Role of Islet Endothelial Cells in \(\beta\)-cell Function and Growth

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Dissertation presented at Uppsala University to be publicly examined in Room B:41, BMC, Husargatan 3, Uppsala, Monday, June 12, 2006 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

The pancreatic islets are collections of endocrine cells, dispersed throughout the pancreas. In adult islets, endocrine cells are closely associated with capillary endothelial cells and receive a high blood perfusion. Transplanted pancreatic islets, on the other hand, have a vascular disturbance, manifested as decreased blood vessel density. Besides impaired islet blood perfusion and oxygenation, this means that the normal close proximity between endothelial cells and \( \beta \)-cell in adult islets is interrupted. The aim of the thesis was to investigate if, and to what extent, \( \beta \)-cells and islet endothelial cells can interact with one another. This hypothesis was investigated during physiological growth of pancreatic islets, following transplantation and in vitro. We observed that islet endothelial and endocrine cell replication coincided immediately after birth, as well as during pregnancy. In pregnant animals, \( \beta \)-cell proliferation colocalized to islets with increased endothelial cell replication, indicating that the two processes were interconnected. The pregnancy hormone prolactin favored endothelial cell replication, and these activated cells could then augment \( \beta \)-cell proliferation. We found that prolactin pretreatment increased blood vessel density and oxygen tension in islets after transplantation. Furthermore, prolactin pretreatment improved endocrine function in a minimal islet transplant model. Partial pancreatectomy performed in association with islet transplantation improved revascularization, oxygen tension and glucose stimulated insulin release from the graft. In conclusion, the findings suggest that endocrine and endothelial cells interact with one another to regulate growth and function in pancreatic islets. This may form the basis for interventions aiming to improve revascularization and function of transplanted islets.

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in vivo veritas
List of Papers

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


IV Improved vascular engraftment and function of autotransplanted pancreatic islets: positive effects induced by partial pancreatectomy. Magnus Johansson, Leif Jansson and Per-Ola Carlsson. Manuscript

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<tr>
<td>BS-1</td>
<td>Bandeiraea (Griffonia) simplicifolia</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CM</td>
<td>culture medium</td>
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<td>ECCM</td>
<td>endothelium conditioned CM</td>
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<td>EcM</td>
<td>endothelial cell culture medium</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<td>ICCM</td>
<td>islet conditioned CM</td>
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<td>IECCM</td>
<td>islet and endothelium conditioned CM</td>
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<td>LI</td>
<td>labeling index</td>
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<td>PRL</td>
<td>prolactin</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>tsp-1</td>
<td>thrombospondin-1</td>
</tr>
<tr>
<td>UE</td>
<td>Ulex europaeus</td>
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<td>VEGF-A</td>
<td>vascular endothelial growth factor-A</td>
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Introduction

Insulin, a hormone produced by β-cells in the pancreatic islets, is the key regulator of carbohydrate metabolism. Diabetes mellitus, characterized by hyperglycemia, ensues when an insufficient amount of insulin is produced. Type 1-diabetes results from an autoimmune destruction of the β-cells, and a total lack of insulin. Type 2-diabetes, on the other hand, is associated with an increased insulin resistance in the liver, skeletal muscles and adipose tissue. When the β-cells cannot compensate for the increasing demand for insulin, type 2-diabetes develops.

Islet anatomy

The pancreas is an organ which is essential both for digestion and regulation of metabolism. The exocrine tissue secretes digestive enzymes and bicarbonate into the intestine. The endocrine cells, constituting 1-2% of the pancreas, are aggregated into the islets of Langerhans, where cell cords surround a dense microvascular network. These cell clusters produce several hormones that jointly participate in the regulation of metabolism (1).

Cellular composition of pancreatic islets

The pancreatic islets are composed of several cell types. The endocrine cells comprise the majority of the cells and have so far attracted most attention. The endocrine cells are, however, crucially dependent on stromal cells for optimal function.

Endocrine cells

β-cell

The β-cells are the most prominent cell type in the pancreatic islets, and usually comprise 50-75% of all islet cells, depending on the species studied. In rodents they occupy the central regions of the islets and communicate with one another through gap junctions. They are polarized with their insulin-containing granula closely associated with capillaries (2). Thus, in view of their close association with capillaries they are uniquely adapted to rapidly respond to changes in nutrients in the blood by the release of insulin into
the circulation, thereby enabling them to continuously keep blood glucose concentrations within strict limits. In addition to insulin, the β-cells also contain other peptides although in smaller quantities, e.g. islet-like amyloid polypeptide (IAPP) and different chromogranins, all of which are secreted in conjunction with insulin. Their exact physiological roles have not yet been clarified.

 α-cells
In rodents α-cells occupy a more peripheral position in the islets, but in e.g. humans the endocrine cells are more scattered throughout individual islets. They make up some 10% of the islet cells, and are preferentially found in the caudal regions of the gland. Their major secretory product is glucagon, which mainly affects the liver to stimulate glycogenolysis and gluconeogenesis to increase blood glucose concentrations. In especially type 1 diabetics their secretion can be increased.

 δ-cells
Also these cells are situated peripherally in rodent islets and usually constitute a few per cent of the islet cells. Their main product somatostatin has an inhibitory effect on both insulin and glucagon secretion, presumably mainly through paracrine interactions. These cells have a branched cytoplasm where their extensions communicate both with other endocrine cells, and capillaries (3).

 PP-cells and ε-cells
PP-cells cells are dispersed throughout the exocrine and endocrine pancreas, especially in the caput region, and produce the hormone pancreatic polypeptide. This hormone is under strong vagal control and a likely function is to serve as a brake on exocrine pancreas secretions (4).

 The presence of a fifth endocrine islet cell, ε-cells has recently been suggested. They secrete ghrelin, are rare and their functional role is as yet not clear (5, 6).

 Nerves
Neurons
The pancreatic islets are richly innervated by the autonomic nervous system. Even neurons themselves can be found in some islets, constituting so called neuro-insular complexes (7). Sympathetic, parasympathetic and enteric neurons are found in the islets and influence the regulation of hormonal secretion. Parasympathetic nerves release mainly acetylcholine and vasoactive intestinal polypeptide in response to vagal stimulation. This stimulates both islet blood perfusion as well as insulin and glucagon secretion through actions on M3 receptors. Noradrenaline, galanin and neuropeptide Y are re-
leased form sympathetic nerves and inhibit both basal and stimulated insulin secretion, mainly through α1-adrenoceptors (8).

Also a rich sensory innervation of the islets, especially with neurons containing substance P and calcitonin-gene related polypeptide, occur. The roles of these neurons are more difficult to study, but it has been suggested that they exert a tonic inhibition on insulin secretion (8), as well as participating in islet blood flow regulation (9, 10).

Schwann cells
Schwann cells are the supportive cells of the peripheral nervous system. The pancreatic islets have been suggested to be enveloped by Schwann cell projections (11). It should be noted that peri-islet Schwann cells become activated following islet injury during autoimmune diabetes in non-obese diabetic (NOD) mice and after streptozotocin treatment (12). Autoimmune destruction of peri-islet Schwann cells also occurs early during islet autoimmunity in NOD mice (13). The role of these cells is normal islet physiology has not been fully elucidated and awaits further studies.

Resident immune cells
Like other tissues also the islets normally contain both macrophages and dendritic cells. These cells are part of the immunologic surveillance of the body, and probably interfere little with normal islet function. During the development of insulitis preceding type 1 diabetes and after allogeneic transplantations they are key players (14).

β-cell proliferation
The regulation of islet β-cell number is still incompletely understood, but it is a subject of crucial importance for understanding normal islet physiology, as well as the progression of diabetes (15, 16). The number of β-cells always reflects a balance between cell replication and cell death, and changes in any of these can influence the number of endocrine cells. Islet mass is relatively constant during adult life and the pancreatic β-cells exhibit a very low degree of mitogenic activity. β-cell proliferation can, however, be augmented in response to an increased demand for insulin, resulting from e.g. obesity, pregnancy or insulin resistance (16).

Several substances are known to stimulate β-cell replication. Glucose is in itself a very potent β-cell mitogen that can induce a 50% increase in β-cell number within 24 hours (17). The gastrointestinal incretin hormone glucagon-like peptide-1 (GLP-1) exerts a multitude of beneficial effects on β-cells, including increased proliferation and neogenesis, decreased apoptosis and improved β-cell response to glucose (18, 19). Placental lactogens and prolactin (PRL) mediate the increased islet mass and β-cell proliferation seen
during pregnancy (20-23), and is known to increase insulin secretion *in vitro* and *in vivo* (24, 25).

