STRATEGIES TO COUNTERACT β-CELL LOSS IN PANCREATIC ISLET TRANSPLANTATION

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Tormod Lund
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CONTENTS

1. ABBREVIATIONS

ANGPT, angiopoietin; ER, endoplasmatic reticulum; GSIS, glucose-stimulated insulin secretion; HLA, human leukocyte antigen; HO, haem oxygenase; Hsp, heat-shock protein; IBMIR, the instant blood-mediated inflammatory reaction; IEQ, islet equivalents; IL, interleukin; IM, intramuscular; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MCP, monocyte chemotactic protein; MIF, macrophage migration inhibiting factor; NF-κB, nuclear factor kappa B; NO, nitric oxide; PARP, poly ADP-ribose polymerase; PDC, pancreatic duct cells; PUFAs, poly-unsaturated fatty acids; RANTES, Regulated upon Activation, Normal T-cell Expressed, and Secreted; ROS, reactive oxygen species; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; RvE1, Resolvin E1; Socs, suppressor of cytokine signalling; SREBP, sterol regulatory element binding protein; STAT, signal transducers and activators of transcription; TAT, thrombin antithrombin complex; TF, tissue factor; TLR, Toll-like receptor; TNF, tumor necrosis factor; TUNEL, terminal dUTP nick end labelling; VEGF, vascular endothelial growth factor.

2. LIST OF PAPERS

Paper I

Lund T, Fosby B, Korsgren O, Scholz H, Foss A. Glucocorticoids reduce proinflammatory cytokines and tissue factor in vitro and improve function of transplanted human islets in vivo. Transplant International 2008; 21: 669-678.

Paper II

Scholz H, Lund T, Dahle MK, Collins JL, Korsgren O, Wang JE, Foss A. The synthetic liver X receptor agonist GW3965 reduces tissue factor expression and inflammatory responses in human islets in vitro. Diabetologia *accepted.*

Paper III

Lund T, Mangsbo SM, Scholz H, Totterman TH, Korsgren O, Foss A. Resolvin E1 reduces proinflammatory markers in human pancreatic islets in vitro. Experimental and Clinical Endocrinology & Diabetes *accepted.*

Paper IV

Lund T, Korsgren O, Aursnes IA, Scholz H, Foss A. Sustained Reversal of Diabetes Following Islet Transplantation to Striated Musculature in the Rat. Journal of Surgical Research *in press*.

3. INTRODUCTION

3.1 TYPE 1 DIABETES

Type 1 diabetes is a disease resulting from specific destruction of the β-cells of the islets of Langerhans of the pancreas (1). As β-cells produce insulin - the hormone that helps to regulate glucose metabolism - loss of β-cells leads to the disordered blood glucose levels that characterize diabetes. Descriptions of diabetes appear in ancient Egyptian and Greek writings, but the causes and mechanisms responsible for the disease are still not fully understood (2).

Pionering studies by David E. Sutherland and his team during the early 80s involving partial pancreas transplantion between monocygotic twins, demonstrated that a nondiabetic pancreas was rapidly destroyed after transplantation into a recipient with type 1 diabetes (3). Histologically, immune cell infiltration of the islets was observed, a finding that suggests an ongoing autoimmune process. Additionally, autoantibodies to β-cell antigens precede clinical symptoms and can predict the risk of developing diabetes (4,5). These, among other observations, strongly suggest that type 1 diabetes is an autoimmune disease. The initiator(s) of this detrimental auto-immune reaction have not been identified, but a combination of genetic susceptibility (6,7) and environmental factors, possibly viral, seem plausible [reviewed in (8)]. It should be noted however, that cases of non-autoimmune, abrupt-onset insulin-dependent diabetes have been reported, and, although rare, they suggest that type 1 diabetes may be a heterogeneous disease (9).

3.2 CAN TYPE 1 DIABETES BE PREVENTED?

Type 1 diabetes currently affects approximately 0.5% of the population in developed countries, with highest rates in Finland (10). Disturbingly, the incidence of the disease has steadily increased since the 1950s (11), with an apparent steep increase in the last decade (10,12-14). Rapid increase in incidence within genetic stable populations implies that nongenetic factors are important in causing type 1 diabetes (15).

Efforts aimed at preventing diabetes has so far been unsuccessful (16). However, once type 1 diabetes becomes clinically evident, there is usually a significant β-cell

reservoir left. Several trials have since the 1970s explored different strategies for preventing the continued destruction of β-cells after diagnosis. Among the more successful attempts, albeit accompanied by significant side-effects, were the trials in the mid- and late 1980's utilizing cyclosporine(17-19), and recent trials involving anti-CD3 monoclonal antibodies (20,21). Both therapies delay, for various lengths (up to several years), but do not prevent the development of full-blown diabetes. As type 1 diabetes seems to be the result of an aberrant immune response, a growing body of evidence is suggesting that two components are needed to stop (further) progression of type 1 diabetes:
1) elimination of aggressive T-cells, and 2) augmentation of regulatory T-cells to achieve long-term tolerance (8). Interestingly, these components are also needed for transplant tolerance. Currently, the only available strategy to induce tolerance involves radiation, chemotherapy and hematopoietic stem cell transplantation (22). This approach has been explored for some time in solid organ transplantation (23), and recently also in patients with recent onset type 1 diabetes (24). The development of less toxic conditioning regimens would allow a broader applicability of such protocols, but at present it is difficult to envision this modality becoming clinical practice in the context of type 1 diabetes.

3.3 TREATMENT OF TYPE 1 DIABETES

Exogenous insulin administration, either as injection or via a subcutaneous pump, is the current treatment for type 1 diabetes. A principal objective is the prevention of long-term vascular complications, both microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (affecting the coronary, cerebral, and peripheral arteries). Intensive diabetes treatment reduces the risk of these complications (25-27), but it carries a two to three-fold increased risk of severe attacks of hypoglycaemia (25). Not surprisingly, fear of hypoglycaemia (28) or development of hypoglymia-associated autonomic failure (hypoglycaemic unawereness) (29) hinders the successful implementation of intensive insulin therapy in many patients.

Data from clinical trials indicate that recent advances, such as the continuous glucose meter, has only modest effect in improving glycemic control; those who appear to gain benefit are those who are actively and skillfully engaged in diabetes self-

management (30). For patients who have difficulties in maintaining glycemic control, early illness and early death is the inevitable result (25).

3.4 TRANSPLANTATION AS A CURE FOR TYPE 1 DIABETES

Transplantation of β-cells by implantation of vascularized pancreata or isolated pancreatic islets is currently the only way to achieve physiological glycemic control, and thereby halt progression of diabetic complications in patients with type 1 diabetes (31). The islets of Langerhans are endocrine cell clusters that represent approximately 1% of pancreatic tissue. Each islet contains different cell subsets specialized in the production and secretion of the hormones (e.g. alpha- and β-cells for glucagons and insulin, respectively) that maintain glucose levels within the physiologic range (31). The principal advantage of β-cell replacement through transplantation is the achievement of glycemic control without the risk of life-threatening hypoglycaemic events associated with intensive insulin treatment (25).

Selecting patients for β-cell replacement is difficult, as the severity of the disease must justify the risks of life-long immunosuppression. However; growing consensus is establishing that there is a small group of patients affected by type 1 diabetes mellitus who suffer from severe glycemic instability with frequent and unpredictable hypoglycemic episodes, despite compliance to intensive and state-of-the art therapy (32). It has been estimated that 5% of patients with type 1 diabetes accounts for more than half of all episodes of severe hypoglycemia (33). Frequent hypoglycaemic episodes is associated with cognitive impairment (34,35), risks of serious accidents (34), and impairs the ability to lead an independent life (36). It can readily be envisioned that the quality of life of these patients is dramatically compromised. Transplantation of β-cells is thus an attractive option for a select subset of patients with type 1 diabetes. Whole-organ pancreatic transplantation involves major surgery and risks of serious complications (36), but when successful, long-term insulin-independence can be obtained. Allogeneic islet transplantation is a gentle procedure, but generally does not produce long-term insulin independence (see below).

3.5 ALLOGENEIC ISLET TRANSPLANTATION

Allogeneic islet transplantation has been explored, with various results, since the early 1980's (37). Sustainable insulin independence was not achieved until the introduction

of the Edmonton protocol in 2000 by Shapiro and co-workers (38). Key points included restrictions on islet recipients with regards to weight and insulin requirements, infusion of islets from up to 4 different donors and avoidance of glucocorticoids in the immunosuppressive regimen (38). Later studies have demonstrated that the results are reproducible (39), however, long-term follow up reveals that most patients (90%) have to resume insulin therapy within 5 years after transplantation (40). In spite of having to resume insulin therapy, most patients are C-peptide positive (intact graft function), are protected from hypoglycaemic episodes and exhibit satisfactory glycemic control at 5 years post transplantation (40). Importantly, the procedure is carried out under local anaesthesia and has low levels of complications.

