Mechanisms and Therapeutic Interventions of Instant Blood-Mediated Inflammatory Reaction (IBMIR)

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Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjöldsväg 20, Uppsala, Saturday, April 28, 2007 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

**Abstract**


Intraportal transplantation of isolated islets of Langerhans is a procedure approaching clinical acceptance as a treatment for patients with type I diabetes mellitus. One major problem with this treatment is that large amounts of cells are lost at the time of infusion into the portal vein, resulting in a low level of engraftment of the islets. One likely explanation for this loss is the instant blood-mediated inflammatory reaction (IBMIR), a thrombotic/inflammatory reaction occurring when islets come in contact with blood. The IBMIR is characterized by coagulation and complement activation, leading to platelet consumption, leukocyte infiltration of the islets, and disruption of islet integrity.

In this thesis, the IBMIR is shown to be triggered by tissue factor (TF), the main initiator of blood coagulation in vivo. TF is expressed in two forms by the endocrine cells of the pancreas, a full-length membrane-bound and an alternatively spliced soluble form. Blocking TF in vitro efficiently reduces the macroscopic clotting, expression of coagulation activation markers, and leukocyte infiltration. This blockade can be achieved by adding either an active site-specific anti-TF antibody or site-inactivated FVIIa that competes with active FVIIa in the blood. TF may be secreted from the islets, since it is colocalized with insulin and glucagon in their granules. The IBMIR has also been demonstrated in vivo in patients transplanted with isolated islets.

There are two ways to block the IBMIR in transplantation: systemic treatment of the patients, or islet pretreatment before transplantation to reduce their thrombogenicity. In this thesis, low molecular weight dextran sulfate (LMW-DS) is shown to reduce activation of the complement and coagulation systems and decrease the cell infiltration into the islets in vitro and in vivo, in both a xenogenic and an allogenic setting. Based on these results, LMW-DS is now in clinical trials.

**Keywords:** Diabetes, Islet transplantation, Islets of Langerhans, Coagulation activation, Complement activation, IBMIR, Tissue factor, Low molecular weight dextran sulfate

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ISSN 1651-6206
ISBN 978-91-554-6851-4
urn:nbn:se:uu:diva-7786 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7786)
Till alla dem som trott på mig
& stöttat mig i vått och torrt
List of publications

This thesis is based on the following publications which will be referred to by their Roman numerals:


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Abbreviations

ADP  Adenosine diphosphate
APC  Active protein C
API  Adult porcine islet
APTT  Activated prothrombin time
asTF  Alternatively spliced TF
AT  Antithrombin III
ATP  Adenosine triphosphate
BMI  Body mass index
β-TG  β-thromboglobulin
C1-INH  C1 inhibitor
Da  Dalton
DAF  Decay acceleration factor
DM  Diabetes mellitus
DS  Dextran sulfate
EC  Endothelial cell
F 1+2  Fragment 1 and 2
FV etc.  Coagulation factor V etc.
FVa  Activated coagulation factor V etc.
FGF  Fibroblast growth factor
Gal  Galα1,3Gal epitope
HGF  Hepatocyte growth factor
HIT  Heparin-induced thrombocytopenia
HMW-DS  High molecular weight dextran sulfate
IBMIR  Instant blood-mediated inflammatory reaction
ICC  Islet-like cell cluster
IEQ  Islet equivalent
Ig  Immunoglobulin
IL  Interleukin
IL-2R  Interleukin-2 receptor
i.v.  Intravascular
LMW-DS  Low molecular weight dextran sulfate
mAb  Monoclonal antibody
MAC  Membrane attack complex
MCP  Membrane cofactor protein
MCP-1  Monocyte chemotactic protein 1
MSC  Mesenchymal stem cell
<table>
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<tr>
<td>PAP</td>
<td>Plasmin antiplasmin complex</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>rFVIIai</td>
<td>Recombinant site-inactivated coagulation factor VIIa</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>TAT</td>
<td>Thrombin-antithrombin complex</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>TM</td>
<td>Thrombomodulin</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
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Introduction

Intraportal transplantation of isolated islets of Langerhans is a procedure approaching clinical acceptance as a treatment for patients with type 1 diabetes. For many years the outcome of islet transplantation has been poor, with insulin independence being achieved in only 10-15% of islet recipients, as compared to up to 85% of those receiving whole pancreases. A recently introduced protocol that combines previously known concepts has improved these results, and today clinical islet transplantation regularly leads to insulin independence \(^1\). However, pancreatic islets from more than one donor are still needed to produce insulin independence, demonstrating that only a small fraction of the transplanted islets successfully engraft in the liver. One likely explanation for this tissue loss is the instant blood-mediated inflammatory reaction (IBMIR), which is characterized by coagulation and complement activation that leads to platelet consumption and leukocyte infiltration of the islets, resulting in disruption of islet integrity and islet loss. It is of great importance to characterize this reaction and find appropriate treatments to increase islet survival after intra-portal transplantation and maintain islet function.

Diabetes Mellitus

Diabetes mellitus (DM) is the general name for metabolic diseases in which the uptake of glucose is obstructed, leading to uncontrolled high glucose levels in the blood. In order for cells to use glucose as a source of energy, they require insulin to act as a key to open up the cells’ influx system. Insulin is a hormone produced and secreted by the endocrine cells (\(\beta\)-cells) of the pancreas as a response to glucose stimulation. If blood glucose levels rise, insulin is released from the \(\beta\)-cells into the blood.

The number of people suffering from DM is increasing at an alarming rate. It is estimated that 4.6%, or 171 million of the world’s population, had diabetes in the year 2000 and that by 2025 the incidence will have increased to 6.0%, or 334 million people\(^2\).\(^3\).

The two most common types of DM are type 1, in which the \(\beta\)-cells are lost as a result of autoimmune disease, and type 2, which involves insulin resistance. In type 1 DM, no insulin is produced; in type 2, the insulin pro-
duction may be normal or elevated but is still not adequate, because the peripheral cells cannot use glucose from the blood.

Type 1 DM is also called juvenile diabetes, since its onset often occurs in childhood or the early teens. Treatment with subcutaneous injections of recombinant, exogenous insulin is required to maintain normoglycemia, indicating that this is an insulin-dependent condition. Type 1 DM is an autoimmune disease in which autoreactive T-cells mediate destruction of the insulin-producing β-cells of the pancreas. Persistent or slow virus infections, such as congenital rubella and cytomegalovirus infections, may be important in the induction of the autoimmune response. Enteroviruses have been shown to be capable of inducing diabetes in experimental animals, and other studies have suggested a role for these viruses in human type 1 DM as well. Patients newly diagnosed with type 1 DM are more frequently positive for Coxsackie viruses (belonging to the enterovirus family), especially Coxsackievirus B4, than are healthy individuals. Viruses can act in two ways, either producing a direct cytolytic effect or triggering an autoimmune effect that gradually destroys the β-cells. The direct viral cytolytic effect can in this case be sufficient to induce type 1 DM, since the β-cells have a limited capacity to regenerate. The presentation of the appropriate self-antigens may result in activation of quiescent self-reactive T-cells that destroy the islets.

The risk of type 1 DM is unevenly spread throughout the world. Finland and Sardinia have a high incidence, while Venezuela and China have two of the lowest incidences in the world. The incidence varies from 0.1 per 100,000/year to 40.9 per 100,000/year.

The complications of type 1 DM are a result of the high blood glucose levels, and most are related to damage to both small and large blood vessels, leading to an increased risk of retinopathy, nephropathy, neuropathy, and microvascular and cardiovascular diseases.

In the case of patients with type 1 DM, it is very important to keep the blood glucose levels stable and within the normal range; otherwise hyperglycemia, resulting in ketoacidosis, or hypoglycemia (insulin coma) may occur, leading to life-threatening conditions.