The factors mentioned above are of major importance for the regulation of β-cell mass *in vivo*. Several other growth factors have been reported to stimulate β-cell proliferation (for a review see (26)). One β-cell mitogen of interest in the context of this thesis is hepatocyte growth factor (HGF). Rat islets express both HGF and its receptor c-met (27), and HGF can stimulate β-cell proliferation, insulin content, and to increase insulin release and islet mass in rodent tissues (28). Furthermore, transgenic mice expressing HGF under the insulin promoter have increased β-cell proliferation and an increased β-cell mass (29). Recently, two independent groups investigated the effects of β-cell specific ablation of c-met expression (30, 31). They confirmed that HGF has an important role in normal glucose homeostasis, at least in rodents.

**Islet microvasculature**

**Islet vasculature and blood perfusion**

The microvasculature of the islets has been described as glomerular-like, since its initial description by Paul Langerhans in 1869 (32). All mammals, *i.e.* also humans, have a direct and separate arteriolar flow to the islets. The islet vasculature is otherwise dependent on the size of the islets. Aggregates of a few endocrine cells are incorporated into the capillary system of the exocrine parenchyma. Otherwise, small islets receive their blood supply from 1 arteriole, and drain through numerous efferent capillaries into a basket-like network around the islets, which subsequently drains into intralobular venules. According to some investigators (see (33)), these efferent capillaries communicate with those around exocrine acini and/or ducts, thereby forming a so-called insulo-acinar portal system. Large islets, on the other hand, possess 1-3 arterioles, and the efferent capillaries drain into postcapillary venules at the edge of the islets, which then empty into intralobular veins. Previously it has been debated to what extent the above arrangements are species-dependent, and if all blood in the pancreas passes through the insulo-acinar network (33, 34).

The microvasculature is of importance to provide oxygen and nutrients, allow for an accurate and continuous glucose sensing and disperse hormones to the systemic circulation. Thus, the capillary blood flow is essential for optimal islet function.

In the pancreas, the islets have five to ten times higher blood perfusion than the surrounding exocrine tissue (35). Islet blood flow is regulated independently from that in the exocrine pancreas (36). A large number of substances have been shown to be of importance in this context, including lo-
cally produced substances such as nitric oxide, angiotensin II and endothelin (36, 37).

The order in which islet endocrine cells become perfused is debated. One hypothesis is the so called “B-A-D hypothesis” that states that β-cells are perfused first, followed by α-cells and δ-cells (33). This would provide an explanation for some of the known hormonal interactions within islets.

Endothelial cells

Endothelial cells line all blood and lymphatic vessels of the body and form a continuous monolayer between the blood and the interstitial fluid. The typical endothelial cell is elongated (approximately 30 µm long, 12 µm wide and 0.3 µm deep) and carries a negatively charged glycosaminoglycan layer on their surface.

Endothelial cells have important functions in the regulation of tissue homeostasis. Thus, quiescent endothelial cells generate an antithrombotic and participate in the regulation of local blood flow through production of vasoactive substances.

Endothelial cells have membrane-bound receptors for a multitude of molecules, including proteins (e.g. coagulant and anti-coagulant proteins, growth factors), lipid transporting particles (e.g. low density lipoprotein), paracrine substances (e.g. nitric oxide, serotonin) and hormones (e.g. endothelins, insulin) as well as adhesion molecules for cell-cell and cell-matrix interactions (38).

Endothelial cell heterogeneity

The endothelial cell population is very heterogeneous. Endothelial cells from different species, small and large blood vessels and from different microvascular beds exhibit different phenotypes and can react very differently to the same stimuli (38). Heterogeneity can also be found in different vascular beds within the same organ. The kidneys contain discontinuous endothelial cells in the glomeruli, fenestrated endothelium in the peritubular region and continuous microvasculature in the other regions.

The differences have been reported to be due to both genetic predisposition and microenvironmental influences.

Differences in phenotype

There are several phenotypically distinct endothelial cells in the body. Continuous endothelium can be thick (>2µm thick) or thin (<1µm thick). The former phenotype can be found in cardiac muscle and bone, whereas thin continuous capillaries are seen in the dermis and brain. Discontinuous endothelium can be found in the liver, spleen and bone marrow. These gaps between the endothelial cells allow for cells to pass between the interstitium and the blood stream (39, 40).
A third form of microvessels is found in the kidney and the endocrine glands, namely the fenestrated endothelium. The endothelial cells have diaphragm-covered pores, that allows for a high permeability for smaller molecules (39, 40).

**Plasticity of endothelial cells in vitro**

Endothelial cells can be isolated and subsequently cultured *in vitro*. The phenotype of these cells is, however, unstable and likely to change when the cells are removed from their microenvironment. Isolated and cultured endothelial cells have been shown to loose tissue-specific characteristics, such as fenestrations (41, 42).

The functional heterogeneity can, however, persist also during identical culture conditions. With few exceptions, the expression most endothelial cell-specific surface markers are unevenly distributed throughout the vascular system (39). The expression pattern is so diverse and so specific for different vascular beds that it has been used to target drugs to specific sites (43). Early studies showed that the endothelial cell marker von Willebrand factor (vWF) is more expressed in venous than arterial endothelial cells, and these differences are maintained when the endothelial cells are cultured (44). A later study examined endothelial cells derived from 14 different locations, but cultured under identical conditions for 10-16 generations. Microarray techniques revealed that these cells still exhibit differences in the expression of more than 3 000 genes (45). To what extent also protein expression is maintained is not known.

Endothelial cell responsiveness to various stimuli is also dependent on the degree of confluence, *e.g.* confluent cultures have a decreased proliferative capacity (46) and a decreased responsiveness to VEGF (47).

**Islet endothelial cells**

Functionally, pancreatic capillaries are very permeable (48), and particularly so those within the islets. Thus, the vasculature is unlikely to pose any restrictions to the diffusion of the islet hormones.

Islet capillaries are wider than those in the exocrine tissue (8-10 µm vs. 4-6 µm. Islet endothelial cells are very thin (approximately 0.18 µm) and about 5% of their surface are constitutes fenestrations (49, 50), *i.e.* almost 10 times as many fenestrae as capillaries in the exocrine and ductular compartments (49). The transition in morphology is very abrupt, and microvessels located to the edge of the islets have four times as many fenestrations on the side facing the endocrine cells. It is likely that vascular endothelial growth factor (VEGF), locally produced within the islets, is responsible for the formation of these fenestrations (49, 51, 52).
Pericytes
Pericytes are elongated multibranched cells that surround microvascular endothelial cells. They are embedded within the same basement membrane as the endothelium and have been shown to produce a multitude of matrix components (53). Different microvascular beds exhibit a great heterogeneity in pericyte coverage (53), and they are rare in pancreatic islets (54-56).

Lymphatic vessels
The lymphatic system is composed of a network of capillaries that drain protein-rich lymph from the extracellular space. These capillaries lack a continuous basement membrane and are highly permeable. The lymph is returned to the systemic circulation through the larger lymphatic vessels.

Lymphatic vessels can be found in the exocrine pancreas among the acini and along the ductal system. There are, however, marked species differences in their extent and distribution (57).

We have previously found that the blood pressure in the islet microvasculature is very low, when compared to the exocrine pancreas (58). The capillary pressure and interstitial pressure is similar, which suggests that islet interstitial fluid is not drained though lymphatic vessels (59). In line with this, lymphatic vessels are not normally found in the pancreatic islets (57). Instead, the hormones diffuse into the blood stream where they are rapidly distributed throughout the body. Overexpression of VEGF-C, a stimulator of lymphangiogenesis, in the pancreatic islets results in a marked increase in the number of lymphatic vessels surrounding the islets. Interestingly, the lymphatic vessels still remain outside the islets (60). Similarly, after islet transplantation lymphatic capillaries are found in high numbers surrounding the implanted islets (61).

Angiogenesis
The formation of new blood vessels is regulated through vasculogenesis or angiogenesis. Vasculogenesis is the formation of new blood vessels from angioblasts, mainly in embryos, while angiogenesis is the predominant way of blood vessel formation in the adult (62).

Angiogenic sprouting from pre-existing vessels is not the only mechanism of blood vessel formation in the adult, but the one most extensively studied. It is a complex process and the regulation has been shown to be redundant and organ specific (63, 64). Nevertheless, some common features have been identified.

Angiogenesis is initiated when the pro-angiogenic stimuli dominate over the anti-angiogenic. This process is usually referred to as the “angiogenic switch”. The first step is characterized by vasodilation and increased blood
vessel permeability. This is part of the destabilization process that is necessary for angiogenic sprouting. This process is regulated by growth factors (e.g. VEGF and angiopoietin 2) and proteinases (65). Blood vessel destabilization is followed by endothelial cell proliferation and migration. Endothelial cells migrate toward a gradient of chemotactic substances, mediated mainly through members of the VEGF-family. Angiopoietin 1 is chemotactic, but does not stimulate endothelial cell proliferation. Interactions with the extracellular matrix and negative regulation by various angiogenesis inhibitors (e.g. tsp-1 and -2, endostatin and angiostatin) are also important (62).