The need for islets from multiple donors and somewhat disappointing long-term results limits the application of the procedure. Progress in the field of islet transplantation warrants implementation of strategies that increase short- and long term survival of pancreatic islets after transplantation.

3.6 LOSS OF β-CELL MASS IN ALLOGENEIC ISLET TRANSPLANTATION

The average human pancreas has between 300,000 and 1.5 million pancreatic islets (41). Although difficult to assess, it has been estimated that patients may loose of up to 90% of their β-cell mass, and still maintain euglycemia (42). Similarily, surgical removal of up to 80% of the pancreas rarely cause diabetic symptoms (43). Thus, only 10-20% of the total β-cell mass is required to maintain euglycaemia (44). It is estimated that an experienced isolation facility can retrieve 50-80% of the islets in pancreas through the process of islet isolation (45,46). If all isolated islets were to survive transplantation and subsequent engraftment, islet transplantation would regularly restore insulin-independence in the recipients. However; in most cases islet transplantation from multiple donors is required (38). Functional evaluation of insulin independent transplant recipient demonstrates that the insulin secretory capacity is only 20-40% of a normal person (47). Taken together, it seems apparent that most of the transplanted β-cells do not survive in the recipient. Several factors, depicted in Figure 1, are thought to be responsible.

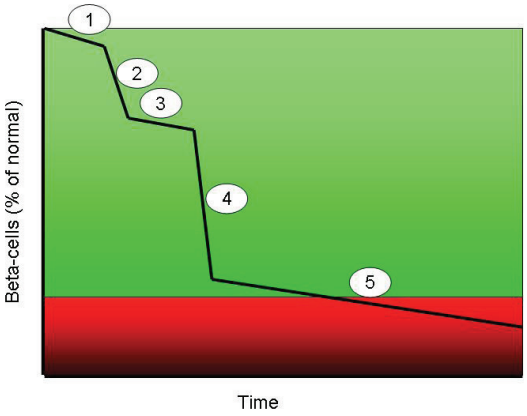

Figure 1: Schematic presentation of loss of β-cells from the time of organ harvest to revascularization in the recipient (modified from Korsgren O et al. Diabetologia 2008). Green area represents insulin-independence, red area insulin dependence.
1. Loss of β-cells during brain death and organ harvest; 2. Loss of β-cells during islet isolation; 3. Loss of β cells during pre-transplant culture; 4. Loss of islets during the islet transplant procedure; 5. Loss of islets during post-transplant period prior to revascularization.

3.7 INFLAMMATORY PROCESSES CAUSING LOSS OF β -CELLS

3.7.1 BRAIN DEATH

Loss of β-cell viability and mass begins with the complex process of brain death in the organ donor. Brain death is initiated by an increase in the intracranial pressure. This increase is usually caused by intracranial bleeding, but could also be secondary to brain swelling (e.g. as a result of cardiac arrest-induced brain ischemia). The skull constitutes a restricted compartment for the brain; increase in pressure will inevitably cause the lower parts of the brain, the temporal lobes, to be forced through the aperture at the base of the skull, the foramen magnum. This herniation of brain tissue compresses the brain stem, cranial nerves and the arterial blood supply to the rest of the brain, causing ischemia and additional swelling, culminating in cessation of blood

flow and brain death (48). In this process, a massive sympathetic autonomic response ensues as the brain stem becomes ischemic, presumably to maintain cerebral perfusion pressure. This “autonomic storm” lasts about 5 to 15 minutes, and causes intense vasoconstriction and ischemia in most organs, including pancreas (49). Herniation is followed by a resolution of the sympathetic surge with vasodilatation and reperfusion injury as result (49).

In addition to ischemia-reperfusion injury, brain death is followed by activation of inflammatory mediators such as the complement system, thromboxanes, platelet, and leukocyte factors (50,51), causing a progressive leukocyte influx into solid organs that enhances the immunogenicity of the grafts (52). Pro-inflammatory gene expression has been demonstrated to be higher in organs at the time of organ procurement than during periods of acute rejection (53), illustrating the magnitude of the pro-inflammatory effects of brain death. This may explain why graft survival from brain-dead donors is inferior to living-unrelated donors, despite the better human leucocyte antigen (HLA) matching (54).

The surge of pro-inflammatory mediators observed in brain dead donors may be explained by at least two factors. First, brain death disrupts the hypothalamic-pituitary-adrenal axis, that normally under stressful conditions produce glucocorticoids. Glucocorticoids inhibit pro-inflammatory- and increase anti-inflammatory signalling, thereby reducing the magnitude of the inflammatory response (55). Administration of glucocorticoids to the brain dead donor has been shown to improve graft function and reduce acute rejection episodes in liver transplantation (56). Second, Tracy and co-workers have demonstrated that the nervous system, via an inflammatory reflex of the vagus nerve, can inhibit cytokine release and thereby prevent tissue injury and death (57,58). This efferent neural signalling pathway is termed the “cholinergic anti-inflammatory pathway”. Disruption of this anti-inflammatory pathway is inevitably the consequence of brain death.

Brain death causes significant pathophysiological alterations in the pancreas, including deterioration of pancreatic microvasculature, inflammation, and histologic damage (59). Studies have demonstrated that brain death is associated with a

significant reduction in islet yield after isolation and impaired islet function in vitro and in vivo (60,61).

3.7.2 ORGAN PROCUREMENT AND COLD ISCHEMIA

Organ procurement arguably represents one of the most invasive surgical procedures conducted today. Because pro-inflammatory response has been shown to correlate with the extent of surgery (62), the surgical trauma associated with the procurement procedure probably contributes to the pro-inflammatory response in the donor.
A subsequent prolonged cold preservation period may exacerbate functional deterioration, an observation widely acknowledged in renal transplantation where prolonged cold storage is associated with poor graft outcome (63,64). Prolonged cold ischemic time is associated with poor graft function in whole-organ pancreas transplantation (36) and islet loss in culture (65). The mechanism responsible may involve generation of reactive oxygen species (ROS) during cold preservation (66).

3.7.3 ISLET ISOLATION

Islet isolation consists of pancreas digestion and purification of the islets. The crucial part of the isolation procedure is the enzymatic dissociation of islets from the surrounding exocrine tissue, by the use of an enzyme blend. The blend consists mainly of collagenase class I and II and neutral protease or thermolysin (67) that is perfused into the pancreas via the main duct. This is followed by 37°C incubation and mechanical dissociation (68).

It has fairly conclusively been demonstrated that some of the components of the enzyme blend are toxic to islets (69-72). Additionally, islets are exposed anoxia, hyperosmolarity, acidity, and mechanical stress during the digestion phase of isolation (73,74). As a consequence, up to 30% of all islets may stain TUNEL (terminal dUTP nick end labeling) positive for apoptosis immediately after isolation (75). Not surprisingly, the length of the digestion process correlate with islet damage (76-78), stressing the importance of an efficient enzyme blend (79). The search for an optimal blend is a work in progress (80).

3.8 THE ROLE OF INNATE IMMUNITY IN THE PERI- AND POST-TRANSPLANT PERIOD

Observations from experimental syngeneic islet transplantation demonstrate that a majority of transplanted β-cells (up to 60%) are lost during the first week after transplantation (81-83). The absence of allo- and auto-immunity in these models implies a dominant role for innate immunity in causing β-cell loss. Moreover, the inflammatory cytokines released during islet transplantation are suggestive of an activated innate immune system (84). The activator of the host immune response may be the islets themselves, as islets become highly inflamed during the process of brain death, organ procurement and islet isolation and secrete multiple pro-inflammatory mediators such as tissue factor (TF), monocyte chemotactic protein (MCP)-1, and interleukin (IL)-8 (85). The innate immunity-driven processes can roughly be divided into the peri-transplant blood-mediated reaction and post-transplant interactions with the host liver.

3.8.1 THE INSTANT BLOOD-MEDIATED INFLAMMATORY REACTION

When islets are dispersed in ABO-identical non-heparinized whole blood, clotting can be observed macroscopically within 5 minutes. This clotting reaction has been termed the instant blood-mediated inflammatory reaction (IBMIR) (86,87). Characterization of the process reveals that when islets come in contact with blood, there is activation and rapid binding of platelets to the islet surface, together with activation of the coagulation and complement systems (86). Within 15 min, leukocytes are found infiltrating the islets, and after an hour, most of the islets are infiltrated by numerous leukocytes resulting in disruption of islet integrity and islet loss (86). The process has been demonstrated in experimental animal models and in the clinical setting (86,87).

