerud for help with endothelial cell isolation and computer assistance, Sara Bohman my sister in arms, for protocols, being a great colleague and friend, Gustaf Christoffersson and Ulrika Pettersson for great instant advising, laughs and for playing in my band.

Peetra Magnusson, for all your help with the endothelial cells and for reading my manuscript. Du är en klippa!

The laboratory technicians, especially, Astrid Nordin, Lisbeth Sagulin, Ing-Marie Mörsare and Eva Thörnelius for invaluable technical assistance and advice.

Agneta Sandler Bäfwe, and Marianne Ljungkvist, for taking care of all the paper work and doing so with a smile. Göran Ståhl for quick assistance.
The physiology people, for sharing their corridor and fika-room with me, even after the move.

All present and former PhD students at the department, for making the workplace more enjoyable.

My sister Hanna and her husband Fredrik, for being there whenever I need you. Hanna, you’re the best sister one can wish for.

My parents, Lars and Christel, for your unconditional love, support and encouragement.

Mikael, for loving me when I don’t deserve it, for wanting what’s best for me at all times, for knowing just what to say to cheer me up. I love you.

Financial support for these studies was provided by: The Swedish Research Council (55X-15043), The Juvenile Diabetes Research Foundation, the EFSD/JDRF/Novo Nordisk Research Programme 2006, the Swedish Diabetes Association, the Swedish Juvenile Diabetes Fund, the Novo Nordisk Foundation, the Anér Foundation, the Åke Wiberg Foundation and the Family Ernfors Fund.
References


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