Initially, endothelial cells form a network of solid cords that subsequently acquires a lumen. This process can be regulated by different isoforms of VEGF-A, angiopoietin 1 and various integrins. Tsp-1 is an endogenous inhibitor of lumen formation (65).

Newly formed blood vessels are vulnerable and require survival signals to avoid regression. When blood vessel have been assembled, endothelial cells produce platelet-derived growth factor (PDGF), and isoforms of this factor, predominantly PDGF-BB, attract supportive mesenchymal cells. Upon contact with endothelial cells, transforming growth factor-β and angiopoietin 1 stabilizes the blood vessel, and the mesenchymal cells differentiate into pericytes. This process stabilizes of the endothelial cells and downregulates VEGF receptor 2 (VEGFR2), after which the vessel can persist for years without survival factors (66).

Islet angiogenesis

The pancreatic islets constitutively express several growth factors that are known to induce angiogenesis. These include VEGF-A, which is a potent endothelial cell mitogen chemoattractant and survival factor. Other known members of the VEGF-family, such as placental growth factor, VEGF-B, -C and -D are also expressed (67). Furthermore, islet endothelial cells express receptors for VEGF, namely VEGFR1, VEGFR2 and VEGFR3 (51, 68-70). Hepatocyte growth factor (HGF) and several isoforms of fibroblast growth factor (FGF) are also expressed in adult rodent islets (27, 67, 71).

Of major importance in this context is VEGF-A, which is a very potent stimulator of endothelial cell proliferation and chemotaxis, and also increases endothelial permeability (72). VEGF-A is constitutively expressed in pancreatic islet β-cells in human rat and mice (27, 50, 67). In line with this, animals lacking islet VEGF-A expression have continuous, instead of fenestrated, capillaries (52, 67). This indicates that locally expressed VEGF-A is necessary for the formation of fenestrations in islets, as has been shown previously in other tissues (56, 73).

Islet endothelial cells remain quiescent in adult animals, despite the constitutive expression of angiogenic growth factors. Normally this is counteracted by several negative regulators of angiogenesis, e.g. tissue inhibitor of
metalloproteinase 1 and 2 (74) and endostatin (75), to help to maintain islet vasculature quiescent.

The large glycoprotein thrombospondin-1 (tsp-1) is of interest in this context. It is present in pancreatic islets (76, 77) and tsp-1 (-/-) mice have increased islet vascular density and develop islet hyperplasia (76). The anti-angiogenic effect is partially mediated by the CD36-receptor, but also through activation of latent TGF-β1 (76). TGF-β inhibits proliferation of both endothelial cells and β-cells (78, 79) and is upregulated in islets post-partum, coinciding with the rapid involution of the islet at this time (80).

Thus, the islets contain numerous factors which may either stimulate or inhibit angiogenesis, but normally, as in most organs in the body, the negative effects prevail, thereby preventing uncontrolled growth of the endothelial cells.

Interactions between endothelial and endocrine cells in the pancreas

Recent studies have suggested multiple interactions between islet endothelial cells and β-cells besides those involved in the regulation of blood perfusion and transport processes over the capillary wall, see below. Many of these studies show a close correlation between increased islet vascularization and islet mass (76, 81, 82), but if endocrine cell growth affects angogenesis, or whether growth of islet vasculature stimulates the endocrine cells to proliferate is more difficult to evaluate.

Early fetal development

The microvasculature seems to play an important role for the organisation of the islets during early pancreatic development, since endothelial cells and pancreatic endoderm co-operate to induce the formation of insulin-producing cells (83). Indeed, the first insulin-positive cells are formed close to blood vessels (1, 83). It has been shown that overexpression of VEGF-A results not only in a hypervascularized pancreas, but also in hyperplasia of the pancreatic islet tissue per se (83).

Late fetal development

The morphology and density of the vasculature in developing pancreatic islets vary during fetal life. After the initial formation of β-cells close to endothelial cells, the vascular density of islets has been reported to be low during fetal development, e.g. in humans and Mongolian gerbils (1, 84). In humans, islets with low vascular density are found from the third month of
fetal development. Referred to as ‘Mantelinseln’ (i.e. mantle islets) they disappear gradually during the first postnatal year (1). In Mongolian gerbils, islet vascularization has been shown to occur almost exclusively after birth (84).

The mechanism responsible for the perinatal increase in blood vessel density has recently been investigated. In transgenic animals it has been shown that when endocrine cell expression of VEGF-A in the islets is repressed, islet vascularization is perturbed, the endothelial cells become less fenestrated and the animals develop glucose intolerance (52, 67). This suggests an important role for mediators produced by the β-cell in the formation of the vasculature in developing pancreatic islets.

Interactions in adult tissues

The importance of endocrine-endothelial interactions in adult pancreatic islets can be illustrated by a recent article (51). In this study, adult mice were treated with different VEGF-neutralizing agents and the effects on different tissues were studied. Of the 17 tissues studied, the most profound effects were observed in the thyroid and in pancreatic islets. Neutralization of VEGF activity resulted in, not only a thickening of the endothelial cells and loss of fenestrations, but also a 45-70% decrease in islet blood vessel density (51). Thus, continuous secretion of VEGF throughout adult life seems to be necessary for maintaining the high islet blood vessel density.

Interactions between endocrine cells and endothelial cells seem to be important also for β-cell function. Two independent reports have shown that islet endothelial cell basement membrane stimulate insulin secretion (85, 86) and β-cell proliferation (86).

Interactions after transplantation

Revascularization of transplanted islets

Since the introduction of the “Edmonton protocol” in 2000 (87), transplantation of pancreatic islets has become a treatment of choice for selected patients with type 1 diabetes. This protocol achieved a 1-year insulin-independence rate comparable to that for whole pancreas transplantation (88). However, in contrast to whole-organ transplantation (89, 90) there seems to be a continuous decline in function of islet transplants and very few patients remain insulin-independent beyond 4 years post-transplantation (91, 92). Since the histocompatibility barrier, the underlying autoimmune disease, and the immunosuppressive agents used are the same for both procedures, it is most likely that also issues related to the adaptation of the implanted islets to their new microenvironment play an important role in this context.
One difference between the two modes of transplantation is that whole-pancreas transplants have an intact endogenous vascular system, which is directly anastomosed to the recipient’s circulatory system, thereby achieving an immediate establishment of blood perfusion. The islets, on the other hand, become disconnected from their vascular supply when they are isolated by collagenase digestion prior to transplantation. Experimental studies have shown that transplanted islets stimulate an angiogenic response from the implantation organ (93), which is concluded within 7-14 days (94, 95). Soluble factors released from the hypoxic islets are likely to be of major importance in this context. The ingrowing blood vessels do, however, rarely enter the clusters of endocrine cells in the grafts, but remain located in the graft stroma (93). As a result of this, the transplanted islets have low oxygen levels (96). The blood vessels are presumably prevented from entering the islets by matrix-bound angiogenesis inhibitors, even though this notion remains to be proven.

During the early phase after transplantation, activation of recipient endothelial cells can have detrimental effects on transplanted islets through production of inflammatory mediators (97-99). During the first days posttransplantation, substantial apoptosis and necrosis occurs in the transplanted islets, resulting in tissue remodelling and decreased insulin synthesis (100).
Aims

The general aim of the thesis was to investigate the hypothesis that β-cell growth and angiogenesis is interconnected in the pancreatic islets. This hypothesis was investigated by investigating the following questions:

1. is physiological islet growth associated with endothelial cell replication?

2. do native pancreatic islets become more pro-angiogenic when β-cell growth is stimulated?

3. can islet endothelial cells stimulate β-cell growth and function through paracrine interactions?

4. does β-cell growth result in improved revascularization and function of transplanted pancreatic islets?
Methods

Animals
Male C57BL/6 and C57BL/6 (nu/nu) mice weighing approximately 25 g were purchased from Bomholtgaard Research and Breeding Center (Ry, Denmark). Adult inbred male Wistar-Furth rats and virgin or pregnant, Wistar rats were purchased from Scanbur AB (Sollentuna, Sweden).

All animals had free access to tap water and pelleted food throughout the course of the study and were housed in a room with a 12-hr light/dark cycle, a humidity of 70%. All experiments were approved by the animal ethics committee at Uppsala University.