It has been demonstrated that human pancreatic islets, but not exocrine tissue, express tissue factor (TF) at the cell surface (87). IBMIR can be abrogated with specific antibodies towards TF, which suggests that TF on the islet surface is the main trigger of IBMIR (87). Moreover, it has been demonstrated that the content of tissue factor in the islets correlates with the strength of the IBMIR (88). Human islets express >50 inflammation-associated genes (85), and several lines of evidence suggest that TF expression is part of general pro-inflammatory status of human islets, induced in a manner analogous to that of other inflammation-related genes (88,89). IBMIR

therefore represents a functional link between inflammation and coagulation. Inhibing IBMIR has been shown to enhances islet survival and improves long-term function in nonhuman primates (90).

Recently, Eich and co-workers have demonstrated, using FDG labeled porcine islets in a pig alloislet transplant model, that following intraportal islet transplantation, only 50% of the radioactivity contained in the injected islets could be found in the liver following intraportal transplantation (the peak occurred at 25 minutes, 5 minutes after completion of the transplantation; all of the infused radioactivity was accounted for) (91). The data imply that 50% of the infused islets are damaged to an extent that they release intracellular FDG, and additionally, that this happens within an extremely short time-period (<20 minutes) (91). Disturbingly, in a series of human islet transplants, the same results were obtained (personal communication O. Korsgren). This may explain the peaks in C-peptide observed during and immediately after clinical islet transplantation (92). Eich and co-workers speculate that the only biological systems capable of such swift destruction are the cascade systems with the complement system as the prime candidate (91). Interestingly, heparin had no effect on this immediate destruction of islets (91), testifying to the suboptimal effects of heparin on complement activation.

3.8.2 INTERACTIONS WITH THE LIVER

As outlined, the events proceeding transplantation are highly stressful to the islets and induce secretion of pro-inflammatory mediators (85). These include chemokines of the CC- and CXC- families, notably MCP-1, macrophage migration inhibiting factor (MIF), IL-8, RANTES (Regulated upon activation, normal T-cell expressed and secreted) and CXCL10 (85). The secretion of these chemokines can recruit monocytes from blood into the islets were they mature into dendritic cells (93), but also attracts and activates macrophages (94). The resident macrophages in the liver (Kupffer cells) appear to be important mediators of cellular injury to the islets, e.g. by secreting pro-inflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α (95,96). Prevention of macrophage activation and the consequent release of inflammatory cytokines preserve islet mass after transplantation (84). Additionally, experimental studies have revealed a significant increase in pro-inflammatory cytokines from neighbouring hepatocytes (97) and intrahepatic endothelial cells (98,99) as a result of

intraportal transplantation. Finally, contaminating ductal (100) and acinar (101) cells that are inherently co-transplanted with the islets are also important contributors to the secretion of pro-inflammatory cytokines. The net result is a peri-islet milieu characterized by high levels of β-cell toxic cytokines (e.g. IL-1β, TNF-α and IFN-γ (102)), hampering the post-transplant survival of islets.

3.9 ALLO- AND AUTOIMMUNITY IN ISLET TRANSPLANTATION

There is extensive crosstalk between the innate and the adaptive immune system. This may be illustrated by the observation that activation of the innate immune system delays or prevents the establishment of peripheral tolerance (103-105). It has been shown that human islets can release high-mobility group box 1 (HMGB1), a protein associated with necrotic cell death (106). HMBG1 activates, possibly through binding with toll-like receptors (TLRs) (107-109), both dendritic (antigen presenting) cells and T-cells (110) and may thus be a link between the innate and adaptive immune system. Accordingly, HMGB1 has been shown to be pivotal in the initiation and progression of allograft rejection (111). Blockade of HMGB1 in NOD mice prevents autoimmune progression and delays diabetes onset (110).

In clinical islet transplantation there is uncertainty regarding the contributions of adaptive immunity to the post-transplant decline in graft function (112), perhaps because intrahepatic islets are not regularly biopsied. However, we (TL, AF and TGJ, unpublished data) and others (113) have witnessed rise in antidonor HLA-levels occurring simultaneously with disappearance of C-peptide in islet-graft recipients, strongly indicating transplant failure due to rejection. Additionally, it has been reported that pre-transplant recipient donor-HLA reactivity is associated with a 4-fold increased risk of graft loss (114). Also, cultured human islets have been reported to cause T-cell responses in mixed islet/lymphocyte cultures dependent upon the degree of HLA mismatch (115). The Edmonton protocol is steroid-free and therefore the potential for alloreactivity may be significant.

Transplantation to patients with type 1 diabetes is a unique situation in that type 1 diabetes is an auto-immune disease. Recurrence of auto-immunity following islet transplantation has been reported, and is associated with graft failure (116-119). It has been suggested that the risk of auto-immune recurrence is particularly high in islet

recipients that have not been subjected to prolonged immunosuppression prior to transplantation (e.g. patients that have not received a kidney graft prior to transplantation) (116) and that a short duration of diabetes, and presumably relative high auto-immune activity, correlates with an increased risk of graft loss (114). Alternatively, the immunosuppressive regimen may play a role, as the Edmonton protocol (daclizumab, tacrolimus and sirolimus) has been demonstrated to increase autoreactive T-cells and to induce autoimmune recurrence after islet transplantion (119).

Some evidence suggest that substitution of monoclonal anti-CD25 antiboides (e.g. daclizumab) with polyclonal T-cell antibodies (e.g. ATG) may reduce the risk of graft failure (112), indicating that the immunosuppression traditionally utilized in islet transplantation is insufficient.

3.10 HYPOXIA AND TOXIC SUBSTANCES IN THE POST-TRANSPLANT PERIOD

3.10.1 REVASCULARIZATION

For the islets that have escaped the IBMIR, and are able to survive in the pro-inflammatory hepatic environment (the presinusoidal capillaries (120)), the journey towards successful engraftment has only just begun. The process of islet isolation and purification has caused complete destruction of the islet capillary network (121), and because revascularization takes about 2 weeks, they are now relaying upon passive diffusion of oxygen and nutrients for a prolonged period (122-124). Islets are normally accustomed to highly oxygenated blood and nutrient rich surroundings (125); in spite of making up 1% of the pancreatic mass, islets receive 15% of the blood flow (126). In whole organ pancreas transplantation it has been shown that the major cause of graft loss and primary non-function during the first days after transplantation is caused by vascular thrombosis (127). This indicates that adequate oxygenation is important for islet survival. A 2 week hypoxic period may thus be detrimental to the transplanted islets (128).

The islets that do survive have adapted to hypoxia (125). Once revascularization is in place, the ability to absorb oxygen is immediately enhanced. This rapid increase in oxygenated blood flow may cause reperfusion injury (44).

Careful observations of intraportally transplanted islets show that, when grafted, the vascular density is only ~25% of that in native islets, accompanied by markedly reduced oxygen tension compared to normal (123,129). This has profound adverse effects on insulin synthesis and glucose-stimulated insulin secretion (122). Additionally, important paracrine signaling from neighbouring endothelial cells is reduced (130), as well as the drainage of secreted hormones, including islet amyloid polypeptide (129). Amyloid deposition is a hallmark of type 2 diabetes, and extensive amyloid depositions has been observed in intraportally transplanted human islets (131).

3.10.2 LIPOTOXICITY

Animal models of type 2 diabetes (e.g. Zucker diabetic fatty (ZDF) rats) have identified overaccumulation of lipids in islets as a cause of β-cell destruction (132). Later studies have confirmed that exposure of isolated human islets to fatty acids damages β-cells and results in apoptosis (133).

The importance of lipotoxicity in clinical islet transplantation is illustrated by the regular occurrence of focal liver steatosis [REF (134) and figure 2]. Islets transplanted to the liver are exposed to high portal vein levels of nutrients and gut hormones, stimulating maximal production and secretion of insulin. High levels of insulin upregulates SREBP1c in hepatocytes, which in turn cause intracellular lipid accumulation (steatosis) and increased secretion of triglycerides (135). Focal liver steatosis is also observed in other scenarios where the liver is exposed to high levels of insulin locally (e.g. in patients with metastatic insulinoma (136) or in peritoneal dialysis when insulin is added (137)). Unfortunately, lipoprotein lipase of the islets (138) may hydrolyze triglycerides released by surrounding hepatocytes, exposing the β-cells to increasing levels of fatty acids. In a murine model of intraportal islet transplantation it has been demonstrated that islets within steatotic leasions display sharp reductions in insulin-positive β-cells and replacement by thick collagen strands reminiscent of the poststeatotic fibrosis observed in islets of ZDF rats (135).