Treatment

Type 1 DM is treated daily with multiple insulin injections of recombinant human insulin. The insulin given is a mixture of 1) rapid or short-acting insulin used before meals to lower the postprandial increase in blood glucose, and 2) long-acting insulin taken to minimize fluctuations in the basal glucose levels between meals and at night.
Transplantation as a cure for type 1 DM

Allotransplantation

The pancreas, located posterior and lateral to the stomach, has both an exocrine and an endocrine portion. The exocrine portion produces and secretes digestive enzymes into the duodenum, while the endocrine cells of the pancreas produce and secrete insulin into the blood in response to rising blood glucose levels. The endocrine portion constitutes only about 1% of the total organ mass.

The insulin is produced as proinsulin, which is cleaved to produce equal amounts of insulin and C-peptide. The C-peptide level in the blood is used as a marker of islet function (insulin production). Since the recombinant insulin used for treatment of the patients contains no C-peptide, all the C-peptide in the patient’s blood should be associated with islet graft function.

The first approach to replacing insulin-producing cells as a means of curing type 1 DM, introduced in 1966, was to transplant whole pancreas from a necro-organ donor. In this type of procedure the whole gland is transplanted, including the exocrine portion of the pancreas. The success rate for whole pancreas transplantation is approximately 85% if transplanted simultaneously with a kidney, but the transplantation is a major surgery and requires a stable patient, leaving the sickest patients untreated. The complications that occur in whole pancreas transplantation are usually severe, including surgical complications and leakage of enzymes from the gland that can cause invasive damage to organs and tissues. Also, the patients often require blood transfusions after their transplants.

The ideal solution for patients with type 1 DM would be to transplant only the endocrine cells. Human transplantation involving the physical replacement of the β-cell mass alone was pioneered by Paul Lacy in 1982. In this procedure, the islets of Langerhans are isolated from a human pancreas. The gland is enzymatically treated with collagenase to digest the connective tissue and then further disrupted by mechanical force applied to the organ in an oscillating metal chamber containing beads. The digest retrieved from this procedure contains a mixture of exocrine and endocrine cells that are separated by density gradient centrifugation. The isolated islets are transplanted transhepatically into an AB0-compatible recipient. This procedure is performed in the radiology department via a catheter placed in the portal vein of the liver. In all, this is a less invasive procedure than whole-pancreas transplantation, with fewer post-transplantation complications. If the number of islets from one preparation is not sufficient, islets from several preparations can be pooled. The duration of hospitalization after transplantation is short, and the procedure can be repeated up to four times in order to obtain insulin independence.
For a long time, the outcome of such transplantations was poor: After transplantation with islets from one donor, only 10-15% of the recipients became insulin-independent. In 2000, Shapiro and coworkers published a modified transplantation procedure (the Edmonton protocol) using a transplantation protocol similar to that of Scharp et al., but with multiple donors (up to 4); this procedure resulted in insulin independence in all 7 recipients. Shapiro and coworkers re-introduced a glucocorticoid-free immunosuppression regimen consisting of anti-rejection medication (sirolimus), an anti-proliferation drug (tacrolimus), and a monoclonal antibody (mAb) against IL-2R (daclizumab). In 2002, the same group published a follow-up study in which 17 patients who had been transplanted with islets were followed for up to 34 months. Their analysis showed that 80% of the patients were insulin-independent after one year, and 67% were still insulin-independent after more than 2 years. The 5-year follow-up from this center showed that a majority of the subjects had maintained C-peptide secretion, but few were off exogenous insulin treatment. The transplantations relieved the glucose instability and problems with hypoglycemia; however, making the islet transplantation more successful still requires major improvements in the islet engraftment, preservation of islet function, and reduction of the immunosuppressive treatment.

Clinical islet transplantation is restricted to patients with severe forms of glycemic lability or recurrent hypoglycemia. Within the Nordic Network for Islet Transplantation, this type of islet transplantation is offered only to patients who have previously received a kidney graft because of end-stage renal disease and are therefore already on immunosuppression.

The number and quality of the islets obtained from a human pancreas depend on many variables. In a retrospective study of 437 donated pancreases used for islet transplantation, Nano and coworkers focused on how donor characteristics, pancreas condition, organ procurement method, digestion characteristics, and purification procedure affected islet yield, purity, and function in vivo. Donor factors, including donor age and high body mass index (BMI), are positively correlated with islet yield; factors such as immunosuppressive therapy and the molecules released by the islets are also important. Refinements in the transportation of the pancreas and the processing, purification, and culturing of the islets have led to improved results. Revascularization is critical for the long-term survival of transplanted islets; endothelial cells (ECs) in the islets have been shown to contribute to this process. The process of islet cell isolation and culture destroys the islet microvasculature, forcing the islets to depend on passive diffusion immediately after intrahepatic islet transplantation in order to obtain the necessary nutrients and an adequate supply of oxygen. This rupture of the surrounding vessels results in ischemic damage to the islets. Islets have been shown to begin sprouting in fibrin gels in response to angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast
growth factor (FGF). Formation of these sprouts, in the form of cellular cords composed of ECs, indicates that revascularization has begun. Revascularization in the kidney subcapsular space has been reported to begin a few days after transplantation and to be complete within 7-14 days; however, this process probably takes longer in the case of intraportally transplanted islets.

Xenotransplantation

One of the obstacles to clinical islet transplantation becoming a general treatment for type 1 DM is a shortage of tissue. The ultimate goal for islet transplantation is to find an unlimited source of cells and to be able to carry out the transplantation without the need for chronic immunosuppressive drug therapy. The shortage of organs has raised questions about alternative sources of insulin-producing cells. One possible alternative is the use of porcine islets, since porcine insulin is identical to the human molecule in 51 of its 52 amino acids and was routinely used as a treatment for type 1 DM until the 1980s, when recombinant human insulin was introduced. In the 1990s, fetal porcine islets were successfully transplanted into 10 diabetic patients who had already received kidney transplants. The study showed no beneficial effect, but porcine C-peptide levels were detected for up to 400 days in the blood of four of the patients, and a kidney biopsy from one of the patients showed surviving porcine islets 3 weeks after transplantation.

Adult versus fetal porcine islets

Both adult porcine islets (API) and islet-like cell clusters (ICCs) from fetal pigs are able to function in animal models and can thus be considered as possible investigational tissues for xenotransplantation into humans. Adult cells are more difficult to isolate but may be expected to function immediately, whereas ICCs are easier to isolate because they require no purification step. The ICCs have a great proliferation potential but need time after transplantation to begin functioning; therefore, one possible approach is to culture the ICCs in vitro before transplantation.

Islets in contact with blood

The instant blood-mediated inflammatory reaction (IBMIR)

Allogeneic islet transplantation (human islets)

One of the major problems in the intraportal transplantation of isolated human islets into type 1 DM patients is the enormous tissue loss. Despite the fact that the tissue is transplanted in large excess, the functional capacity of the transplanted islets corresponds to only about 20% of that found in non-
diabetic persons, indicating that only a small fraction of the transplanted islets successfully engraft in the liver. As a consequence, there is generally a need for multiple donors in order to obtain insulin independence in a patient. During transplantation, the contact between the intraportal islets and the blood induces a thrombotic/inflammatory reaction called the instant blood-mediated inflammatory reaction (IBMIR). The IBMIR is characterized by a rapid activation of the coagulation and complement systems, recruitment and infiltration of the islets by leukocytes, and rapid binding and activation of platelets. The islet integrity and morphology are disrupted in the forming thrombus, entrapping the islet graft. This damaging effect of the IBMIR is a possible explanation for the magnitude of the tissue loss and the intraportal thrombosis that may occur in clinical islet transplantation. Even without clinical signs of intraportal thrombosis, clotting occurs in the large branches of the liver vessels, entrapping the islets and preventing them from reaching the smaller vessels of the portal tree where they can engraft. If the IBMIR could be efficiently abrogated, a higher proportion of the islets could successfully engraft, leading to a reduction in the number of donors needed for each patient. The optimal situation would be a 1:1 ratio, in which the islets from one necro-organ donor are sufficient to cure one patient.