Fetal and newborn rats (Paper I)
Fetuses of Wistar rats at day 20 of gestation (referred to as day -1) were investigated. Day 0 of pregnancy (gestational length 21-22 days) was defined as the day on which spermatozoa were found in the vaginal smear. In addition, some pregnant rats were allowed to give birth, after which the newborn rats were studied on days 2 or 7 postpartum, respectively. For pups studied at day -1 the mothers were injected through the tail vein with 3H-thymidine, whilst those studied day 2 or 7 after birth were injected i.p. with 3H-thymidine, and killed 2 h later. The pancreatic glands were then carefully dissected, weighed, and prepared for histological examination.

Pregnant rats (Paper II)
Pregnant Wistar rats were investigated at days 5, 10, 15, 18, or 20 of gestation with 12-week-old virgin female rats serving as controls. In addition, some animals were allowed to give birth before being studied on days 2 or 7 postpartum. Blood glucose concentrations were measured at 8 A.M. on the day of the experiment. The pancreatic glands were carefully dissected and prepared for histological examination.
Chemicals

All chemicals were purchased from Sigma-Aldrich (Irvine, UK) if nothing else is mentioned.

In vivo protocols

$^{3}$H-Thymidine treatment (I-II)

The animals were injected intravenously with approximately 100 µl of $^{3}$H-thymidine (0.5 µCi/g body weight; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and killed 2 h later. The organs were then processed as outlined in detail below.

Partial pancreatectomy (IV)

Wistar-Furth rats were anesthetized with sodium pentobarbital (Mebumal®; Apoteket, Göteborg, Sweden; 60 mg/kg i.p.), whereas athymic C57BL/6J (nu/nu) were anesthetized with avertin (0.02 ml/g i.p. of a 2.5 % [vol/vol] solution of 10g 97% [vol/vol] 2,2,2-tribromoethanol in 10 ml 2-methyl-2-butanol (Kemila, Stockholm, Sweden)). The animals were then placed on a heated operating table to maintain body temperature at 38°C. The tail region of the pancreas was gently dissected with cotton tip applicators through a midline abdominal incision. All parts of the pancreas not attached to the stomach or duodenum, hereafter referred to as the splenic part of the gland, were then removed and the bleeding was controlled by diathermy. The detached splenic part of the pancreas has in our previous experiments been shown to constitute approximately 60% of the whole gland (101, 102). Separate animals were sham-operated. In this case, the splenic portion was mobilized, but otherwise not treated. Following partial pancreatectomy or sham-operation, the surgical wound in the abdominal wall was closed by sutures.

Islet isolation (II-IV)

Pancreatic islets from male C57BL/6 mice or Wistar-Furth rats were isolated as previously described (103, 104). Groups of 100-150 islets were maintained free-floating in RPMI 1640 supplemented with 11.1 mmol/l D-glucose, 2 mmol/l L-glutamine and benzylpenicillin (100 U/ml; Roche Diagnostics, Bromma, Sweden), streptomycin (0.1 mg/ml), and 10% vol/vol fetal calf serum (FCS) in 95% air/5%CO$_2$ at 37°C; hereafter denoted as islet culture medium (CM). Culture medium was changed every 48h.

In paper III, autotransplantations were performed using islets isolated from the extirpated splenic part of the pancreas of the partially pancreatec-
tomized Wistar-Furth rats. Other rats received syngeneic islets from control Wistar-Furth rats. In some additional control animals, the islets from the splenic and duodenal parts of the pancreas were isolated and transplanted separately.

Human islets (Paper IV)

Human islets from 9 heart-beating donors (age 51±4 years, 4 males/5 females) were isolated at the Human Islet Isolation Core Facility for the Nordic Countries located at the Department of Clinical Immunology, Uppsala University, Uppsala, Sweden, and kindly provided by Professor Olle Korsgren. The β-cell function in islet preparations were investigated prior to delivery by islet perifusion giving a mean glucose stimulation index of 11.1±1.6 (n=9) when changing from low (1.67 mmol/l) to high glucose concentration (16.7 mmol/l) in the perifusion medium. The human islets were kept in culture for 4-7 days in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5.6 mmol/l D-glucose, 2 mmol/l L-glutamine (Sigma-Aldrich) and 10% (vol/vol) fetal calf serum prior to transplantation (105, 106). In order to avoid influence of variable exocrine contamination on the revascularization process (cf. (107), apparently clean islets were manually selected for transplantation. All experiments involving human islets were approved by the local human ethics committee for Uppsala University.

Islet transplantation (III-IV)

Mouse islets (III)

Groups of 250 C57BL/6 islets were packed in a braking pipette after 4-5 days of culture, and implanted beneath the left renal capsule, of syngeneic C57BL/6 mice anesthetized with an intraperitoneal injection of avertin. Some of the islets were treated with ovine PRL (oPRL; 500 ng/ml; ProSpec-Tany Technogene, Rehovot, Israel) added to the culture medium during the last 24 h before transplantation. Controls were cultured in normal culture medium with the corresponding volume saline.

The transplanted animals in another group were injected intraperitoneally with oPRL (50 µg dissolved in 0.1 ml saline and 0.1% bovine serum albumin (BSA)) from the day before transplantation, until day 7 post-transplantation. Controls were injected with the corresponding volume saline.

Weight matched non-transplanted controls were used for determination of oxygen tension and blood vessel density in endogenous islets.
Rat & human islets (IV)
Groups of 250 Wistar-Furth islets, or 200 human islets, were packed in a braking pipette and implanted beneath the left renal capsule, of syngeneic pentobarbital-anesthetized Wistar-Furth rats or athymic avertin-anesthetized C57BL/6 (nu/nu) mice, respectively. Some of the islet recipients (rats) had been partially pancreatectomized or sham-operated (see above) 4 days before transplantation. In one experimental group, islets obtained from the extirpated splenic portion of the pancreas following partial pancreatectomy were autotransplanted into these rats.

Some C57BL/6 mice were given an intravenous injection of alloxan (75 mg/kg; Sigma-Aldrich) 5 days before transplantation and were considered to be diabetic if their non-fasting blood glucose concentrations exceeded 16.7 mmol/l at this time.

Isolation of islet endothelial cells (II)
Outgrowth of islet stromal cells on a collagen matrix was stimulated using a modification of a previously described protocol (108). Briefly, 20 hand-picked apparently clean islets were transferred onto a collagen matrix (1.8 mg collagen type I/ml; Collagen GmbH, Nutacon, Leimuden, Netherlands) in each well in a 24-well culture dish. Islets were cultured at 37°C (air/CO2 95:5) in 1 ml CM but supplemented with 20% (vol/vol) fetal calf serum and 100 µg endothelial cell growth supplement; hereafter denoted as EcM. Vascular sprouts grew out from the islets and the islets were removed before expanding cells reached confluence.

Cells were detached with 0.25% (wt/vol) trypsin (Gibco, Gaithersburg, MD, USA) for <5 min at 37°C. The suspension was washed twice in CM, and the endothelial cells were extracted from the cell suspension by BS-1 coated Dynabeads as previously described (75, 109). After incubation, the BS-1-coated Dynabeads with bound endothelial cells were separated using the magnetic particle concentrator for 2 min. The cells were reseeded in collagen-coated plastic culture dishes in EcM. By the use of Bandeiraea (Griffonia) simplicifolia (BS-1) coated Dynabeads endothelial cells were separated from contaminating cells, and a purity of >90% was achieved; this characterization has been described in detail elsewhere (75).

Oxygen tension measurements (III-IV)
Mice were anesthetized with avertin, and rats with thiobutabarbital (Inactin®; Research Biochemicals, Natick, MA; 120 mg/kg body weight i.p.), and subsequently placed on an operating table maintained at body temperature (38°C), and tracheostomized. Polyethylene catheters were inserted into the right carotid artery and left jugular vein. The former catheter was connected
to a Statham P23dB pressure transducer (Statham Laboratories, Los Angeles, CA, USA) to monitor mean arterial blood pressure, whereas the latter catheter was used for continuous infusion of Ringer solution (5 ml/kg⁻¹·h⁻¹) to substitute for loss of body fluids.

A left subcostal flank incision was performed, and the graft-bearing left kidney was immobilized in a plastic cup. Oxygen tension was measured in the islet graft and adjacent renal parenchyma with modified Clark microelectrodes (Unisense, Arhus, Denmark) as previously described (110, 111). The electrodes (outer tip diameter, 2–6 µm) 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. For oxygen tension measurements in endogenous pancreatic islets, the pancreas was exposed and immobilized, and its islets were visualized in a similar manner (58, 112). Measurements of oxygen tension were performed in three or more superficial pancreatic islets and in the surrounding exocrine parenchyma of each animal.