There are several possible explanations for lipid-induced β-cell damage. First, excess lipid induces stress on the membranes of lipid-metabolizing organelles (e.g. the endoplasmic reticulum (ER) and mitochondria) (139), thereby increasing the production of ROS (140). Second, fatty acids can directly elicit a pro-inflammatory response by binding to Toll-like receptors (TLRs) (141). TLRs belong to a family of pattern-recognition receptors that activate nuclear factor-kappaB (NF-κB) and initiate synthesis of pro-inflammatory mediators (142). TLRs are found on β-cells, and suppression of TLRs is associated with improved islet survival (143).

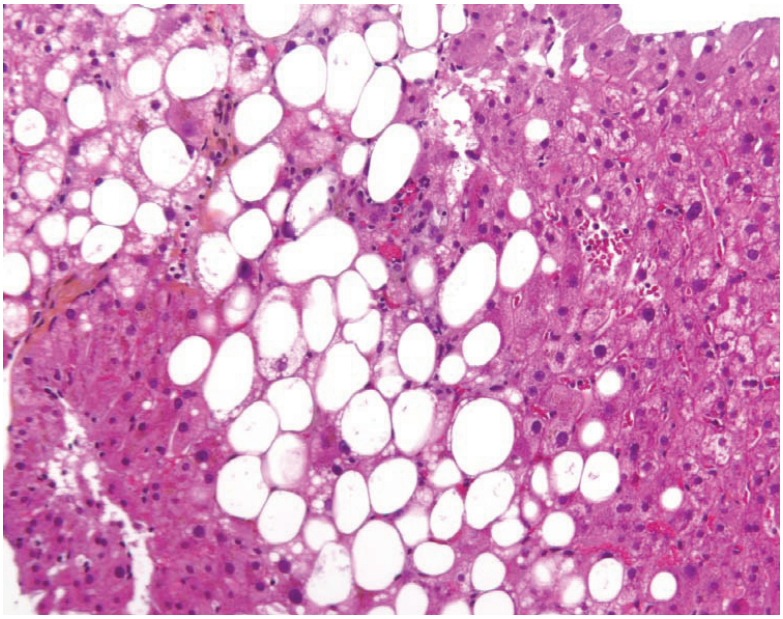

Figure 2: Focal liver steatosis 5 months after a second islet transplant procedure. The two transplants were performed within a 3-week period in August 2003. Ultrasonography and magnetic resonance imaging 1 month after the second transplant were normal, indicating that focal steatosis needs time to develop. The patient was initially insulin independent, but resumed low dose insulin within 3 months after the second transplantation. 1 year after biopsy-proven liver steatosis, the patient received a third islet infusion, after which the patient enjoyed a 3 month period of insulin independence, with subsequent reinstatement of low-dose insulin therapy. Follow-up at 4 years indicates partial graft function with a 50% reduction in insulin requirement

compared to pre-transplant levels, with decent glycemic control (HbA1c levels persistently < 10) and absence of hypoglycaemic episodes.

3.10.3 TOXIC IMMUNOSUPPRESSION

Allogeneic islet transplantation warrants immunosuppression to prevent rejection. Unfortunately, all immunosuppressive agents utilized in the maintenance regimen after transplantation are islet toxic (144-146). Experiences from allogeneic kidney transplantation reveal that 15% of transplanted patients develop diabetes due to β-cell toxic immunosuppression (147), although it must be noted that glucocorticoids are avoided in islet transplantation (38). Still, intraportally transplanted islets are in close proximity to the the portal blood where the peak drug concentrations of e.g. calcineurin inhibitors are reported to be 2-3 times that of the peripheral circulation (148). This may potentiate the diabetogenic effects of the drugs (149).

3.11 MECHANISMS OF β-CELL DESTRUCTION

The principal mediators of β-cell damage in the context of islet transplantation are pro-inflammatory cytokines, oxidative stress and toxic substances. Regardless of the mediator, the mechanism of damage relates in large measure to the activation of intracellular transcription factors, most notably NF-κB, the Janus kinase–signal transducer and activator of transcription 1 (STAT1) pathway and the mitogen-activated protein kinases (MAPK). The chain of events that promote activation of these intracellular transcription factors is essentially unknown (150), although a highly complex picture is emerging (151).

3.11.1 PRO-INFLAMMATORY CYTOKINES

Following receptor binding of IL-1β, TNF-α and IFN-γ distinct signalling cascades are activated in β-cells, as shown in Figure 3. IL-1β mediates activation of the transcription factor NF-κB pathway, a pathway partly shared by TNF-α. The IL-1β and TNF-α signalling cascades also cause the activation of members of the MAPK. IFN-γ signalling involves activation of the STAT1 pathway. The concerted action of these transcription factors alters the expression of multiple genes, ultimately causing deleterious effects on β-cells following islet transplantation (152,153).

A growing body of evidence indicates that there is considerable cross-talk between the STAT and NF-κB signalling pathways [reviewed in (154)]. In human islets in vitro, both activation of STAT1 (e.g. by IFN-γ) and NF-κB (e.g. by IL-1β) are required for inducing apoptosis (155).

3.11.2 OXIDATIVE STRESS

During normal cellular metabolism, ROS are formed. The primary source of ROS production is probably mitochondria (156). All cells are equipped with multiple enzymes (e.g. superoxide dismutases) and anti-oxidants (e.g. vitamins) to neutralize ROS. However, during hypoxia the mitochondrial respiratory chain is inhibited, and as the antioxidant defence systems become overwhelmed, ROS accumulates (157). The result is oxidative stress. Oxidative stress is characterized by depletion of ATP, mitochondrial membrane depolarization, cytochrome c release, and activation of apoptotic and necrotic pathways (75,156,157). Islets have been shown to be particularly prone to ROS accumulation because of low levels of antioxidant enzymes (158). ROS are able to activate multiple signalling transduction pathways, notably MAPK (159) and NF-κB (160).

3.11.3 NITRIC OXIDE

As a consequence of activation of the intracellular pro-inflammatory signalling pathways, multiple deleterious events may be initiated, such as upregulation of the pro-apoptotic cell-surface receptor Fas (161), upregulation of pro-inflammatory mediators (162-164), transcription of apoptotic genes, caspase activation (165,166) and suppression of antiapoptotic signalling (167). However, the most important mediator of cellular damage is possibly the enzyme inducible nitric oxide synthase (iNOS) (168) that causes formation of nitric oxide (NO). NO has been shown to be highly toxic to human islets, causing apoptosis and necrosis (106,155,169,170). NF-κB, STAT1 and MAPK signalling all induce iNOS expression, and conversely, the improved islet viability observed by inhibiting these signalling cascades may, at least partially, be attributed to reduced NO production (171-173). Selective inhibition of iNOS have been shown to prevent diabetes in the NOD mouse (174), protect rat islets from hypoxia-induced apoptosis (175) and to protect human islets from cytokine-induced cell-death (106).

The deleterious effects of nitric oxide (NO) are mainly based on its, and its derivates, ability to deplete the cells of energy (ATP) (176). Both the energy status of the cells prior to NO exposure, as well as the concentration of NO are crucial determinants. NO may, in combination with compromised ATP production (e.g. hypoxia) cause necrosis (177), but if ATP production is sufficient, apoptosis may ensue or there may not even be cell death at all (176). Similarly, pro-inflammatory cytokines may induce necrosis or apoptosis in a NO-dependent manner in human islets (106), possibly dependent upon the energy status of the islets.

NO depletes the cell of ATP via inhibiting mitochondrial respiration, inducing mitochondrial permeability transition, inhibiting glycolysis and finally by activating poly ADP-ribose polymerase (PARP) (178). Glucose-stimulated insulin secretion (GSIS) is dependent upon mitochondrial oxidation of glucose to CO_2 (179), and consequently, NO may be one mechanism responsible for reduced GSIS under pro-inflammatory conditions (169). Also, the NO stresses the endoplasmic reticulum (ER) (180), causing inappropriate folding of proteins in the ER, a mechanism involved in β-cell apoptosis (181-184).

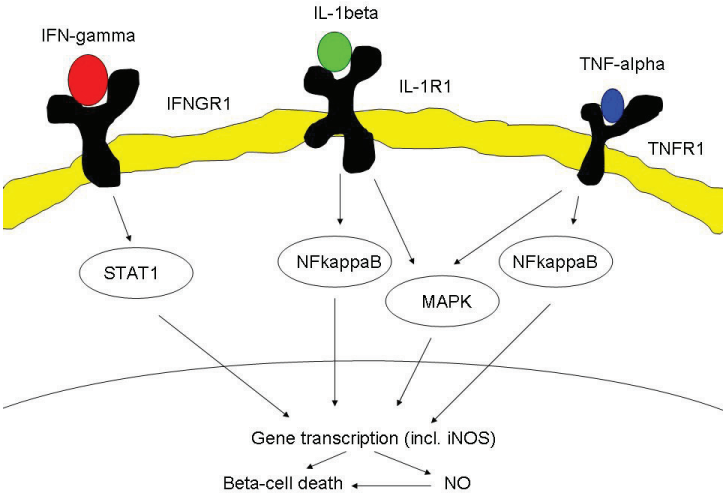

Figure 3: Following binding with membrane bound receptors, pro-inflammatory cytokines activate intracellular signalling cascades, culminating in inflammatory gene

transcription and production of NO. The net result may be apoptosis or necrosis. The complexity of the signalling pathways is grossly simplified.