Xenogeneic islet transplantation (porcine islets)

The IBMIR induced by porcine islets in human blood is most likely very similar to the allogeneic IBMIR. Natural antibodies recognizing modified epitopes on the islet structure are present, but that is true for both human and porcine islets\textsuperscript{[34,35]}. The additional risk in the case of porcine islets in human blood involves the presence of naturally occurring antibodies against a carbohydrate moiety, Gal\textsubscript{\alpha}1,3Gal (Gal), that is expressed on cells of most mammals but not on cells of humans, apes, or Old World monkeys\textsuperscript{[36]}. In humans, these antibodies constitute 85-95\% of all the natural xenoreactive antibodies\textsuperscript{[33]}. Formation of such antibody-antigen complexes will activate the complement system, resulting in a rapid destruction of the graft. The Gal epitope is expressed on porcine fetal islets but only at very low levels on APIs\textsuperscript{[37]}. The extent to which the Gal epitope is expressed by the duct cells, and ECs of the blood vessels in the adult porcine pancreas is a matter of debate, but the endocrine cells have been shown to lack expression of the Gal epitope\textsuperscript{[33,38]}. Since islet grafts are initially non-vascularized, and their vascularization involves cells of recipient origin, this activation of the complement system may possibly be avoided\textsuperscript{[39]}. Instead, a reaction very similar to the allogeneic IBMIR occurs.
The Blood Defense Systems

The immune systems of the body consist of two interactive systems, innate immunity and adaptive immunity.

Innate immunity involves recognition mechanisms that the host uses immediately or within hours of exposure to an antigen. This congenital defense system is the initial response that eliminates microbes and prevents infection in the body, but unlike adaptive immunity, it does not provide long-lasting or protective immunity to the host. The innate immune system involves phagocytic cells (neutrophils and monocytes/macrophages) that release inflammatory mediators, natural killer cells (NK cells), and molecules such as complement and coagulation proteins, acute phase proteins, and certain cytokines.

Adaptive, or acquired, immunity refers to antigen-specific defenses that take several days to become protective and are designed to react with and remove specific antigens that stimulate antibody production. This type of immunity involves both B-lymphocytes, which produce antibodies that promote phagocytosis, and T-lymphocytes, which enhance the phagocytic and microbicidal functions of antigen-presenting cells. This defense system is highly specialized and is responsible for immunological memory, by which each pathogen is remembered. The memory cells that are generated can then be recruited to quickly eliminate the pathogen if subsequent infection occurs.

Blood coagulation, with special reference to tissue factor (TF)

Coagulation of the blood is a critical process that protects the body against bleeding, preventing the potentially lethal consequences of excessive blood loss.

Blood coagulation involves plasma, platelets, TF-bearing cells, and vascular components. At the site of injury, a series of reactions take place: The primary reaction involves contraction of the vessel wall to minimize the blood loss, platelet adhesion, and formation of a platelet plug, exposing binding sites for coagulation factors. The primary processes are often insufficient to stop the bleeding, and a secondary reaction then takes place, which involves the activation of the coagulation factors (serine proteases) and leads to the generation of thrombin and fibrin formation.

Blood coagulation may be considered as a cell-based model that involves three steps: an initiation stage on TF-bearing cells, amplification on platelet surfaces, and a propagation step on platelets that leads to the generation of thrombin and formation of a fibrin clot. The initiation step is localized to cells that express TF; normally, these cells are outside the vessels. TF is the main physiological initiator of blood coagulation, since it is a high-affinity receptor for FVII/FVIIa. It consists of three domains: a large extracellular domain, a membrane-spanning domain,
and a short cytoplasmic domain. In the vessels, TF is expressed only in the adventitial layer of the vessel wall and is thus exposed to the blood only if the vessel is ruptured. The extracellular domain of the exposed TF rapidly binds circulating FVII, facilitating its cleavage to generate the proteolytically active TF/FVIIa complex. This complex is an efficient enzyme that activates small amounts of FIX and FX. FXa associates with FVa to form prothrombinase complexes on the cell surface. The source of FV can vary; for example, it can be released from the α-granules of platelets as a response to partial activation after the platelets adhere to collagen or other extracellular matrix proteins at the site at which coagulation is initiated. The FVa is released in its partly activated form and is fully activated by thrombin. The FXa/FVa complex generates trace amounts of thrombin from prothrombin.

In the amplification step, the reaction moves from the TF-bearing cells to platelets, and the small amounts of thrombin formed on the TF-bearing cells increase the platelet adhesion. The platelets are activated and begin to accumulate activated FV, FVIII, and FIX on their surfaces, leading to the formation of more thrombin. FVIII is normally found in complexes with von Willebrand factor (vWF) and is localized to the vascular space. When platelets are activated, the vWF/FVIII complexes bind to the platelets and are cleaved by thrombin to form active FVIIIa, setting the stage for the large-scale production of thrombin.

During the propagation phase, the platelet surface coordinates the assembly of FIXa/FVIIIa and FXa/FVa complexes. The FIXa/FVIIIa complexes are formed when the FIX that has been activated on TF-bearing cells diffuses to the platelets where FVIIIa is being released from vWF. These new FIXa/FVIIIa complexes activate FX to FXa; the activated FXa forms a complex with FVa, thereby cleaving prothrombin to generate thrombin on a large scale. Thrombin is produced in large enough quantities to cause the conversion of fibrinogen to fibrin, and a hemostatic plug is formed (Figure 1).
Blood-borne TF

TF is constitutively expressed in the advential layer of the vessel wall, but its expression may also be induced in cells of the blood (e.g., monocytes) and on ECs. In addition, α-granules of resting and activated platelets contain TF, and functionally active TF has been shown to be present in plasma in circulating microvesicles originating from activated platelets. Panes and colleagues have shown that human platelets not only assemble the clotting reactions on their surface but also supply their own TF for thrombin generation. Blood-borne TF may be essential for thrombus formation, since it has been shown that within minutes TF antigen can be found in the growing thrombus.

Full-length TF is very hydrophobic in aqueous solutions because of its transmembrane domain; it needs to be inserted into a membrane or phospholipid vesicle to be active in suspension. This requirement has led to the assumption that TF is only found bound to cells. However, in 2001 a shorter alternatively spliced TF (asTF) lacking the membrane-spanning exon 5 was isolated from human promyelocytic leukemia cells and sequenced, and in 2003 the presence of this soluble form was reported in various human tissues. High levels were found in the lungs, and moderate levels in the pancreas and placenta. It was also detectable in kidney, brain, and heart. This form of TF is present in the blood, and it may be recruited to growing
thrombi\textsuperscript{50}. Up to 30% of the TF found in circulating blood has been proposed to be derived from this alternative splicing \textsuperscript{47,50}.

Mediators of inflammation are known to induce activation of coagulation. A connection between inflammation and TF was made when interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-\(\alpha\)) were shown to induce rapid expression and release of asTF from EC\textsuperscript{51}. Treatment of ECs with these cytokines also led to decreased levels of tissue factor pathway inhibitor (TFPI). This induction of asTF in ECs is therefore considered a marker for and contributor to imbalanced hemostasis. It is not clear whether asTF has procoagulant activities under physiological conditions\textsuperscript{50}; some studies have suggested that asTF acts as a competitive inhibitor of unaltered TF, forming an inactive complex with FVIIa\textsuperscript{52}. A third form of TF has recently been reported in tumor cells; this form has a 495-bp insert, but no mature TF translated from this transcript has been detected\textsuperscript{53}.

**Regulation of blood coagulation in vivo**

The activation of blood coagulation is tightly regulated. Antithrombin (AT) inactivates many of the serine proteases involved coagulation, including FIXa, FXa, FXIIa, and thrombin. The AT-protease complexes that are formed are then rapidly cleared from the plasma by the liver. The complex formed with thrombin, the thrombin-antithrombin complex (TAT), is often used as marker of coagulation activation, since it reflects thrombin generation.