Blood flow measurements (III-IV)
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 mm tip; outer diameter, 0.45 mm; Perimed) as previously described (112). Mice were anesthetized with avertin and rats with thiobutabarbital. The flow probe was positioned perpendicular to the immobilized tissue surface by the use of a micromanipulator, and care was taken to avoid compression of the tissue. Because it is difficult to calibrate the instrument in physical units of blood flow, all such values are given as arbitrary tissue perfusion units. The blood perfusion of endogenous islets cannot be determined by laser-Doppler flowmetry due to their small size.

Measurements of blood parameters (III-IV)
Blood glucose concentrations were determined with test reagent strips (Medisense, Baxter Travenol, Deerfield, IL, USA) from samples obtained from the cut tip of the tail. A blood sample was collected after performing the blood flow and oxygen tension measurements, and analyzed for hematocrit and blood gases. pH less than 7.30, pO₂ less than 10 kPa, pCO₂ more than 6.8 kPa, or hematocrit less than 40 were used as preset exclusion criteria from the study.

Perfusion of graft bearing kidneys (IV)
Grafts from transplanted Wistar-Furth rats were investigated one month post-transplantation for glucose- and arginine-stimulated insulin secretion
using a perfusion protocol previously described in mice (113, 114). At different times during the perfusion, the medium contained 2.8 or 16.7 mmol/l D-glucose or 5.6 mmol/l D-glucose + 10 mmol/l L-arginine. Medium was administered at a rate of 3 ml/min without recycling for 60 min with a perfusion pressure of approximately 40 mmHg. The perfusion experiments started with a 15-min period using medium containing 2.8 mmol/l glucose, followed by 20 min using 16.7 mmol/l glucose and 15 min using 2.8 mmol/l glucose. Then medium containing 5.6 mmol/l glucose and 10 mmol/l arginine was given for 10 min, before the perfusions were concluded by a 15-min perfusion with medium containing 2.8 mmol/l glucose. A 3.0 ml sample of the effluent medium was collected and the insulin concentrations of the samples were measured by ELISA (Mercodia, Uppsala, Sweden). The rate of insulin secretion was calculated by multiplying the insulin concentration in the sample by the flow rate, giving values of insulin expressed as ng/min. The area under the curve (AUC) was then determined from these values.

Minimal islet mass model (III)
Alloxan-diabetic C57BL/6 mice were used as recipients. The number of transplanted islets (200) was chosen on the basis of our previous studies in this strain, e.g. (115, 116), and aimed to implant an islet mass insufficient for full reversal of hyperglycemia in most of the diabetic recipients. Cure from diabetes was defined as non-fasting blood glucose concentrations lower than 11.1 mmol/l. The graft-bearing kidneys were removed on all cured animals 1 month post-transplantation to ascertain that they would subsequently become hyperglycemic (blood glucose >16.7 mmol/l).

Histological protocols
All samples, i.e. pancreas, islet grafts, isolated endothelial cells, were fixed in 10% (vol/vol) formaldehyde, dehydrated and embedded in paraffin before further processing. Sections, 5 µm thick and randomly chosen from all parts of the samples, were prepared, mounted on poly-L-lysine glass slides and stained for vascular endothelium, insulin, tsp-1 and HGF.

Visualization of endothelial cells (I-IV)
Blood vessel endothelium was visualized with the lectins Bandeiraea (Griffonia) simplicifolia (BS-1) or Ulex europaeus (UE), as described in detail previously (117). The slides were counterstained with hematoxylin. Positive control slides comprised paraffin-embedded rat intestine for BS-1 and paraffin-embedded human intestine for UE. We have previously shown that BS-1
produces consistent staining of all blood vessels in rat, mouse and human islets, whereas UE only stains blood vessels of human origin (110, 117).

As an additional control of endothelial cell purity (II), samples of endothelial cells were trypsinized, spun onto poly-L-lysine glass slides, fixed in 4% (vol/vol) paraformaldehyde for 2 h and subsequently dehydrated. The slides were stained to detect endothelium with a monoclonal rat anti-CD31 antibody (clone MEC7.46; HyCult Biotechnology., Uden, The Netherlands), using EnVision+ System (Dako, Glostrup, Denmark). Antigen retrieval was performed by boiling for 20 min with Target Retrieval Solution (pH 9.0; Dako) in a microwave oven (750 W). The slides were counterstained with hematoxylin.

Insulin staining (I-IV)
Islet β-cells were stained with a monoclonal guinea-pig anti-insulin antibody (ICN Biomedicals, Aurora, OH, USA), as previously described in detail (117). These slides were then used, as outlined below, to autoradiographically estimate islet endothelial and β-cell proliferation as well as to measure islet vascular density and pancreatic islet volume.

Cell proliferation

**Autoradiography (I-II)**
Wet slides with sections from the pancreas stained with either BS-1 or for insulin were dipped in 50% film-emulsion (Autoradiography emulsion®, Kodak, Rochester, NY, USA), in 0.75 mmol/l ammonium-acetate, and kept in a light-proof chamber to dry overnight. The films were then exposed for 3 weeks at 4°C, before being developed, fixed and counterstained with hematoxylin.

**Ki67 staining (IV)**
Consecutive sections (5 µm) of graft-bearing kidneys from partially pancreatectomized or sham-operated recipients were stained for the cell proliferation marker Ki 67 according to the manufacturer’s instructions (Lab Vision Corporation, Fremont, CA, USA) after antigen retrieval. The slides were finally developed with 3,3’-diaminobenzidine tetrahydrochloride (DAB).

**Thrombospondin-1 staining (II)**
The slides were incubated for 10 min with 8% (wt/vol) hydrogen peroxide in tris-buffered saline (TBS), and washed. Antigen retrieval was performed by boiling with citrate buffer (Lab Vision) in a microwave oven (750 W) for 20 min. The sections were washed in TBS and thereafter incubated with normal
rabbit serum (Dakopatts, Glostrup, Denmark) diluted 1:20 with TBS containing 0.1\% (wt/vol) bovine serum albumin (ICN Biomedicals) for 1 h. Primary antibodies against tsp-1 (A6.1; Lab Vision), diluted 1:50 in TBS, were applied to the slides overnight at 4ºC. The slides were washed (TBS; 3 X 5 min) and incubated for 30 min with a secondary rabbit anti-mouse antibody (Dakopatts) diluted 1:100 in TBS. The slides were then washed again. Mouse peroxidase anti-peroxidase antibody (Dakopatts), diluted 1:100 in TBS, was applied to the slides for 30 min. The slides were washed and developed with DAB for approximately 8 min before being counterstained with hematoxylin. Negative control slides were incubated with normal rabbit serum instead of primary antibodies.

Double fluorescence staining for HGF and endothelium (II)

Deparaffinized slides were incubated with neuroaminidase type X in TBS for 2 hours at 37ºC. The sections were washed in TBS and thereafter incubated with normal goat serum (Dakopatts) diluted 1:20 with TBS containing 0.1\% (wt/vol) BSA for 1 h. Primary antibodies against HGF (1:20; clone Hyp-T6942; Institute of Immunology, Tokyo, Japan) and Fluorescein isothiocyanate-labeled BS-1 (1:5) diluted in TBS, were applied to the slides over night at 4ºC. The slides were washed (TBS; 3 X 5 min) and incubated for 1 h with a secondary Texas Red conjugated goat anti-rabbit antibody (clone: 111-075-144; Jackson Immunoresearch, West Grove, PA, USA) diluted 1:50 in TBS. The slides were then washed again and mounted with a mounting medium containing 4’,6-Diamidino-2-phenylindole (Vectashield + DAPI; Vector Laboratories, Burlingame, CA, USA).

Morphological evaluations

Islet endothelial, \(\beta\)-cell and endocrine cell proliferation (I-II, IV)

Tissue sections were evaluated in a light microscope (400X) by an observer unaware of the origin of the samples. All investigated islets were digitally photographed and stored as computer files. Cells with \(\geq 10\) black silver grains over their nuclei were considered to be in the S-phase of the cell cycle (118). Background thymidine incorporation was, in general, less than 3 grains/nucleus.

The fraction of endothelial cells, insulin-positive and other endocrine cells with \(^3\)H-thymidine incorporation in their nuclei was counted. In Paper II, 44±2.4 islets, corresponding to 3370±223 \(\beta\)-cells, (n=53) were counted in each pancreas, as determined by using a computerized system for morphometry (Scion Image, Scion Incorporation, MD, USA).
In order to determine whether the proliferation of endothelial cells was associated with endocrine cell replication we performed a linear regression analysis of the labelling index (LI) of endocrine and endothelial cells for each animal. We also wanted to investigate whether endocrine LI was higher in islets where \(^{3}\)H-thymidine labelled endothelial cell nuclei were found. To achieve this, we divided the islets from each animal into two groups. The first group contained islets where at least one labelled endothelial cell nucleus was identified in the sections, while islets without detectable endothelial cell proliferation were referred to the second group. The means for the LI of endocrine cells in the two groups were calculated for each animal and considered as one observation in the subsequent statistical analysis.