3.12 ENDOGENOUS PROTECTIVE MECHANISMS

3.12.1 GLUCOCORTICOIDS

An inflammatory insult is followed by systemic release of anti-inflammatory glucocorticoids (cortisol) from the adrenal glands. Despite continuous efforts of the pharmaceutical industry to design anti-inflammatory compounds that can circumvent side effects while maintaining effectiveness, no other drug has (yet) approached the therapeutic benefits of glucocorticoids (185). Glucocorticoids efficiently block leukocyte migration towards sites of inflammation, by inhibiting relevant NF-κB-driven cytokines and chemokines and thereby ensues quick dampening of inflammation (186). NF-κB is a key regulator of pro-inflammatory gene expression (187) and chemokines of the CC- (e.g. MCP-1) and CXC- (e.g. IL-8) family are dependent on NF-κB (188-191). NF-κB activation, and subsequent secretion of pro-inflammatory mediators may directly harm the β-cells by drawing immune cells into the islets in the donor during brain death, increase cytokine production from intraislet macrophages during isolation and in pretransplant culture (155) and cause immune activation post-transplantation in the recipient. Additionally, NF-κB induces PARP activation (70), and thereby apoptosis (192). An increasing body of evidence implicates NF-κB in β-cell apoptosis (193,194), and agents interfering with NF-κB binding to DNA have been shown to increase β-cell survival and improve insulin release following islet isolation (70,195-197). In addition to interfering with NF-κB, glucocorticoids mediate an increase in anti-inflammatory cytokines such as IL-10 (198). IL-10 overexpression (in combination with low-dose CyA) has been shown to prolong allograft survival in a murine islet allotransplant model (199).

3.12.2 THE LIVER X RECEPTORS

Inflammation and metabolism are tightly linked processes. For example, injurious signals (e.g. ROS or toxins) may activate intracellular danger sensors (the inflammasomes (200)) that via activated caspase-1 subsequently alters lipid (201), glucose (202) and protein (203) metabolism, presumably to protect the cell and promote homeostasis after injury. Additionally, caspase-1 processes pro-inflammatory

cytokines (204). Alternatively, an injurious signal may activate NF-κB, and thereby cause profound effects on lipid (205), glucose (206) and protein (207) metabolism, in addition to the well-documented effects on chemokine and cytokine secretion. Thus, injurious stimuli cause cells to alert their surroundings using inflammatory mediators, but also to change their metabolism to maximize the chance of survival.

Conversely, nutrients, especially lipids and their metabolites alter the inflammatory profile of the cell. Lipids are sensed and regulated by intracellular nuclear hormone receptors, notably the liver X receptors (LXRs) and the peroxisome proliferator-activated receptors (PPARs) (208). These lipid-sensing receptors heterodimerize with the retinoid X receptor (RXR) to activate a feed-forward metabolic cascade that maintains lipid homeostasis (139). Simultaneously, these receptors also suppress inflammatory gene transcription, perhaps through interference with NF-κB (209,210). Due to their effects on metabolism and inflammation, LXR and PPAR agonists represent promising anti-diabetic agents (211-213). Some PPAR agonists (e.g. the glitazones and the fibrate class of hypolipedemic drugs) have been used clinically for decades (211).

LXRs primarily act as cholesterol sensors: when the cellular concentration of oxysterols (oxygenized cholesterol) increases, LXR induces the transcription of genes that protect the cell from cholesterol overload. In islets, they promote the transcription of genes for ATP-binding cassette transporters, ABCA1 and ABCG1 (214) that transport lipids out of the cell. At the same time, LXRs also up-regulate sterol regulatory element binding protein (SREBP)1c (215,216). SREBP1c boosts synthesis of poly-unsaturated fatty acids (PUFAs) for use in e.g. the cell membrane and organelles, and thus has cytoprotective effects (200). However, other lipids are also synthesized and prolonged SREBP1c activity is associated with lipotoxicity in β-cells (214,217). Importantly, LXRs suppress NF-κB target genes (209,218), and dampens both innate (209) and adaptive immune responses (219). The effects of LXRs on β-cells are schematically represented in Figure 4.

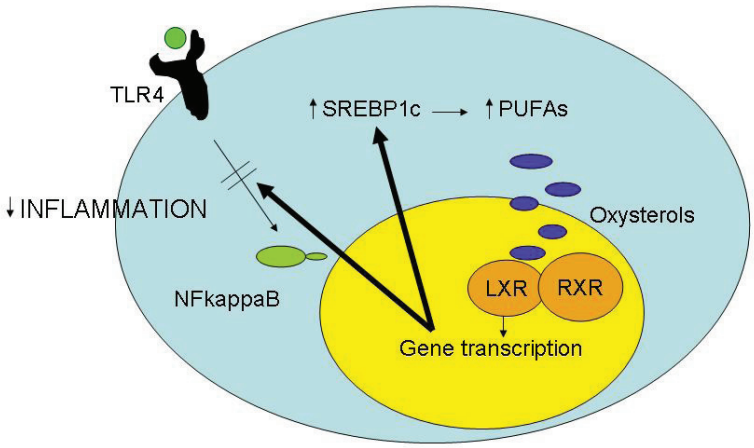

Figure 4: The LXR-RXR complex is activated by endogenous oxysterols and initiate transcription of products that inhibit downstream TLR4 signaling. Additionally, LXRs increase transcription of SCREBP1c that in turn increases cellular lipogenesis (e.g. production of PUFAs).

3.12.3 LIPID SIGNALING – THE POLY-UNSATURATED FATTY ACIDS

Injury prompts cells, via cyclooxygenases or lipoxygenases, to synthesize eicosanoids (e.g. prostaglandins and leukotrienes) from PUFAs. Prostaglandines and leukotrienes have been recognized for their pro-inflammatory effects for more than seven decades (220,221), and are involved in the development acute inflammation. Recent studies indicate that as inflammation proceeds, immune cells stop producing eicosanoids and instead convert PUFAs into lipoxins, protectins and resolvins (222,223). These lipid mediators actively terminate inflammation and promote resolution of the inflammatory process (222,223).

Interestingly, PUFAs downregulates SREBP1c, and thereby limit their own production (224). This has been demonstrated to occur through interacting with the binding domain of LXR in the SREBP1c promoter (225). A recent study has

demonstrated that PUFAs, and especially the PUFA-derived product Resolvin E1 (RvE1), decreases liver steatosis and increases insulin sensitivity in adipose tissue (226). It is conceivable that some of these effects are due to downregulation of SREBP1c.

3.12.4 SUPPRESSOR OF CYTOKINE SIGNALLING PROTEINS

A different mechanism for negatively regulating the pro-inflammatory loop is the production of suppressor of cytokine signalling (Socs) proteins (227). Transcription of Socs genes is induced by NF-κB activation, and the Socs proteins act in a negative feedback-loop that attenuates pro-inflammation (228). For example, Socs-3 inhibits inflammation-induced NO production and apoptosis in a β-cell line (229), as well as several chemokines and the death receptor Fas (230). Moreover, Socs3 transgenic islets have been demonstrated to be protected in an allogenic transplant model (231).

3.12.5 HEAT SHOCK PROTEINS

Heat-shock proteins (Hsps) constitute another protective mechanism. Hsp70 confers protection against NO induced necrosis (232) and inhibit iNOS expression (233). Additionally, the level of protection against IL-1 induced apoptosis has been reported to depend upon the level of Hsp70 expression (234). Hsp32, or haem oxygenase-1 (HO-1), has been shown to protect against pro-inflammatory cytokine and Fas induced β-cell dysfunction, and HO-1 upregulation resulted in improved islet function in vivo (235). HO-1 has also been shown to be prolong islet allograft survival (235-237).

4. AIMS OF THE STUDY

The common denominator causing loss of β-cells in islet transplantation seems to be inflammation and the single most important inflammatory reaction may occur in the recipient upon transplantation: IBMIR. Our main hypothesis is that anti-inflammatory strategies, applicable to the donor, islets in pre-transplant culture and to the recipient, could limit the loss of β-cells. The purpose of the present study was to expand our knowledge on anti-inflammatory strategies and their effect on human islets, and thereby hopefully identifying safe and effective tools for improving β-cell survival following islet transplantation. Finally, we reasoned, a powerful tool for reducing loss of β-cells would be to develop a transplant technique that avoids IBMIR.