FXa localized to the cell surface is effectively protected from inactivation by plasma protease inhibitors, but if it disassociates from TF-bearing cells, it is quickly inactivated by TFPI or AT\textsuperscript{54}. Thus, FXa activity is generally localized to the surface of the cell on which it was formed. Low levels of the activated coagulation factors are probably present in the blood of healthy individuals, but they do not lead to clot formation under normal circumstances, since TF-bearing cells and activated platelets are usually not found in close proximity, and the diffusion rate of the factors is slow. TFPI inhibits TF-FVIIa-FXa and FIXa and is produced by microvascular ECs, among others\textsuperscript{55}. It can either directly inhibit FXa in TF-FVIIa-FXa complexes or form a complex with free FXa, which then forms a complex with TF-FVIIa, competing with unactivated FX\textsuperscript{56}. Thrombin binds to thrombomodulin (TM) on the EC surface, and the thrombin/TM complex converts protein C to its active form, active protein C (APC). APC then leaves the complex and inactivates FVa and FVIIIa in the presence of protein S.

**Anti-coagulant therapy**

Heparin is composed of polysaccharide chains that typically range in molecular weight from 3000 to 30,000 Daltons (Da). Heparin acts directly on the proteases generated by the intrinsic and extrinsic pathways of the coagulation cascade, inhibiting the thrombin-mediated conversion of fibrinogen to
fibrin and thereby potentiating the effect of AT. One major risk of heparin treatment is the immune-mediated, heparin-induced thrombocytopenia (HIT) that can occur after only days of treatment. HIT is more often caused by unfractionated heparin than by low molecular weight heparin. HIT mediated by heparin-platelet/factor 4 antibodies leads to the formation of immune complexes that activate the classical pathway of the complement system, bind to platelets, and stimulate platelet aggregation and thrombin generation. These reactions produce a rapid decrease in the platelet count and are often associated with thromboembolic complications. Heparin also affects the complement system by binding its regulators, such as factor H and C1 inhibitor (C1-INH).

Low molecular weight dextran sulfate (LMW-DS, MW 5000) is a polyanionic derivative of dextran that is a polymer of anhydroglucose (Figure 2). LMW-DS is prepared by sulfation of dextran (MW 1000), followed by careful purification. The result is a LMW-DS with an average molecular weight of 5000 Da and a range of 3000-8000 Da. LMW-DS contains approximately 17% sulfur. Each glucose unit in the dextran chain has approximately two sulfate groups, generally located at the second and fourth carbons (C2 and C4) of the glucose units. DS (≥10,000 Da) is optimally stable between pH 6 and 7. Because it inhibits both the complement and the coagulation systems, LMW-DS has been used clinically in antiviral therapy against HIV and for the treatment of acute cerebral infarction, and immobilized DS has been used to treat patients with hyperlipidemia by plasmapheresis.

Figure 2. The chemical structure of low molecular weight dextran sulfate (LMW-DS).

Melagatran is a small molecule inhibitor that directly blocks the active site of thrombin. It binds irreversibly to thrombin, but its half-life in plasma is short (3h). In clinical trials, Melagatran has been used for subcutaneous treatments and has been administered orally in the formulation known as Ximelagatran. Melagatran and Ximelagatran have been used as anticoagu-
lants in studies of total hip or knee replacement, with good results. Unfortunately, its manufacturer, AstraZeneca, announced in 2006 that Ximelagatran would not be marketed because of hepatotoxicity that occurred during clinical trials.

Recombinant site-inactivated coagulation factor VIIa (rFVIIa) is obtained by inactivating FVIIa (NovoSeven, Novo Nordic, Denmark). The inactivated rFVIIa can be obtained by treating FVIIa with 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone. Inactivation of the catalytic site of FVIIa creates a molecule that competes with FVIIa for binding to TF, preventing coagulation initiation from proceeding.

The Roche inhibitor, Ro69, is a 391-Da low molecular weight FVIIa inhibitor that inactivates the active site of FVIIa in the blood in a manner similar to that of chloromethyl ketone.

Monitoring anticoagulant therapy
Activated prothrombin time (APTT) is a performance indicator that assesses the contact activation and common coagulation pathways by measuring the activity of FV, FVIII, FIX, FX, FXI, FXII, prothrombin, and fibrinogen. In addition to its use in detecting abnormalities in blood clotting, it is also used to monitor the treatment effects of anticoagulants, such as heparin. For example, ellagic acid is used to activate FXII of the intrinsic pathway in citrated plasma, and Ca$^{2+}$ is added to start the clotting reaction. The time elapsed until a clot is formed is measured in seconds.

Prothrombin time (PT) is used to evaluate the TF pathway and the common pathway by measuring thrombin, FV, FVII, FX, and fibrinogen. PT is used to help detect bleeding disorders and liver damage, to assess vitamin K status, and to monitor the treatment effects of anticoagulants such as warfarin. Citrated plasma is mixed with thromboplastin (containing TF) and Ca$^{2+}$ in order to trigger clotting in the plasma. The clotting time is measured in seconds.

The complement system
The complement system is one of the most ancient components of the immune system, involving a series of proteins circulating in the blood and the fluids surrounding tissues. It consists of approximately 30 different cell-bound and circulating proteins. The soluble constituents circulate in their inactive forms and become sequentially activated in response to the recognition of molecular components of microorganisms, starting a cascade in which the binding of one protein promotes the binding of the next protein in the cascade. There are three activation pathways: the classical pathway, the alternative pathway, and the lectin pathway. The classical pathway is activated by antibody-antigen complexes, the alternative pathway by C3b binding to microbial surfaces, and the lectin pathway by the interaction of micro-
bial carbohydrates with mannose-binding proteins in the plasma or tissue fluids. Each of the three pathways thus differs in the way it is activated, but all three lead to the production of the key enzyme, C3 convertase (Figure 3).

**Figure 3.** The complement cascade. (DC: Dendritic cell; MBL: Mannan Binding Lectin; Mo: Monocyte; Mø: Macrophage)

Complement has many roles: It triggers inflammation, attracts phagocytes to sites of infection, promotes attachment of antigens to phagocytes (opsonization), causes the lysis of gram-negative bacteria and human cells displaying foreign epitopes, plays a role in the activation of naïve B-cells, and removes harmful immune complexes from the body.\(^{67,68}\)

Activation of the complement cascade results in the formation of a terminal complement complex called the membrane attack complex (MAC, sC5b-9), which penetrates the cell membrane and causes cell lysis. All normal human cells are protected from the complement system by the exposure of membrane regulatory proteins, such as membrane cofactor protein (MCP), decay acceleration factor (DAF) and CD59, on their surfaces.\(^{68}\)

**Complement activation triggers an upregulation of TF**

Cross-talk occurs between the complement and coagulation systems in many ways; for example, upon complement activation, C5a induces TF expression by leukocytes\(^ {69}\) and human ECs.\(^ {70}\) sC5b-9 has procoagulant activity, inducing the exposure of negatively charged phospholipids on the platelet surface\(^ {71}\), which is important for TF-induced coagulation and the activation of platelets. sC5b-9 also induces TF activity in human vein endothelial cells.\(^ {72}\) TF expression can be induced in intravascular cells, such as monocytes, in a variety of ways: by endotoxin, complement activation, C-reactive protein, or inflammatory cytokines such as TNF-\(\alpha\).\(^ {73,74}\)
Aims of this study

General aims
The occurrence of the IBMIR in clinical islet transplantation is detrimental to the survival and function of the pancreatic islets. The general aims of this thesis were to investigate the mechanisms that trigger the IBMIR and to find pharmaceutical targets to prevent this detrimental reaction and improve the survival of islet grafts in clinical islet transplantation.

Specific aims
Paper I
The aim of the work described in paper I was to investigate the mechanisms of the IBMIR, to find ways to inhibit the detrimental reactions to the islets, and to determine whether the IBMIR occurs in vivo in transplanted patients.

Paper II
In paper II the aim was to characterize the TF activity associated with pancreatic islets, to further investigate the effect TF inhibition on the IBMIR in vitro, and to identify ways of predicting the outcome of islet transplantation.

Paper III
The aim of paper III was to investigate the ability of LMW-DS (MW 5000) to prevent the IBMIR in vitro and in vivo in a pig-to-mouse model.