**Islet mass and islet fraction (I-II)**

Total islet volume was measured by a direct point-counting technique (119) at a magnification of 200X. In Paper I, a total of 10 different fields were viewed in each pancreas (corresponding to \(~1200\) points). The islet mass was estimated by multiplying the pancreatic weight with the islet volume fraction of the whole pancreas assuming similar densities of the tissues. Indeed, the densities of the exocrine and endocrine pancreas differ by less than 1\% (120).

In paper I, the fetal pancreata were not weighed and therefore islet mass was not possible to estimate. The volume fraction of islets in the pancreas was estimated by the direct point-counting technique in the same histological sections. The number of intersections overlapping islets was counted in a light microscope (200X). A total of 10 different fields were counted in each pancreas (corresponding to \(1023 \pm 14\) points; \(n=5-6\) animals in each group).

**Endocrine graft volume (III-IV)**

Tissue sections of the islet grafts were stained according to van Gieson. The total graft volumes and the fractions constituting endocrine cells were estimated from these sections by using a computerized system for morphometry (Scion Image). In order to estimate the transplanted islet volume, the area of 250 cultured islets was measured by computerized morphometry (ScionImage). The islets were not aggregated, but measured singly.

**Islet vascular density (I-IV)**

The blood vessel density in the pancreatic islets was determined by a direct point-counting method on the histological sections stained with BS-1 at a magnification of 400X (119). In paper I, 20 islets (corresponding to \(624 \pm 23\) intersections) were evaluated in each animal. In paper II, \(657 \pm 25\) intersections were evaluated in each pancreas (\(n=5-7\) animals in each group).
In paper IV, tissue sections stained for BS-1 from all parts of the rat and human pancreata, or rat or human islet transplants, were chosen for the evaluation of islet vascular density. Separate tissue sections of the human islets transplants were stained for UE and used to estimate the proportion of new blood vessels in the islets derived from the donor. The rationale for this is that if all new blood vessels were derived from the host (mouse), all blood vessels in the human islet grafts would be UE-negative. Vice versa, if all new blood vessels arose through the expansion of the remnant donor endothelium, the number of blood vessels quantified following BS-1 and UE staining would be expected to be similar. We have previously observed that the estimated vascular density of endogenous human islets is identical following quantification of BS-1 and UE-positive blood vessels (110). The blood vessel density in pancreatic islets (both endocrine tissue and stroma, respectively) was determined by a direct point-counting method at a magnification of 400X (119). A total of 5 sections, corresponding to approximately 600 intersection points were counted in each transplant or pancreas. The total graft volumes and the fractions constituting endocrine cells in the grafts were estimated using a computerized system for morphometry as given above (Scion Image).

Tsp-1 expression in endocrine cells (II)
Tsp-1 stained sections were examined in a blinded fashion in a light microscope at a magnification of 400X. The presence of tsp-1 staining was ranked by a dichotomic arbitrary scale: 0 indicated islets with very weak or unidentifiable tsp-1 staining in the cytoplasm of endocrine cells, whereas + denoted clearly tsp-1 positive islets. Tsp-1 positive islets exhibited a uniform cytoplasmic staining pattern in endocrine cells. A total of 29.2±2.5 islets were examined in each animal (n = 3-7 in each group).

Distance from endocrine cells to islet capillaries (I)
BS-1 stained pancreatic slides from day 2 postpartum were evaluated. The distance from the centre of endocrine cell nuclei to the closest intra-islet capillary was measured. This distance was determined for every nucleus in the islet sections and the cells were divided into two groups, depending on whether they had incorporated $^3$H-thymidine or not. A total of 47.8 ± 13 thymidine incorporated and 344 ± 100 non-incorporated endocrine nuclei were evaluated per animal (n=4-5 in each group).
In vitro studies

In vitro stimulation with ovine PRL (II-III)

Duplicate groups of 20 freshly isolated islets from adult animals were transferred to a 24-well plate with 1 ml CM per well (Ø 16 mm). Recombinant ovine prolactin (oPRL; 250-500 ng/ml; ProSpec-Tany Technogene, Rehovot, Israel) was added to some of the wells. Control wells only contained CM and islets. Control wells only contained CM and islets. The medium was removed and frozen after 24 h of culture. The VEGF-A contents in the medium were determined with ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Islet-conditioned medium (II)

Islets were isolated, handpicked and cultured in groups of 150 islets in 5 ml CM for 4 days as given above to minimize contamination of exocrine tissue and passenger leukocytes. Groups of 150 islets were thereafter cultured in 5 ml fresh CM for 24 h. The medium was then centrifuged for 2 min at 2 000 rpm to remove cells. The supernatant was collected and is hereafter denoted ICCM.

Endothelium-conditioned medium (II)

Dynabead-purified endothelial cells were cultured for 4 days in EcM as given above. At this time-point the endothelial cells were in exponential growth, but had not reached confluence. The wells were washed with CM to remove all EcM. Fresh CM was then added to some of the wells with endothelial cells, whereas ICCM was added to others. Additional wells without endothelial cells were used as controls. Some of the wells containing endothelial cells exposed to ICCM were supplemented with neutralizing VEGF-A antibody (1 µg/ml; NeoMarkers, Fremont, CA, USA) or neutralizing HGF antibody (1 µg/ml; R&D Systems), whereas some of the wells containing endothelial cells exposed to CM were supplemented with recombinant rat VEGF-164 (3-40 ng/ml; R&D Systems). After 12 h the medium was collected after centrifugation for 2 min at 2 000 rpm to remove the cells. The samples were used either for analysis of HGF concentration (Enzyme Immunoassay; Institute of Immunology, Tokyo, Japan) according to the manufacturer’s instructions, or for the incubation experiments described below. Culture medium obtained from wells with endothelial cells+CM and endothelial cells+ICCM, without addition of VEGF or VEGF antibody, was denoted endothelium conditioned medium (ECCM) and endothelium conditioned ICCM (IECCM), respectively.
Islet incubation in conditioned medium (II)
A total of 1 ml CM, ECCM or IECCM was put into wells in 24-well plates. Neutralizing goat anti HGF-antibody (1 µg/ml; R&D Systems) or rabbit anti-mouse antibody as a control antibody (1 µg/ml; Dakopatts) were added to wells with ECCM or IECCM. Thereafter, 20 islets cultured for 5 days in CM were placed in each well. After 6 h of exposure to a conditioned medium, 3H-thymidine (10 µCi/ml) was added for an additional 60 min. The islets were then washed repeatedly in ice-cold PBS, fixed in 4% (vol/vol) paraformaldehyde for 2 h and subsequently dehydrated in alcohol. The islets were embedded in paraffin, sectioned and stained for insulin. The β-cell proliferation rate was then determined by autoradiography as described above.

Semi-quantitative real time RT-PCR (III)
The effect of oPRL on tsp-1 levels was analyzed by semi-quantitative real-time RT-PCR. A total of 200 islets from male C57BL/6 mice were handpicked and cultured for 4 days, as described above. During the last 24 h, half of the islets were incubated with oPRL, as described above, whereas the remaining half served as untreated controls. Total RNA was extracted using Ultraspec® (Biotex Laboratories, Houston, TX, USA), and first strand cDNA was synthesized as previously described (121). PCR reactions were carried out with the Lightcycler real-time PCR instrument (Roche Molecular Biochemical, Mannheim, Germany), using the DyNAamo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The primers used for amplification were: tsp-1 forward: 5'- GGAACGGAAGACAACACTG, tsp-1 reverse: 5'- GCTCTGGGCTCTAGCACCC, beta-actin forward: 5'- GCACCTGAGGGTGTTACTTG, beta-actin reverse: 5'- CCACCGATCCACAGAGTACTTG, VEGF-A forward: 5'- ACTTGAGGTGGTGTTAATCGG Samples were run in duplicates. Tsp-1 mRNA levels were normalized against beta-actin mRNA expression and given as a fraction of the value obtained in islets cultured for 4 days under normal islet culture conditions.