5. METHODS

5.1 ETHICS

Human islets were isolated from deceased donors after consent was obtained from the organ donor registry or relatives, and experiments were performed in accordance with local institutional and Norwegian rules and regulations. The animal procedures and housing were in accordance with institutional guidelines and national legislation conforming to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication no. 85-23, revised 1996). All animal experiments were approved by the Institutional Animal Care Committee.

5.2 HUMAN ISLET ISOLATION AND CULTURE (PAPER I-III)

Islets were isolated according to the automated Ricordi method (238), refined by the Nordic Network for Islet Transplantation (68). The isolations were performed in Oslo and in Uppsala. Pancreata were digested using Liberase until May 2007 and thereafter SERVA Collagenase. To ensure comparable results, quality testing using dynamic or static insulin secretion was performed on hand-picked islets, only using preparations were an insulin stimulation index > 2 was obtained. Additionally, aliquots of ~100 islets were stained with dithizone and purity calculated, and generally, only preparations with purities >50% were used. Islets were cultured at 37 °C (5% CO_2) in CMRL 1066 medium supplemented with 10% ABO-compatible human serum or heat-inactivated foetal calf serum. The medium was changed every second day.

5.3 TRANSPLANTATION OF HUMAN ISLETS TO SCID MICE (PAPER I)

Male inbred SCID mice were chemically rendered diabetic and used as recipients. Human islets were cultured for 48 h prior to transplantation, and subsequently batches of 600 IEQ human islets were washed twice and transplanted under the left kidney capsule. The nonfasting blood glucose levels of the animals were measured twice a week during the first week. Thereafter, blood glucose levels were assessed once a week. At 4 weeks, cured mice were subjected to an intraperitoneal glucose tolerance test. Two to three days later, grafts were removed to confirm the recurrence of diabetes.

5.4 SYNGENEIC INTRAMUSCULAR ISLET TRANSPLANTATION (PAPER IV)

Lewis rats were used as donors and recipients; the recipients were chemically rendered diabetic, thus allowing a transplant model devoid of allo- and autoimmunity. Islets (2000 IEQ) were dispersed in 0.15 mL of transplant medium, collected in a syringe, and transplanted in the fibre direction of the m. biceps femoris bilaterally, to obtain a pearls-on-a-string distribution of islets in the muscle. Post-transplant, blood glucose was measured daily in the recipients, until>3 consecutive measurements were below 10 mmol/L. After that, blood glucose was measured once a week. Eight weeks after transplantation, graft function was assessed by an intravenous glucose tolerance test.

5.5 DYNAMIC INSULIN SECRETION (PAPER I-III)

For dynamic insulin secretion, 20 islets were handpicked and added to each of six perfusion chambers, layered between inert polystyrene beads. A Krebs–Ringer bicarbonate buffer was used at a flow rate of 0.2 ml/min. The perfusion system was initiated after a 30-min equilibration period in 1.67 mM glucose, followed by a 60-min stimulation period with 20 mM glucose and finally back to 1.67 mM glucose. Samples were collected at 6-min intervals, immediately ice-chilled, and stored at -20°C until analyzed. In paper II and III, the hand-picked islets were photographed prior to perfusion, analysed with Cellimage® and following calculation of total islet size, the insulin secretion was normalized to islet area.

5.6 TUBING LOOPS (PAPER III)

To measure IBMIR in vitro we used a modification of a tubing loop model previously described (87), designed to resemble a blood vessel. In brief, loops made of polyvinylchloride were treated with a heparin surface. 6 µL (about 6000 IEQ) islets were preincubated, washed and resuspended in 150 µL CMRL 1066. Thereafter, fresh ABO-identical blood (7 mL) was added to the loops and incubated for 15 minutes on a rocker inside a 37°C incubator for 15 min, before addition of the islets. We also included a control loop containing blood supplemented with 150 µL CMRL 1066 but no islets. The rocker was set to generate a blood flow of about 45 mL/min (to mimic portal blood flow). Blood samples were collected into tubes containing EDTA before perfusion and at 5, 15, 30, and 60 min after the addition of islets. Platelets were

counted with a Coulter AcT diff analyser and plasma concentrations of thrombin-antithrombin complex were quantified with a commercial EIA kit.

5.7 RNA ISOLATION

RNA was isolated from frozen islet pellets (paper I-III) with RNA isolation kit using the MagNA Pure LC instrument according to the manufacturer's description.
RNA from frozen muscle biopsies (paper IV) were isolated first by disruption and homogenization using mortar and pestle, subsequently solubilized in lysis buffer followed by three cycles in a rotor-stator homogenizer. RNA was then extracted according to the manufacturer's protocol using the RNeasy kit from Qiagen. In all cases, RNA concentrations were determined with a spectrophotometer, and a standardized amount of RNA was reverse transcribed.

5.8 ASSAYS

Enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and the low-density array (LDA) are standardized and commonly used methods of analysis and will not be described in further detail. Similarly, commercial kits for ATP content, ADP/ATP ratio, Caspase 3 and 7 activity, mitochondrial dehydrogenase, DNA content activity were used. The procedures are described in the methods section of each paper and are generally performed according to the manufacturer's instructions.

5.9 METHODOLOGICAL CONSIDERATIONS

In general, data generated in vitro are simplified approximations of the complex biological diversity encountered in vivo. For example, the blood loop model consisting of heparin coated polyvinylchloride tubes do not harbour the biological activities of endothelial cells in vivo. Additionally, air enclosed in the tubes affects blood-activation. The results obtained must therefore be interpreted carefully, and rather the relative differences between the groups, than the absolute values, are important. The in vivo models utilized have been devoid of allo- and autoimmunity and represent different islet:organism size ratios. These factors will inherently affect post-transplant islet engraftment, survival and function.

6. SUMMARY OF RESULTS

6.1 PAPER I: Glucocorticoids reduce pro-inflammatory cytokines and tissue factor in vitro and improve function of transplanted human islets in vivo.

In vitro, we showed that glucocorticoids reduced human islet mRNA and protein levels of the CC- (MCP-1) and CXC- (IL-8) chemokines and TF with ~50%, and increased IL-10 mRNA. Islet levels of TF remained lower than control during a 3 day period after drug removal. The anti-inflammatory effect was abrogated by high glucose. Furthermore, glucocorticoids had a profound dampening effect on glucose-stimulated insulin secretion (~60%), but the reduction was accompanied by an increase in intracellular insulin content. Furthermore, this effect was reversible within 5 days after drug removal from culture medium, at which point the glucocorticoid treated islets performed better than controls. Apart from increased intracellular ATP levels (~40%), there were no effects on islet viability. When transplanted to diabetic SCID mice, glucocorticoid treated islets reversed diabetes more effectively than controls.

6.2 PAPER II: The synthetic liver X receptor agonist GW3965 reduces tissue factor expression and inflammatory responses in human islets in vitro.

In this paper we demonstrated, in vitro, that the transcription factors LXRα and -β are expressed in human islets, and that the expression is upregulated by LPS and a synthetic LXR agonist (GW3965), but not by glucocorticoids. Mapping the effect of LXR stimulation in human islets, we showed that GW3965 has pleiotropic effects on human islets. It downregulated CC-(MCP-1 and CCL-5) and CXC-(IL-8) chemokines and TF, in addition to the transcription factor STAT1. The efficacy approached that of glucocorticoids. Additionally, treatment resulted in upregulation of vascular endothelial growth factor (VEGFA) as well as the zinc transporter SLC39A1. On the other hand, GW3965 also had pro-inflammatory properties, evident by upregulation of Toll-like receptor 4 (TLR4) and CXCL10. However, GW3965 had, in contrast to glucocorticoids, no effects on insulin secretion. Nor could we detect effects on islet viability.

6.3 PAPER III: Resolvin E1 reduces proinflammatory markers in human pancreatic islets in vitro.

Resolvin E1 (RvE1) has two identified receptors; BLT1 and ChemR23. In vitro, we showed that human islets weakly express BLT1, but not ChemR23. Still, we showed that RvE1 has anti-inflammatory properties on human islets, reducing mRNA and protein levels of MCP-1, IL-8 and TF with an efficacy approaching that of glucocorticoids. Additionally, the expression of the CC-chemokine receptor and ligand 5 (CCL- and CCR5) and MMP9, and culture supernatant levels of CXCL10, were reduced. RvE1 upregulated TGF-β. At very high concentrations, RvE1 demonstrated pro-inflammatory properties. No adverse effects of RvE1 on insulin secretion or islet viability were observed. We additionally evaluated the effect of RvE1 on IBMIR in vitro, and found a modest inhibition of platelet consumption and complement activation at 5 minutes following islet-blood contact, indicating a modest inhibition of the initial phases of IBMIR.