Paper IV
In paper IV the goal was to further characterize the effect of LMW-DS on the allogeneic IBMIR in vitro and to develop a tentative administration protocol for LMW-DS in clinical islet transplantation.
Study design and methods

These experiments were designed to analyze and inhibit the strong reactions elicited by isolated human islets in contact with AB0-identical blood or adult porcine islets (API) in contact with A- or non-A-matched human blood. More detailed information can be found in Papers I-IV.

Ethics

All animal experiments were approved by the Research Ethics Committee of Uppsala University, Karolinska University Hospital or Université Catholique de Louvain (Brussels, Belgium). Human islets were isolated after informed consent was given for multiorgan donation and research.

Islet isolation

Human islets (Papers I, II and IV) were isolated with Liberase by a modified, previously described semiautomated digestion-filtration method 75-77. The isolated islets were maintained in suspension culture, and the medium was changed every other day.

APIs (Paper III) were isolated according to the Ricordi method 78 79. from pancreata of 2- to 3-year-old Swedish Landrace sows weighting 200-300 kg. The APIs were cultured for 1 to 4 days, and the medium was changed every other day.

Experimental blood models in vitro

Tubing loops as a model (Papers I-IV)

The tubing loop model has been developed to mimic the transplantation situation in which islets come in contact with human blood in the portal vein and for use in investigating the effects of potential inhibitors of the IBMIR. The model consists of loops of polyvinyl chloride with an inner surface furnished with immobilized heparin, in which islets are incubated with fresh AB0-identical (papers I, II, and IV) or A- or non-A-matched (paper III) hu-
man blood for up to 60 min. Samples are withdrawn during incubation and transferred to EDTA for blood cell counts and subsequent quantification of coagulation and complement activation markers. After the incubation, macroscopic clots or islets are recovered and snap-frozen pending immunohistochemical analysis.

The effect of TF blockade on isolated islets was investigated using anti-TF monoclonal antibodies (mAbs), recombinant active site-inactivated FVIIa (Paper I), or the FVIIa inhibitor Ro69® (Paper II). In addition, the ability of LMW-DS to inhibit allogeneic IBMIR (Paper IV) and xenogeneic IBMIR, in which APIs were incubated with human blood (Paper III), was tested in the loop model.

Clotting time determination by ReoRox (Papers I and III)

The pro-coagulant activity in culture medium from human islets of Langerhans (Paper I) and the effect of various kinds of dextrans on API-induced clotting (Paper III) were investigated using a free oscillating rheometer, the ReoRox 4. The ReoRox can be used to measure the viscosity, viscoelasticity, and clotting time of plasma or whole blood in a sample cup that is set to nearly free oscillation (about 10Hz). When clotting occurs, the sample changes properties, and software is used to determine the clotting time, measured to the point at which the frequency is decreased and the damping is increased 80.

Blood and plasma functional analyses (Paper I-IV)

APTT, which measures the effect of the contact activation and common coagulation pathways, was used as a tool to monitor the effects of LMW-DS on the bleeding times of healthy volunteers. The values were compared to those obtained in the presence of heparin, which is the anticoagulant of choice in clinical islet transplantation today.

The function of platelets in the presence of LMW-DS or heparin in whole blood was analyzed by PFA-100®. In this screening test, which is sensitive to abnormalities in platelet function, the blood passes through a capillary tube under high shear stress onto a coated membrane, and the time for total closure of the membrane as a result of platelet aggregation is determined 81.

Clinical islet transplantation (Papers I and II)

Major inclusion criteria for the study were: long-standing type 1 DM, frequent uncontrollable hypoglycemic attacks and unawareness of these problems, and a previous transplantation with a kidney graft because of end-stage renal disease. A modified Edmonton steroid-free protocol including da-
cluzimab, sirolimus, and tacrolimus was used. The islets were transplanted intraportally through a catheter placed in the portal vein, and blood samples were collected before, during, and after transplantation to monitor the IBMIR. In paper I, four consecutive patients were transplanted with islets from a total of nine donors, and in paper II the study was extended to nine patients transplanted 20 times with islets from a total of 26 donors.

Animals and transplantation procedure
(Papers III and IV)

Transplantation of APIs to diabetic, athymic mice (Paper III)

Intraportal transplantation of APIs into diabetic, athymic mice induces the IBMIR and is therefore a useful small animal model for studying xenogeneic IBMIR, although the anti-Gal antibody is not present. This model was used as a tool to study the effect of LMW-DS on the IBMIR elicited by APIs in the livers of athymic, diabetic mice in which diabetes had been induced by streptozotocin treatment.

Infusion of LMW-DS in cynomolgus monkeys (Paper IV)

The pharmacokinetics of LMW-DS were studied in six anesthetized cynomolgus monkeys. LMW-DS was administered i.v. into the jugular vein via an indwelling catheter or i.v. into the portal vein, and the animals were continuously monitored by electrocardiogram and blood pressure and pulse measurements. In addition, blood samples were drawn from a femoral vein catheter for APTT analysis as described above.

Immunochemical techniques

Immunohistochemical staining (Papers I-III)

Pieces of whole pancreas and isolated islets, collected in embedding medium and snap-frozen, were cryo-sectioned and stained for full-length TF (Paper I) using an mAb or for asTF (Paper II) using a polyclonal antibody raised against a peptide that is specific for asTF and includes the last 11 amino acids at the C-terminus of the molecule. In paper III, the sections from the loop experiments were stained for platelets (CD41) and for infiltrating granulocytes and monocytes (CD11b). In addition, the API transplants in the athymic mouse livers from the in vivo study were stained for insulin.
Electron microscopy (Papers I and II)
The islets and precipitated culture medium were examined by electron microscopy. In order to preserve antigenicity, the specimens were processed by the low-temperature method\textsuperscript{83}. Ultrathin sections placed on nickel grids were immunolabeled using the immunogold technique\textsuperscript{84}. Colloidal gold particles were used as electron-dense markers on the antibodies tested. Normal pancreatic tissue specimens and isolated islets were sampled and stained for TF in paper I. In paper II, the pellets of precipitated culture medium were stained for TF, insulin, and glucagon.

Immunoprecipitation (Paper I)
Isolated islets of Langerhans were lysed, the cell debris was removed, and TF was precipitated with polyclonal antibodies. The samples were subjected to SDS-PAGE and Western blot analysis, and TF was visualized by incubation with an anti-TF mAb.

Detection of complement and coagulation activation markers
Activation markers of complement (C3a and sC5b-9), coagulation (e.g., TAT, prothrombin fragment 1+2, FVIIa-AT, FXIa-AT, FXIa-C1INH, FXIIa-AT, FXIIa-C1INH), and fibrinolysis (D-dimer, plasmin-antiplasmin complex [PAP]), platelets (β-thromboglobulin), and a marker of islet function (C-peptide) were measured by the appropriate enzyme immunoassays in samples collected from patients who had undergone clinical islet transplantation and from tubing loop experiments.

Statistics
All results were expressed as means ± standard error of the mean (SEM). Detailed information regarding statistical calculations is given in the papers.
Results and Discussion

The role of TF as a trigger of IBMIR (Papers I and II)

Intraportal transplantation is approaching clinical acceptance as a treatment for type 1 DM patients. However, although many attempts have been made to improve the outcome of these procedures, islets from more than one donor are still needed to produce insulin independence. Thus, only a small fraction of the transplanted islets successfully engraft in the liver. When the islets come into contact with the blood, they elicit the IBMIR, characterized by coagulation and complement activation that lead to platelet consumption and leukocyte infiltration of the islets and ultimately result in disruption of islet integrity and islet loss.

In study I our investigation of the mechanism(s) by which the IBMIR is triggered revealed that an inhibitory anti-TF antibody could efficiently block the IBMIR in vitro in both the ReoRox and tubing loop systems. This finding was confirmed by using site-inactivated FVIIa (ASIS) and Ro69®, a low molecular weight FVIIa inhibitor, both of which showed a dose-dependent inhibition of the IBMIR in the tubing loop model, thereby minimizing platelet and leukocyte consumption and the levels of coagulation activation markers in the blood. Thus, the effect of these TF inhibitors strongly indicated that TF is the trigger of the IBMIR.