Statistical analysis
All values are given as means±SEM. All statistical comparisons were made with SigmaStat® (SPSS Science Software, Erfurt, Germany). Comparisons between two groups were performed with Student’s unpaired t-test. Multiple comparisons of parametric values with control values were performed by ANOVA with Dunnett’s post-hoc test. Multiple comparisons of non-
parametric values (such as fractions) were performed by non-parametric ANOVA and Dunn’s post-hoc test. For comparisons of morphology score, or when only two groups were compared, Wilcoxon signed rank test was used. Coefficients of correlation were obtained by simple linear regression followed by evaluation of statistical significances of correlation by ANOVA. For all comparisons, a probability of chance differences <0.05 was considered to be statistically significant.
Results and Discussion

Aim 1 – is physiological islet growth associated with endothelial cell replication?

To address this question we studied perinatal (I) and pregnancy-induced (II) growth of the endocrine pancreas, both of which are limited in time and morphologically well characterized (21, 122-124).

Perinatal growth (I)

The β-cell labelling index was much higher perinatally than in adult animals, with a maximal value being observed on day 2 postpartum. Insulin-positive cells were also more frequently found in fetal ductal epithelium than in adult rats, suggesting that β-cell neogenesis also occurred. These findings are in line with previous studies showing that postnatal islet growth reflects a balance between endocrine cell proliferation, neogenesis and apoptosis (80, 125, 126). The fraction of the pancreas comprising islets was also higher in all perinatal animals when compared to adult rats, which also confirms previous findings (127).

Islet vascular density the day before birth was only 40% of that seen in adult animals. Furthermore, most of the blood vessels were found in the periphery of the islets, whereas the central insulin-positive islet core contained much fewer capillaries. The low vascular density may reflect the fact that fetal islets do not need to monitor blood glucose levels, nor deliver insulin rapidly to the systemic circulation to the same degree as in adults (128).

Microvascular density increased by 50% from the day before birth to the second postnatal day and reached adult levels at day 7 postpartum. The proliferating endocrine cells were closer to islet capillaries than quiescent cells. One explanation for this may be that these endocrine cells are exposed to higher concentrations of nutrients, oxygen and growth factors and therefore will be more prone to proliferate. This does not exclude the possibility that growth factors are produced locally by stromal or endothelial cells as well. Recently, the endothelial cell basement membrane has been found to be a niche that is beneficial for β-cell proliferation (86). The endothelial cells may also directly stimulate proliferation through production of substances directly stimulating β-cell replication. One such candidate is HGF, which is a potent mitogen for both β-cells and endothelial cells, and is released from
fetal pancreatic fibroblasts (129-131) and from islet capillary cells during pregnancy (Paper II). It may be that before birth, the high local HGF-production by fibroblasts dominate over that produced by endothelial cells, whereas the latter becomes more important later in life.

Growth during pregnancy (II)

Both islet β-cell and total endocrine cell proliferation were significantly increased at day 15 of pregnancy. At all other time points, β-cell LI was similar to control animals. This confirms results from previous studies (21, 123). In parallel to increased cell proliferation, there was an increased islet mass at day 15 of pregnancy, which then normalized during late pregnancy and the early lactation period.

Islet endothelial cell proliferation rates, on the other hand, were increased at days 10, 15, 18 and 20 of pregnancy when compared to that of virgin rats, but returned to normal immediately after delivery. Endothelial cell proliferation was associated with an increased blood vessel density at days 15 and 18 of pregnancy.

Total endothelial cell LI correlated to the degree of endocrine cell LI when individual animals were compared. Furthermore, when individual islets were investigated we found that islets with established endothelial cell proliferation had a higher endocrine cell proliferation than other islets.

In conclusion, physiological growth of the pancreatic islets seems to be associated with an increased endothelial cell replication, at least after birth. During pregnancy, β-cell replication preferentially occurs in islets with active replication of endothelial cells.

Aim 2 – do native pancreatic islets become more pro-angiogenic when β-cell growth is stimulated?

In order to address this question, we studied expression of growth factors with angiogenic activity during physiological growth and after stimulation with PRL in vitro (II-III).

We focussed our interest on tsp-1, a potent angiogenesis inhibitor, which has been reported to be expressed in pancreatic islets (76, 77). The islet vascular density increases when tsp-1 is deleted (76), which suggests a role for tsp-1 as an important negative regulator of islet angiogenesis.

In line with this, we found that approximately 90% of the islets in virgin rats were tsp-1 positive. The tsp-1 staining was restricted to the cytoplasm of endocrine and endothelial cells. The fraction of tsp-1 positive islets transiently decreased to between 40 and 55% at days 10 and 15 of pregnancy,
coinciding with the increase in endothelial and β-cell proliferation. It should be noted that the endothelial cell LI remained high at day 18 and 20 of pregnancy, that is, at a time point when the fraction of tsp-1 positive islets had increased again. Tsp-1 expression alone does, therefore, not seem to be enough to prevent endothelial cell proliferation. It should, however, be noted that blood vessel mass did not increase at day 18 and 20 of pregnancy. Tsp-1 induces apoptosis in activated endothelial cells (132) and one possible explanation is that tsp-1 counteracts endothelial cell replication during late pregnancy to prepare for the post-partum involution of the islets.

The majority of the effects on islets caused by pregnancy, including β-cell proliferation, has been shown to be mediated by PRL (24, 133). Therefore, we aimed to further investigate the effects of PRL on islets. Addition of ovine PRL to CM increased β-cell proliferation in rat islets in vitro approximately 2.5-fold, confirming previous reports (133). Incubation of rat islets for 24 h with oPRL increased the VEGF-A release to the culture medium with approximately 50%. Confirmatory results were obtained in mouse islets, where PRL increased VEGF-A mRNA expression by approximately 35%. PRL-induced VEGF-A production has previously been reported in mammary glands and macrophages (134, 135). PRL treatment also decreased islet tsp-1 mRNA expression by 40% in mouse islets.

In conclusion, the findings suggest that PRL may contribute to an angiogenic switch in islets, favoring endothelial cell replication and, as a consequence of this, β-cell growth.

Aim 3 – can islet endothelial cells stimulate β-cell growth and function through paracrine interactions? (II)

In preliminary experiments, we found that co-culture of islets and islet-derived endothelial cells induced β-cell proliferation (data not shown). In order to elucidate the mechanisms behind this, we exposed β-cells to conditioned culture media in different combinations. Fresh culture medium, culture medium conditioned with islets or culture medium conditioned with endothelial cells did not induce β-cell proliferation. However, when the culture medium was first conditioned with islets and after that conditioned with endothelial cells, β-cell proliferation increased. HGF was shown to be present in the latter medium in higher concentrations than in the others, and the mitogenic response decreased when a neutralizing HGF antibody was added.

The next goal was to try to determine the islet-derived stimulus responsible for the induction of HGF expression. HGF expression in liver endothelial cells has previously been shown to be VEGF-dependent (136). In line with this, we could inhibit the expression of HGF by addition of a VEGF-
neutralizing antibody to the islet conditioned culture medium. However, addition of VEGF-A alone to the islet-derived endothelial cells did not induce HGF expression, but we found that a combination of insulin and VEGF-A could induce HGF expression in islet derived endothelial cells.

We developed a fluorescence staining to evaluate whether endothelial cells expressed HGF during pregnancy. We found a co-localization of HGF and our endothelial cell marker BS-1 in a subpopulation of islets at day 15 of pregnancy. This opens the possibility that endothelium-derived HGF stimulates β-cells through paracrine effects during growth. In virgin rats, such a co-localization was very rare (data not shown).

In conclusion, islet derived endothelial cells can be stimulated to secrete the β-cell mitogen HGF after combined stimulation with insulin and VEGF-A.

Aim 4 – does β-cell growth result in improved revascularization and function of transplanted pancreatic islets?

During culture, the endothelial cells dedifferentiate and disappear rapidly from the pancreatic islets (137). This means that islets need to stimulate ingrowth of new endothelial cells and microvessels to ascertain an adequate blood perfusion. Alternatively, dedifferentiated intra-islet endothelial cells may be stimulated to participate in angiogenesis.

The interventions chosen to evaluate if β-cell growth per se affects islet grafts, namely partial pancreatectomy and PRL treatment, have previously been shown to be associated with islet growth (21, 133, 138, 139). We also used syngeneic, normoglycemic recipients in order to isolate the effects of the intervention from those evoked by changed carbohydrate metabolism and rejection.

Endocrine graft volume (III-IV)

The endocrine volumes of one-month-old grafts were increased by approximately 50% in the transplants preincubated with PRL, and almost doubled when the animals were injected with PRL. In the latter group it was actually similar to the estimated transplanted endocrine volume. Thus, the usual decline in graft volume seen post-transplantation could to a large extent be prevented by PRL. At present it is unclear if this reflects a decreased death of β-cells after implantation or an increased replication. PRL has also been reported to have immunomodulatory effects and these may also contribute to the favorable effects on endocrine graft volume, by affecting to non-specific inflammatory response seen after islet implantation (140, 141).
The endocrine volume of one-month-old grafts was higher also in rats that were pancreatectomized before transplantation when compared to control recipients receiving syngeneic rat islets, with or without previous sham-surgery. A difference in graft volume was found already at day 5 after transplantation, indicating that the beneficial effects of pancreatectomy occur during the first days after transplantation.