6.4 PAPER IV: Sustained Reversal of Diabetes Following Islet Transplantation to Striated Musculature in the Rat.

As our anti-inflammatory strategies were ineffective in reducing IBMIR we sought to develop a strategy that would avoid islet-blood contact altogether. We transplanted islets into the m.femoris biceps of Lewis rats, carefully avoiding bleeding, but showed that twice the amount of islets were required to reverse diabetes, compared to intraportal transplantation. However; when successful, the median time to reversal of diabetes and post-transplant weight development was similar to intraportal transplantation. Intravenous glucose tolerance test at 8 weeks posttransplantation demonstrated comparable insulin responses between the transplanted groups (both delayed compared to non-transplanted, healthy controls), and similar effects on temporal blood glucose decline were observed. PEGylation of islets were evaluated as a tool to improve efficacy, but was ineffective. Histological examination of the grafts revealed significant peri-islet fibrosis surrounding the intramuscular grafts, but also a significant amount of ductal-like tissue. mRNA analysis of biopsies taken 1 hour after intramuscular transplantation showed induction of IL-6 and IL-8 expression. Sham and islet transplantation induced similar inflammatory response, and higher transplant volume injected elicited a larger inflammatory response.

7. DISCUSSION

THE INFLAMMATORY BARRIER IN ISLET TRANSPLANTATION

Inflammation is a primordial response that functions to protect us against invasion by pathogens. However, the effects on human islets as they venture through brain death, procurement, cold ischemia, isolation and encounter with blood in the recipient show that aggressive and prolonged inflammatory responses can be detrimental.

Humans, along with most animals, have evolved mechanisms to ensure that the inflammatory response is limited in time and space. Many endogenous anti-inflammatory and pro-resolving mediators function to counteract the properties of pro-inflammatory factors. During the progression towards brain death, and subsequently during procurement, cold ischemia, islet isolation and pretransplant culture, these mechanisms are severely disrupted or absent.

This work has explored three different endogenous anti-inflammatory mechanisms for reducing inflammation in human islets. As it became apparent these strategies were insufficient in protecting the islets when encountering blood, we additionally sought to identify a way to circumvent blood-contact in islet transplantation.

THE EFFECTS OF GLUCOCORTICOIDS

Glucocorticoids are the first class of endogenous anti-inflammatory mediators that have been successfully used for therapeutic purposes. During inflammation, secretion of cortisol from the adrenal cortex dampen local and systemic inflammatory events (239). Glucocorticoids have substantial effects on gene expression; ~1% of the genome might be influenced (239). In Paper I we explored the effects of glucocorticoids on human islets, and showed a potent inhibition of the pro-inflammatory markers TF, MCP-1 and IL-8. TF expression is part of general pro-inflammatory status of human islets (85,89) and the principal initiator of IBMIR (87). Inhibition of TF has been demonstrated to improve islet transplant outcome in an allogenic non-human primate model of islet transplantation (90) and TF content in human pancreata correlates with clinical outcome (89). MCP-1 is a potent chemattractant for macrophages, and likely participates in the non-specific immune reaction that destroys islets after transplantation. MCP-1 content in islets has been

demonstrated to correlate with clinical outcome (240). IL-8 is a potent chemoattractant of eosinophils and neutrophils (241) and may synergize with the effects of MCP-1 in promoting immune cell infilatration following transplantation. Glucocorticoids may exert the inhibiting effects through transrepression of NF-κB (242). Additionally, we found elevated transcription of IL-10 following glucocorticoid treatment of human islets. IL-10 is an anti-inflammatory cytokine that also activates T-regs (243). Viral IL-10 induction has been shown to prolong allograft survival in a murine islet transplant model (199). Glucocorticoids have been demonstrated to transactivate IL-10, which may be the mechanism responsible (244).

Insulin secretion was blunted by glucocorticoids, accompanied by an increase in the intracellular insulin content (Paper I), suggesting that glucocorticoids interfere with the secretory process of insulin release, and not the transcription. This observation is in line with previous studies (245). Additionally, the effect was reversible; 5 days after drug removal, glucocorticoid-treated islets responded better to glucose than controls. Collectively, these findings may suggest that glucocorticoids do not have durable detrimental effects on insulin metabolism.

Glucocorticoids did not affect islet viability in our study. On the contrary; it increased intracellular ATP levels. Intracellular ATP levels have been shown to positively correlate with transplant outcome in a pig-to-nude-mice transplant model (246). These findings contradict previous studies on murine islets and β-cell lines, where dexamethasone has been shown to induce apoptosis (247). However; the conditions in our study were different in that we did not use hyperglycaemic conditions. Alternatively, species specific differences may be responsible (248,249).

Possibly as a result of these anti-inflammatory effects, we found that glucocorticoid treated human islets reversed diabetes more efficient when transplanted to SCID mice. In a recent study it has been shown that glucocorticoid treatment of the brain-dead donor improves graft survival and reduces acute rejection episodes (56). Collectively, these data suggest that glucocorticoid treatment of the donor and/or islets in culture may be beneficial.

However, glucocorticoids have diabetogenic properties in vivo (250-254). Preliminary observations indicated that glucocorticoid treatment of the recipient severely complicates post-transplant blood glucose control (T. Lund, unpublished observations). We therefore wanted to explore other anti-inflammatory substances, with the identification of a non-diabetogenic, potent anti-inflammatory agent as target.

LIVER X RECEPTOR AGONISM AND HUMAN ISLETS

Liver X receptors (LXRα/β) are transcription factors belonging to the nuclear receptor superfamily and act as key regulators of lipid metabolism and inflammation [reviewed in (255)]. LXRs are activated by endogenously produced oxysterols. In paper II we showed that LXRs are expressed in human islets, and that the expression is upregulated by LPS. This suggests that activation of LXRs during stress constitutes an endogenous protective mechanism in human islets, as has been found to be the case in macrophages (256) and human monocytes (257). Supporting this, we found that GW3965, a synthetic LXR agonist, upregulated the expression of LXRs, and downregulated the pro-inflammatory mediators TF, IL-8 and MCP-1. Moreover, we also found that LXR activation significantly decrease the transcription factor STAT1, known to be involved in cytokine-induced β-cell death (172,258).

Interestingly, the data also showed significant upregulation of two known proangiogenic factors; angiopoietin-1 (ANGPT1) and vascular endothelial growth factor (VEGF) in GW3965 exposed islets. These factors have been shown to promote graft angiogenesis and enhance islet revascularization, contributing to improved glycemic control and better preservation of islet mass in mice (259,260).

On the other hand we observed upregulation of toll-like receptor-4 (TLR4), a receptor associated with enhanced bacterial removal during infection (261), and CXCL10, a pro-inflammatory cytokine involved, via TLR4 signaling, in β-cell destruction (262). These data may illustrate the endogenous effects of LXRs; on one hand limiting excessive cytokine production and on the other hand enhancing removal of gram negative bacteria during infection [reviewed in (263)]. However, TLR-4 has also been implicated in β-cell death and graft rejection after transplantation (143).

Furthermore, we showed that GW3965 significantly induced the expression of the typical LXR lipogenic target gene, SREBP1c, as well as the cholesterol efflux genes, ABCA1 and ABCG1, in LPS-stimulated islets. Prolonged activation of lipogenic gene expression can result in accumulation of free fatty acids and triglycerides, causing ß-cell dysfunction (264,265). Chronic stimulation of LXRs by the synthetic agonist T1317 has been shown to induce a marked upregulation of genes involved in lipogenesis in pancreatic β cells (266,267), and lipid overloading may cause serious β cell dysfunction and apoptosis (265,268). So even though it has been established that LXRs are important for maintaining normal β cell function (269), there also seems to be compelling evidence that supraphysiological activation of LXR could induce islet lipotoxicity (267,270).

In our study, however, we found no evidence of storage of excess lipids in GW3965 exposed islets, and consequently, we could detect no adverse effects on islet viability. On the contrary, we found a tendency towards lower basal insulin secretion from GW3965 treated islets, resulting in an increased insulin stimulation index. Increased basal insulin secretion is associated with cytokine induced islet dysfuncition (271), and these data may imply that GW3965 induced reduction in inflammatory milieu has positive effects on human islets. A possible explanation for the diverging results may be differences in the LXR agonist used, dosage and exposure time, glucose concentrations, or species specific differences (human versus murine islets).

Taken together, activation of LXRs with the synthetic LXR agonist GW3965 has potent dampening effects on key inflammatory mediators in human islets in vitro without adverse effects on insulin secretion or islet viability. However, not all inflammatory markers were reduced (e.g. TLR4 and CXCL10). Additionally, lipotoxicity remains a concern, especially under high glucose concentrations and/or prolonged vigorous LXR stimulation. Although probably safe as an ex-vivo tool to reduce immunogenicity of human islets prior to transplantation, in vivo studies need to exclude potential detrimental effects of GW3965.