Immunohistochemical staining with anti-TF antibody revealed the presence of TF in the endocrine cells of the islets of Langerhans and the ECs of the pancreatic vessel walls, but not in the exocrine, acinar cells of the human pancreas. This staining pattern suggested that TF was located in the cytoplasm of the cells of the pancreatic islets. A similar distribution of TF was observed in isolated islets, demonstrating that TF expression was not induced by the isolation procedure. Immunoprecipitation followed by SDS-PAGE and Western blotting identified a polypeptide of 47 kDa, identical to the size of TF, in the pancreatic islets. RT-PCR confirmed the expression of TF at the mRNA level. The TF production in isolated islets, as measured by TF content, increased temporarily after 2 days of culture but was considerably lower after 7 days. Ultrastructural examination of the islets in situ revealed the presence of TF in both α- and β-cells. TF was detected in the β-cell granules, particularly in the core; however, it was present in higher concentrations, in a random distribution, throughout the matrix of the α-cell granules. TF was also localized in the smooth endoplasmic reticulum, in the
Golgi stacks, and in transitory vesicles budding from the trans-Golgi stacks. Thus, it appeared that TF was synthesized in the \(\alpha\)- and the \(\beta\)-cells. There was no TF immunoreactivity in either the \(\delta\)- or PP cells.

The procoagulant activity found in ultracentrifuged culture medium from isolated islets could be abrogated by anti-TF monoclonal antibody directed against the active site, indicating that TF released from the islets is bound to microparticles and cell debris. Immunohistochemical investigation of islets by electron microscopy showed that TF was localized to the \(\alpha\)- and \(\beta\)-cell granules. TF in pellets from ultracentrifuged culture medium was also associated with electron-dense material that included unidentified microparticles, granules, and granule debris of both \(\alpha\)- and \(\beta\)-cell origin. Consistent with the finding that TF is present in the endoplasmic reticulum, Golgi complex, and cytoplasmatic granules of the \(\alpha\)- and \(\beta\)-cells, we found that TF could be secreted with glucagon and insulin into the supernatant.

Although TF was first described as a transmembrane molecule, the alternative asTF form was shown to contain a unique 40-amino acid residue C-terminal sequence from which exon 5 is deleted, resulting in the loss of the transmembrane portion of the protein. Therefore, the resulting protein is water-soluble. AsTF is expressed by various tissues, including the pancreas. It is not clear, however, whether asTF acts as a procoagulant in the presence of phospholipids or as a competitive inhibitor of TF by binding FVIIa. The results of RT-PCR and immunohistochemical analyses revealed that islets express asTF. Since we have seen TF expression only in the endocrine cells, and to some extent in the ECs of the vessels in the human pancreas, it is likely that most of this TF variant is expressed by the endocrine cells of the islets of Langerhans.

Unlike TF, which activates the coagulation system, monocyte chemoattractant protein (MCP-1) has been shown to produce negative clinical outcomes in islet after kidney transplantation by promoting inflammation. MCP-1 is secreted by isolated islets in response to stress, causing the islets to become chemoattractive to the macrophages that infiltrate the islets. Melzi and coworkers have shown a significant correlation between MCP-1 release and the identity of the surgical team in charge of organ harvest as well as the enzyme used for isolation.

Even in the absence of clinical signs of the IBMIR, we have been able to demonstrate that this deleterious reaction occurs in vivo in the portal vein at the time of islet transplantation. This conclusion was clearly demonstrated by the rise in coagulation activation markers, such as TAT and FVIIa-AT complexes, that occur in patients’ plasma at the time of transplantation. The maximum concentrations of TAT demonstrated during the first 15 to 60 min after transplantation were seen to be comparable to those during sepsis. Generation of D-dimer that peaked on day 1 after the islet infusion, reflecting fibrinolysis, further strengthened the conclusion that the IBMIR occurs after islet infusion.
Even if the IBMIR is likely to occur, a crucial question is whether this reaction has any impact on the outcome of clinical islet transplantation. The transplanted patients are treated with insulin during the initial weeks after transplantation in order to let the transplanted islets rest. The fasting C-peptide levels at 7 days after the transplantation therefore reflect the basal production of endogenous insulin and can be used to follow the function of the transplanted islets in the liver. The negative correlation that we observed, in which the high TAT levels and FVIIa-AT levels at the time of transplantation never coincided with the large increases in C-peptide levels on day 7 after transplantation (and vice versa) indicates that if an immediate, strong IBMIR takes place, the islets are efficiently destroyed by the immune system of the recipient and the production of insulin a week later is reduced. In addition, clot formation in a large liver vessel prevents the islets from reaching a small branch of the portal tree, where they can engraft. In the few cases in which the islets induced a low level of TAT generation in combination with low C-peptide levels, the islets may have been non-functional (e.g., because of apoptosis).

In conclusion, both the isolated islets and cell debris from the islet cultures contain TF. This material is subsequently infused into the portal vein of the patients and is assumed to trigger a detrimental IBMIR during clinical islet transplantation, since only approximately 15-20% of the patients become insulin-independent when tissue from only one organ donor is used. Blocking the IBMIR may improve clinical islet transplantation and reduce the number of donors needed for each patient. In order to protect the islets from the IBMIR, they can be pretreated with agents that block TF expression or synthesis. The use of a new culturing medium, final wash culture medium, has yielded improved results in terms of reducing the proinflammatory activity observed, by reducing the release of MCP-1 and TF production. The anti-inflammatory effects of this medium seems to be due in part to the presence of pyruvic acid, which has been shown to participate in the process of islet engraftment, function, and survival. The anti-inflammatory antioxidant N-acetylcysteine is a promising drug that has previously been shown to inhibit the activation of coagulation by downregulating the translation of TF mRNA and interfering directly with coagulation factors. Beuneu and coworkers have demonstrated that N-acetylcysteine at clinically relevant concentrations can inhibit the procoagulatory activity of recombinant TF, primary duct cells, and human islet preparations without causing cellular toxicity.

Our laboratory has shown that TF expression is significantly lowered when the islets are co-cultured with nicotinamide, an antioxidant. In a clinical study, oral administration of high-dose nicotinamide to transplanted patients favorably affected the outcome of clinical islet transplantation. Use of nicotinamide in the processing medium during islet isolation has resulted in an improvement in the isolation outcome, allowing a higher percentage of...
the preparations to qualify for clinical islet transplantation\textsuperscript{95}. These findings suggest that TF expression should be quantified before transplantation: If the TF levels are high, the islets can be maintained in culture under optimal conditions in the appropriate concentration of nicotinamide until the TF content has dropped to acceptable levels. Then, to further reduce the TF expression \textit{in vivo}, the patients can be given oral nicotinamide treatment.

Low molecular weight dextran sulfate (LMW-DS), a candidate drug for inhibition of the IBMIR in clinical islet transplantation (Papers III and IV)

In a recent paper from one of the leading centers of clinical islet transplantation, Inverardi and coworkers stated that it is of critical importance to reduce the IBMIR in order to succeed in clinical islet transplantation\textsuperscript{96}. Although immunosuppression is used at the time of transplantation, this treatment modality is not effective in protecting the islets from the immediate destructive effect of the innate immune system. Development of other means of protection is crucial if better success rates are to be achieved. LMW-DS (MW 5000) is a good candidate drug to for improving the outcomes in clinical situations, and unlike other specific inhibitors of the IBMIR, LMW-DS is available for clinical use. Furthermore, it has been established that LMW-DS has no toxic effects on the islet function or viability of either human or porcine islets, and insulin release in response to a glucose challenge is unaltered. The morphology of the cells is also unaffected by LMW-DS. In order to evaluate the effects of LMW-DS on the IBMIR, we used a xenogeneic system both \textit{in vitro} and \textit{in vivo}. The rationale for using a xenogeneic system is that the IBMIR induced by porcine islets in contact with human blood is very similar to that produced by human islets\textsuperscript{97}. For our \textit{in vivo} studies, we used athymic mice transplanted with porcine islets, since mouse islets have in our hands been poor triggers of the IBMIR. Using the ReoRox to assess API-induced clotting of human plasma in the tubing loop model with APIs and in human islets in contact with human blood, we have shown that LMW-DS protects the islets from the IBMIR. In the tubing loop model, LMW-DS prevented macroscopic clotting, inhibited blood cell consumption, and reduced both coagulation (TAT) and complement activation (C3a, sC5b-9). LMW-DS had a suppressive effect on the contact system (FXIIa-AT) and fibrinolysis (PAP), but FXIIa-C1-INH was unchanged. A beneficial effect on most parameters was seen with concentrations of LMW-DS as low as 0.01 g/L. When the APIs retrieved from the loops were sectioned and stained for platelets (CD41) and granulocyte/macrophage infiltration (CD11b), the control islets from untreated blood showed a capsule of fibrin and platelets, surrounding the islets with infiltra-
tion of CD11b positive cells into the islets; in contrast, the highest dose of LMW-DS produced a complete inhibition of clot formation, and the number of infiltrating CD11b positive cells was considerably decreased. A similar effect was observed with the second-highest dose of LMW-DS, but here a thin layer of platelets adhering to the islets was observed. LMW-DS has been shown to have a direct inhibitory effect on the complement activation in human plasma.