The β-cell proliferation in two- and five-day-old grafts were paradoxically decreased in partially pancreatectomized compared to sham-operated rat recipients. The increased endocrine mass in grafts in partially pancreatectomized recipients can therefore not be explained by growth induced by signals from the surgically manipulated pancreas. A better vascular support is an alternative reason for the increased islet cell survival. Our current findings are not supported by a previous study that reported increased β-cell replication in islets transplanted to a pancreatectomized recipient (142). The main difference between the two studies is that we used a model of partial pancreatectomy where the pancreatectomized control group remained normoglycemic at all time-points. This is in contrast to the work by Montaña and co-workers, who used a model of 95% pancreatectomy where the pancreatectomized control group became hyperglycemic (142). We believe that the differences in β-cell replications can be explained by the different metabolic demand in the two models.

Islet revascularization (III-IV)

In paper III, the oxygen tension was shown to be approximately 30 mmHg in endogenous mouse pancreatic islets, but less than 10 mmHg in one-month-old syngeneic control transplants. However, there was a threefold increase in oxygen tension in the transplanted islets derived from pretreated with PRL.

In paper IV, the oxygen tension of native rat islets was found to be 40-45 mmHg, but only 4-7 mmHg in one-month-old syngeneic control rat islet transplants. However, the oxygen tension in the islet transplants was increased three- to fourfold in autotransplanted recipients. Partially pancreatectomized recipients of syngenic islets had a graft oxygen tension similar to that of autotransplanted recipients, whereas sham-operated recipients of syngeneic islets had low graft oxygen tension values. Furthermore, when human islets were implanted to partially pancreatectomized C57BL/6 (nu/nu) mice, the graft oxygen tension was markedly increased, as compared to sham-operated controls. Thus, the improved oxygen tension seemed to be directly related to the pancreatectomy procedure.

In line with the findings on graft oxygen tension, also the blood perfusion of one-month-old mouse islet grafts was increased by approximately 40% when PRL was used and by approximately 60% when rats were pancreatectomized before islet transplantation.
Blood vessels constituted approximately 11% of the volume in endogenous mouse islets and 12% in endogenous rat islets, but only 5-6% in one-month-old syngeneic control transplants in both species. The vascular density in transplanted islets of PRL-treated mice was increased by approximately 50% when compared to their respective controls. The graft vascular density after partially pancreatectomized in rats was, however, completely restored one month post-transplantation, and was thus markedly higher than in the control grafts. Sham-operated rats receiving syngeneic islets had values similar to those of control animals.

Approximately 8% of the islet volume in endogenous human islets was blood vessels, and this was similar when evaluated as BS-1 or UE positive structures. In contrast, blood vessels only constituted 4-5% of human islet transplants in sham-operated recipients. However, human islets implanted into partially pancreatectomized recipients had a vascular density similar to that of native islets. This increase was due to a combination of increased ingrowth of recipient blood vessels and an expansion of donor endothelial cells. This notion is supported by previous experimental studies (137, 143).

Thus it has been demonstrated that, in addition to inducing an increased graft volume, PRL treatment and partial pancreatectomy in the recipient also stimulates the revascularization, blood perfusion and oxygen tension in transplanted islets. The mechanism for this improvement caused by PRL is likely to reflect the stimulation of VEGF-A and HGF referred to above. How partial pancreatectomy exerts its effects remains more elusive, but the procedure has been shown to increase the systemic concentration of VEGF (144), which may stimulate islet angiogenesis (52, 67, 145). Alternatively, VEGF can mobilize endothelial progenitor cells from the bone marrow (146), which has previously been shown to increase revascularization of transplanted pancreatic islets (147). However, further studies are warranted to clarify this issue.

Evaluation of graft function (III-IV)

To investigate to what extent the improved graft volume and revascularization affected also transplant function we performed additional experiments encompassing a minimal islet mass model and perfusions of graft-bearing kidneys. We found in the former model that all alloxan-diabetic animals that were transplanted with islets preincubated with oPRL were normoglycemic 15 days after implantation, which should be compared to only 43% in the control group. All these mice reverted to hyperglycemia after nephrectomy, confirming that no regeneration of the endogenous islets had occurred.

The partially pancreatectomized rats were normoglycemic and a minimal islet mass model could therefore not be used. Instead, we chose to perfuse the graft-bearing kidneys. Distinct first- and second-phase insulin release in response to high glucose stimulation was observed from one-month-old rat
islet grafts of sham-operated control recipients. These grafts also responded with an even more pronounced insulin release upon stimulation with arginine. However, corresponding rat islet grafts implanted to partially pancreatectomized recipients released much more insulin in response to both these stimuli. Especially the first phase of insulin release during high glucose was markedly improved with peak values more than doubled when compared to those in control grafts.

In conclusion, islet revascularization and function can be improved by PRL administration or the performance of a partial pancreatectomy. This improvement is probably due to the increased revascularization. Understanding the mechanisms behind this improvement would provide an opportunity to develop interventions that increase vascular engraftment and clinical transplantation success rate.
Conclusions

Physiological β-cell growth was associated with an increased endothelial cell replication in the immediate postnatal period and during pregnancy.

The pancreatic islets increase their production of the angiogenesis stimulator VEGF-A and decrease the synthesis of the angiogenesis inhibitor tsp-1 during β-cell growth.

Islet endothelial cells can produce β-cell mitogens in response to stimuli from islets. The functional importance of this remains to be elucidated.

Transplantation of islets to recipients subjected to partial pancreatectomy or treated with PRL is associated with improved graft revascularization and function and an increased islet transplant volume.
Swedish summary


I det andra delarbetet studerade vi celldelningen av β-cell och endotelceller under graviditeten, samt under den första veckan efter födseln. Vi fann att tillväxten av kärlträdet föregick tillväxten av den endokrina vävnaden, vilket var oväntat. Vi såg också att β-cellerna delade sig oftare i de öar där vi fann prolifererande endotelceller.


Transplantation av insulinproducerande vävnad är en metod som i speciellt utvalda fall kan användas för att behandla typ-1 diabetes. Tyvärr kräver detta stora mängder insulinproducerande vävnad, och en patient behöver vanligen öar från flera donatorer. Det finns flera orsaker till detta; avstötning av transplantatet, autoimmun attack av immunsyssvaret och skadliga effekter av avstötningssmedicine. Dessutom har man funnit att en stor mängd av den transplanterade vävnaden dör kort tid efter transplantation på grund av ospecific inflammation, samt brist på syrgas och näringsämnen. Vår grupp
har tidigare funnit att återinväxten av blodkärl i de isolerade Langerhanska öarna är störd efter transplantation. Detta resulterar i låga syrgasnvärdel och en defekt insulinsekretion. I det tredje delarbetet byggde vi vidare på fynden i delarbete II och stimulerade öarna med graviditetshormonet prolaktin före transplantation. Genom detta ökades uttrycket av det blodkärlstimulerande ämnet VEGF och uttrycket av hämmaren thrombospondin-1 sänktes. Därmed kunde vi öka inväxten av blodkärl i de Langerhanska öarna, öka de lokalas syrgasnvärdel och minska det antal öar som behövdes för att bota försöksdjur från experimentell diabetes.

Kliniskt har man funnit att patienter som genomgår en pankreatektomi (Px; borttagande av buksarrkörteln) med efterföljande ötransplantation klarar sig bättre än de som transplanteras på vanligt sätt. I det fjärde delarbetet fann vi att Px inverkar positivt på transplantationsresultatet. Transplantaten hade en ökad blodkärlsdensitet, ökad genomblödning, samt högre syrgasnivärer. Transplantatet frisatte även mer insulin när det stimulerades med glukos. Transplantaten innehöll också en ökad mängd insulinproducerande vävnad, antagligen tack vare en ökad överlevnad under de första dagarna efter transplantationen. Vi kunde dessutom konfirmera våra resultat med humana öar transplanterade till möss.

Sammanfattningsvis har vi kunnat visa att blodkärlen är viktiga för att de Langerhanska öarna ska fungera optimalt. En ökad mängd blodkärl är associerad med en förbättrad funktion och dessutom verkar kommunikationen mellan endotelcellerna och den insulinproducerande vävnaden vara viktig för att de Langerhanska öarna ska fungera optimalt.
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