RESOLVIN E1 - ANOTHER LINK BETWEEN LIPID METABOLISM AND INFLAMMATION

Lipid mediators derived from ω-3 PUFAs have emerged as endogenous suppressors of inflammation (222,272-274), and include protectins, maresins and resolvins (275). Resolvin E1 (RvE1) belongs to the E-group of resolvins and has potent anti-inflammatory and pro-resolving properties in many tissues and cells (276-280). In Paper III, we demonstrated that human islets express BLT1, the receptor for RvE1, but at a low level. However, other studies have confirmed effects of RvE1 in the absence of BLT1 (277), and in line with this, we found that RvE1 reduced IL-8, MCP-1 and TF in human islets. Additionally, we found reduced levels of CCL5 and its receptor CCR5. Disruption of CCL5/CCR5 and MCP-1/CCR2 signaling pathways has been reported to delay islet allograft rejection (281). In culture supernatant, we also found reduction of CXCL10, a chemokine possibly involved in the pathogenesis of type 1 diabetes (262). Although these receptors/chemokines, except MCP-1, are expressed at low levels in islet preparations, the findings may imply that RvE1 downregulates several important aspects of the inflammatory response. Additionally, we found no effect on insulin secretion or islet viability.

RvE1 can be synthesized in an aspirin-dependent pathway, and it has been demonstrated that healthy volunteers taking aspirin and ω-3 PUFAs have physiologically relevant doses of RvE1 in peripheral blood (282). This may indicate that RvE1 is a safe compound that could soon be commercially available. A recent study has demonstrated that RvE1 decreases liver steatosis and increases insulin sensitivity in adipose tissue (283) in a murine model. Since islet transplantation inherently produces (transient) liver steatosis, and insulin resistance is highly unfavourable in the context of minimal β-cell replacement, these properties of RvE1 could be relevant. Combined with the observation that RvE1 efficiently blocks ADP- and tromboxane-induced platelet aggregation (279) led us to investigate whether RvE1 could have an effect on IBMIR. Disappointingly, RvE1 added to blood only modestly dampened IBMIR, and only during the first 5 minutes after islet-blood contact (~20% reduced platelet consumption, ~50% reduction in thrombin-antithrombin (TAT) complexes). This may illustrate the potency and strength of the reaction elicited by blood upon contact with foreign tissue. Indeed, heparin dosages need to be increased to 4 IU/mL to have an effect on IBMIR (a dosage corresponding

to 20.000 IU in an adult). However; because islets reach the liver within 5 minutes after blood contact (91), RvE1 may still offer some benefit.
Based on the observations that anti-inflammatory treatment alone has only modest effects on IBMIR, we next set out to identify a strategy which allowed us to avoid IBMIR altogether.

AVOIDING IBMIR WITH INTRAMUSCULAR ISLET TRANSPLANTATION

In order to avoid IBMIR, we reasoned, the islets need to be transplanted into a site where they are protected from direct blood-contact. An available, well vascularised transplantat site is striated musculature, which has been employed successfully in autotransplantation of parathyroid tissue for decades (284). Similarities between the parathyroid tissue and pancreatic islets, as well as a few experimental and clinical observations, could indicate that the intramuscular (IM) site would be a feasible site for islet transplantation (285-288). In Paper IV we developed a simple and reproducible IM islet transplantation model and showed that IM islet transplantation to m. biceps femoris is feasible in a syngeneic rat model. Key points included pearls-on-a-string distrubion of islets in the muscle and utilizing as small transplant volumes as possible. However, in spite of an optimized transplant technique, twice the number of islets was required for reversal of diabetes compared to intraportal transplantation. We identified several possible reasons. First, in spite of an improved transplant technique, we observed clusters of islets with disrupted morphology in muscle, surrounded by fibrosis. This indicates a suboptimal transplant technique, as single islets located between muscle fibres appeared to have normal morphology. Second, the transplant procedure induced a volume dependent increase in the expression of IL-6 and -8 in myocytes (that is, larger transplant volumes elicited a larger pro-inflammatory response). The expression of IL-1β and TNF-α was not significantly upregulated, but that could possibly be due to the timing of the biopsy (1 hour after transplantation). These locally secreted pro-inflammatory mediators may recruit and activate immune cells to the transplant site, and thereby contribute in causing islet loss.

However, we believe there are several reasons for optimism. First, when IM graft function was established, it was durable (>100 days). Second, the insulin response to glucose as well as the temporal decline in blood glucose in the intravenous glucose

tolerance-test was comparable to intraportal grafts, indicating that the grafted islets could deliver insulin to the circulation in a similar fashion as intraportal islets. Third, examination of IM islet grafts, contrary to intraportal grafts, revealed a relative abundance of pancreatic duct cells (PDC). The suggestion that PDCs may differentiate into insulin producing cells has been proposed (289); the IM transplant model may represent a useful tool for evaluating PDC function in islet transplantation. Clinically, the purity of islet preparations transplanted into patients averages 50% to 60% (290), and the total proportion of PDC has been reported to approach 40% (291).

The rat model may not be appropriate for a comparative study between intraportal and IM islet transplantation. This is because rat islets have similar size as human islets, and the engraftment of islets in the considerably smaller rat liver is not comparable to humans [reviewed in (45)]. Additionally, rat muscle is small, and it is technically difficult to obtain a dispersed distribution of islets in the muscles.

7. FUTURE PERSPECTIVES

In light of research conducted in this thesis new possibilities arise.

First, combinatory approaches of the tested substances may enhance efficacy. For example, glucocorticoids exert their non-genomic effects through mobilization of annexin 1 [reviewed in (239)]. The secretion of annexin 1, however, is performed by the ATP-binding cassette (ABC) transporter system (292). As the expression of the ABCs is potently upregulated by LXRs, LXR activation may enhance the effect of glucocorticoids. Additionally, glucocorticoids upregulate BLT-1, a pro-inflammatory receptor partly responsible for steroid-resistance, in for example asthma (293). Some of the anti-inflammatory effects of RvE1 occurs through functional antagonism of BLT-1 (277). This may possibly explain why ω-3 PUFAs have steroid-sparing effects, in for example ulcerative colitis (294). The effects of RvE1 appear to be more potent than their ω-3 precursors (295), suggesting that the anti-inflammatory effects of glucocorticoids may be enhanced by RvE1 through inactivation of BLT-1.

Second, combinatory approaches may reduce unwanted effects. For example, RvE1 has been reported to potently enhance peripheral insulin sensitivity (295), an effect that may reduce the increased insulin resistance occuring when glucocorticoids are administered. Additionally, the unwanted lipogenic effects of LXR activation, occuring via upregulation of SREBP1c, may be reduced by RvE1. This because PUFAs downregulate SREBP1c (224) by interaction with the binding domain of LXR in the SREBP1c promoter (225).

Third, the studies suggest pro-tolerogenic effects of glucocorticoids (upregulation of IL-10) and RvE1 (upregulation of TGF-β) that may be explored in an allograft transplant setting.

Fourth, the experimental IM transplantation model would allow studies on bioengineered matrices carrying growth factors that potentially could improve islet engraftment and revascularization. Additionally, IM islet transplantation would allow for serial biopsies to be taken. As we know very little about islet engraftment, this model could be a valuable tool for enhancing our understanding of this complex process. Finally, preclinical studies conducted in Uppsala indicate that islet

revascularization in muscle may be close to the native pancreas, while in liver it is sharply reduced (personal communications P.O. Carlsson). This may imply that the long-term islet function may be better in muscle.

8. CONCLUSIONS

We have identified several endogenous strategies that potently reduce inflammation in pancreatic islets in culture, and shown that reduced inflammation can be translated into superior function following transplantation. Of the tested anti-inflammatory strategies, glucocorticoids represent the most powerful. However, due to their diabetogenic effects, glucocorticoids are probably best avoided as chronic treatment of the recipient.

The LXR agonist GW3965 has anti-inflammatory properties in human islets, with an efficacy that approaches that of glucocorticoids, without adverse effects on insulin secretion or islet viability. However, some aspects of the inflammatory response were upregulated, and concerns regarding lipotoxicity and overall safety need to be addressed prior to clinical application.

Resolvin E1 displayed exclusively anti-inflammatory properties on human islets, albeit with less efficacy than glucocorticoids. In spite of downregulating the inflammatory response, only modest effects on IBMIR was observed.

Intramuscular transplantation is a feasible strategy to avoid IBMIR, but it is less efficient compared to intraportal islet transplantation in rats. The IM transplant technique needs refinement.

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