LMW-DS treatment of diabetic athymic mice intraportally transplanted with APIs allowed these mice to maintain normoglycemia for a significantly longer period than did untreated animals transplanted with APIs. LMW-DS also protected the islets from the IBMIR in vivo; the liver grafts retrieved the islets from untreated animals were entrapped in clots, whereas few islets from LMW-DS-treated mice were entrapped. Immunohistochemical staining of liver sections showed an infiltration of CD11b-positive leucocytes into islets from untreated animals, while the islets retrieved from the LMW-DS-treated animals showed markedly fewer infiltrating leucocytes.

In addition to abrogating the IBMIR, LMW-DS potentiates the mitogenic effect of hepatocyte growth factor (HGF) on hepatocytes. This activity favors the engraftment of the islets and has a protective effect on the intrahepatic islets in vivo. HGF is important for liver growth and regeneration and is a crucial factor in angiogenesis. Liver injury at the time of intraportal transplantation triggers the production of HGF, which in combination with DS can regenerate the liver.

The effect by which LMW-DS inhibits the coagulation and complement system is not fully understood, but it likely involves a potentiating effect on C1-INH, which may affect the activation of both systems. As shown earlier by Ozmen et al., complement activation, at least in the allogeneic setting, is secondary to coagulation activation. In their study, Melagatran, a direct thrombin inhibitor, was used to inhibit the coagulation activation in vitro in the loop model and was also seen to abrogate complement activation completely. In earlier studies, complement activation during the IBMIR was shown to play a central role in the infiltration of CD11b-positive leucocytes, since the inhibition of complement by sCR1 in combination with heparin also inhibited cell infiltration.

As early as 1954, Walton showed that the acute toxicity of dextran sulfate is closely associated with its molecular weight. LMW-DS causes a low level of acute toxicity, whereas high molecular weight dextran sulfate (HMW-DS; MW 47,000 to 460,000 Da) administered i.v. causes hemorrhage, emboli, and thrombi as a result of cell and plasma protein agglutination. These effects clearly make HMW-DS less suitable for use in clinical trials. HMW-DS has also been shown to activate the fibrinolytic system and to have direct effects on cell interactions by inhibiting E-selectin-mediated adhesion of neutrophils to ECs. In both the ReoRox and tubing loop models, LMW-DS was far more effective than HMW-DS (MW
500,000) in preventing the clotting reaction. Two non-sulfated forms of dextran (LMW-dextran and HMW-dextran) were also tested. The non-sulfated dextrans gave a very slightly prolonged clotting time in the ReoRox when compared to the APIs alone, and when tested in the tubing loop with APIs, they had only a marginal effect on the IBMIR. Thus, sulfation was crucial for the inhibitory effect of dextran on the IBMIR triggered by islets.

At present, immobilized DS is used in treating hyperlipidemia by plasmapheresis. Immobilized DS has been shown to have effects on the contact system, but our investigations in paper III indicated that no such problem occurs when soluble LMW-DS is used at the doses we tested. LMW-DS (MW 8000) has been used in clinical trials as an anticoagulant in combination with urokinase in stroke patients and as an antiviral agent for the treatment of HIV. Severe thrombocytopenia with bleeding (e.g., epistaxis) has been reported as a side effect after 7 days of treatment by infusion or after weeks of peroral treatment with LMW-DS (MW 8000). Perorally administered LMW-DS (MW 8000) has low bioavailability, so high doses are needed in order to achieve systemic effects. Growing rats, rabbits, and guinea pigs treated i.v. with 15 to 30 mg LMW-DS (MW 7000)/kg for up to 8 weeks developed osteoporosis and brittle bones. Similar adverse effects (fatigue, osteoporosis, and epiphyseolysis) were seen when growing rats were treated i.v. with 20 mg LMW-DS (MW 3000)/kg five times a week for 4 to 8 weeks. These side effects are not expected in our proposed protocol, since DS will only be used for the first 6 h post-transplantation. This assumption that thrombocytopenia will not occur after such a short treatment is supported by the fact that our experimental cynomolgus monkeys maintained adequate platelet counts after receiving LMW-DS intraportally. The in vitro bleeding times measured in the PFA-100 did not show any influence of either the LMW-DS or heparin, indicating no negative effects on platelet function.

There is always a risk of bleeding when blood coagulation is inhibited, and therefore we compared LMW-DS to the clinically used dose of heparin. Both LMW-DS and heparin concentrations in blood had previously been shown to have a direct relationship with APTT in normal individuals. This finding was confirmed in paper IV, in which citrated blood was also shown to be applicable, facilitating bedside monitoring. LMW-DS produced APTT values in the same range as those obtained with the currently used clinical dose of heparin.

If LMW-DS is to be used clinically as an IBMIR antagonist in islet transplantation, it is of great importance to establish the pharmacokinetics of the compound. We therefore performed pharmacokinetic studies in cynomolgus monkeys. In the initial experiment, the APTT reached >900 s but rapidly dropped back to normal levels in the first animal, which had received a bolus dose of 3 mg LMW-DS/kg, according to the protocol of Lorentsen and co-workers. From this experiment, the half-life of LMW-DS was estimated to
be 5 to 10 min. In the next two experiments, we tested a higher bolus dose of 4.5 mg/kg combined with a continuous infusion of 0.3 to 0.6 mg/kg/h. The APTT remained constant at 50 to 60 s in the first animal and 70 to 80 s in the second. From these results and an assumed distribution volume in humans of 6.4 L, the half-life of the drug in humans was estimated to be approximately 15 to 20 min, as compared to up to 1.6 h in humans given DS (MW 8000). The difference in half-life can be explained by the molecular weight of DS, 5000 Da vs. 8000 Da. In i.v.-treated rats\textsuperscript{113}, only a small fraction of the infused LMW-DS (MW 8000) was eliminated by the kidneys; the rest was most likely eliminated by the liver ECs\textsuperscript{114}. The cut-off for elimination by the kidneys was <4000 Da. The average molecular weight of our LMW-DS preparation is 5000 Da (ranging from 3000 to 8000 Da), close to this cut-off, suggesting a greater elimination by the kidneys. In the last three monkeys, the bolus dose was divided in two portions given intraportally, the first 1.5 mg/kg given as a bolus dose and the following 3 mg/kg given over 20 min. The dose of the continuous infusion ranged from 0.75 to 1.5 mg/kg/h. The platelet count, liver enzymes (ALT and AST), and creatinine levels were all within normal range throughout the experiments. No bleeding or other adverse events were observed. This pharmacokinetic study of LMW-DS in cynomolgus monkeys further demonstrated that a continuous infusion of DS after an initial bolus dose could maintain the target APTT at a constant level.

Based on these experimental data and additional studies of LMW-DS, we developed a protocol for use in a clinical trial to downregulate the IBMIR at the time of islet transplantation. It is probably most important to inhibit the IBMIR during the first 3 to 6 hours after islet infusion into the portal vein. This initial protocol involves administration of a bolus dose of 3-6 mg LMW-DS/kg, one-third given before the transplantation to raise the APTT and give a protective effect before transplantation, and two-thirds given together with the islets intraportally, to protect the islets when they come into contact with blood in the portal tree. This protective effect (or elevated APTT) should allow the islets to extend higher up in the portal tree before a continuous infusion of 0.3 to 1.2 mg/kg/h is immediately started to maintain the APTT for up to 6 h post-transplantation. In this protocol, the APTT will be monitored and the dose of LMW-DS will carefully be adjusted to yield an APTT of 50 to 100 s; according to our in vitro studies, this level corresponds to a concentration of LMW-DS of 0.05 to 0.1 g/L. A comparison of LMW-DS with heparin has shown that administration of LMW-DS has an effect on hemostasis similar to that of heparin treatment, and it will therefore not increase the risk of bleeding any more than does the currently used dose of heparin. The short half-life of LMW-DS offers the possibility of a quick corrective response in case an unexpected bleeding event should occur.
General discussion

Blocking the IBMIR may improve clinical islet transplantation and reduce the number of donors needed for each patient. This blockade could be approached in three ways: treatment with systemic IBMIR antagonists, coating of the islets to make them less recognizable to the immune system, and pretreatment of the islets to make them less thrombogenic. Pretreatment of the islets before transplantation, for example with agents that can block TF expression or are capable of blocking TF synthesis (e.g., anti-sense reagents), has a clear clinical benefit compared to systemic inhibition, since it would have no adverse effect on hemostasis in the recipient.

Many candidates are under consideration for use in systemic IBMIR inhibition, but unlike the other candidate antagonists, LMW-DS (MW 5000) is currently available for clinical use. LMW-DS has been shown herein to inhibit all components of IBMIR in vitro and in vivo in a pig to mouse model. Because of its availability, LMW-DS is the first candidate drug to be used in clinical trials for preventive treatment of the IBMIR in intraportal islet transplantation into type 1 DM patients. Clinical trials using LMW-DS instead of heparin treatment in islet transplantation have already started, with LMW-DS being tested in healthy volunteers.
Conclusions

- TF is the main trigger of the IBMIR in vitro and in vivo.

- Islets intended for islet transplantation produce TF in two active forms, a full-length transmembrane form and a soluble, alternatively spliced form lacking the membrane-spanning region. TF present in both the islets and the culture medium elicits a thrombotic reaction, which affects the function of the transplanted islets.

- The endocrine, but not the exocrine, cells of the pancreas synthesize and secrete active TF. Electron microscopy reveals TF expression by both the α- and β-cells but not by δ- or PP cells.

- The IBMIR has been shown to occur in vivo in transplanted patients and to be negatively correlated with outcome, as reflected by C-peptide generation 7 days after transplantation.

- LMW-DS can abrogate all the components of the IBMIR in in vitro and in vivo model systems.

- LMW-DS is a promising drug to for inhibiting the IBMIR in clinical islet transplantation.
Acknowledgments

This thesis work was carried out in the Division of Clinical Immunology, Department for Oncology, Radiology, and Clinical Immunology, Uppsala University. I would like to express my sincere gratitude to all who, in one way or another, have contributed to this thesis. I especially would like to thank:

Bo Nilsson, my supervisor, for giving me the opportunity to do this, for always being around to provide guidance and discussions, for sharing your extensive knowledge and enthusiasm.

Olle Korsgren, my co-supervisor, for always seeing the possibilities in things rather than the difficulties; for inspirational talks, especially during harvest in the islet lab; and for hard judgments - because no matter how much data we have to support our theory, you always find the weak link that we haven’t thought about 😊! And, finally, for support and encouragement when I decided to have a baby in the middle of this.

Kristina Nilsson-Ekdahl, my co-supervisor, for kindly giving me so much of your time, for helping me to get back on track after my maternity leave, for proofreading this thesis, and for many talks, especially during my first time here. What would I have done without you?!

Lisa Moberg, for leading the way in the field of tissue factor in islets and for statistical guidance.

Masafumi Goto, for leading me into the field of low molecular weight dextran sulfate, for lots of inspiration, for showing interest in my work and for showing enthusiasm as well as a never-ending commitment in everything you do!

Linda Mathsson, for always taking time to discuss things and proofread my abstracts and posters, for sharing all the good and bad things with me during these years, and also for opening your home to me, always offering a place to stay in the "Pensionat Mathsson". You have been a tremendous support to me!
Jenny Tjernberg, for being a friend in the lab as well as outside the lab. For many discussions, support, funny stories, and laughs.

The Ö-lab team, including Bumsan (Margareta Engkvist) and Ulrika Johansson, for letting me stay a few nights in your homes and for teaching me so many things that has been invaluable to me; Magnus Ståhle, for always having a joke in your back pocket; Torsten Eich and Annaika Olsson, for nice company when skiing in Igls; Sanja Cabric, for company in Vienna; Viola Selling, Christina Andersson, Andrew Friberg, José Caballero, Hideyuki Yamaya, Naomi Forslund, Erik Åhlin, Daniel Brandhorst, Heide Brandhorst, Anna Svensson, and Nina Mikkola, for the hours we have spent together in the islet lab.

Past members of the islet lab, including Anette von Malmborg, for all the laughs; Peter Schmidt, one of the worst morning persons I have ever met (if it does not include birds 🐦); Izabela Jönsson, who shares many ways of thinking; Selina Parvin, a former worker in the lab with a contagious laugh; Henrik Krook, for insight into the world of companies; and Megumi Goto, for creating such a good atmosphere around KlinImm, and together with Masafumi having nice dinners for all of us.

Graciela Elgue, for running a whole bunch of ELISAs for me, nice summer BBQ at your place, and for sharing your great knowledge of the field of blood coagulation; Javier Sanchez, for help with the confocal microscope and “contact information” in the coagulation area; Jonas Andersson, for good help with smaller computer issues and for nice company in the PhD room; Rolf Larsson, Ulf and Margita Nilsson, Susanne Lindblom, Jaan Hong, AnnaKarin Lidehäll, Adil Babiker, Lillemor Funke, Osama Hamad, Mohammad Mullazehi, Petra Magnusson, Ida Rasmusson, Jennie Bäck, Anna Andersson and Jonas Leijon, for talks, lunches, and nice coffee breaks.

Mona Persson, for helping me with various things and keeping track of all of us; Christl Richter-Frohm, for administrative help with issues concerning this thesis and my Licentiate thesis; Ann-Sofie Lindberg, for administrative help and nice talks.

The GIG group, for nice talks and company at dinners at Norrlands and GH. I never reached my goal of visiting all nations…

Elisabeth Wijkström, the extra mum at the lab, for always seeing and looking after all of us, for listening to what we have to say, and for great support.
All the other people coming and going in the lab, for creating a nice environment.

All my co-authors, for being so cooperative, for quick responses and nice input.

Deborah McClellan, for always at the last moment reading and translating my papers and my thesis into proper English.

My friends, for quality time outside the lab.

Mum and Dad, for loving me no matter what I do, for believing in me, supporting me financially during my studies, and for enormous moral support. My sister Therese, for always looking after me and for time and time again telling me how good I am. My brother Henrik, the little brother growing big, for many talks about life in general—we usually understand each other; thanks for the support. Grandma, for trying her best to understand what I do and for asking me when is it time for me to finish, since her goal in life for many years has been to make it until I finish… The Forssell family, for letting me be the person I am, taking good care of me with nice dinners, and quality time relaxing in your summer houses.

Finally, I would like to thank the following two for being my family and reminding me of the true values in life: Anders, for loving me unconditionally… Thanks for all the support and for giving me the space to finish this; I’ll cite one of my favorites - Per Gessle - by saying ”Det är över nu…”!

Alexander, Mummy’s sweetheart, for all the hugs and kisses, for giving so much of yourself and all the ”bus” that has forced me to think of other things than work. You are the light of my life, and I love you tremendously (despite all the gifts you bring home from day-care: colds, stomach flu, eye inflammation, etc. 😊).
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Acta Universitatis Upsaliensis

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Editor: The Dean of the Faculty of Medicine

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