The Role of Innate Immunity in Islet Transplantation

Clinical and Experimental Studies

BY

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Abstract

Clinical islet transplantation is an emerging procedure to cure type 1 diabetes. The graft is implanted by infusion into the liver through the portal vein. A major obstacle that still needs to be overcome is the requirement for islets from multiple donors to achieve insulin independence.

An innate inflammatory reaction, the IBMR, is elicited when islets are exposed to blood. The IBMR has been described as a clotting reaction culminating in disruption of islet morphology and is a plausible cause for loss of tissue during the early post-transplant period.

In this thesis, the underlying mechanisms of the IBMR were characterized. The IBMR was first demonstrated in patients undergoing an islet transplant, and a number of clinically applicable strategies to limit this reaction were identified.

The thrombin inhibitor melagatan completely blocked the IBMR in an in vitro tubing blood loop system, indicating that thrombin is the driving force in the reaction. Interestingly, islets were shown to produce and secrete tissue factor (TF), the physiological trigger of coagulation. Inactivated FVIIa, a specific inhibitor of TF, successfully blocked initiation of the IBMR. An alternative approach to limit the IBMR was to pre-treat islets in culture prior to transplantation. Nicotinamide added to the culture medium effectively decreased the level of TF in human islets. Infiltration of immune cells, also a part of the IBMR, was characterized in detail. The predominant cell types infiltrating the islets were neutrophilic granulocytes and, to a lesser degree, monocytes. Both cell types may exert direct cytotoxic effects, and the antigen-presenting monocytes may also be important for directing the specific immune system to the site of inflammation.

These findings have provided new insight into the nature of the IBMR and offer several new strategies to improve the outcome of clinical islet transplantation.

Keywords: diabetes, islet transplantation, islet of Langerhans, coagulation activation, IBMR, tissue factor, thrombin, inactivated FVIIa, melagatan, neutrophilic granulocyte

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


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* Authors contributed equally to the work

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ABBREVIATIONS

ANOVA Analysis of variance
AP-1 Activator protein 1
APAAP Alkaline phosphatase anti-alkaline phosphatase
APC Activated protein C
AT Antithrombin
β-TG β-thromboglobulin
CTL Cytotoxic T-lymphocyte
D Damping
EIA Enzyme immunoassay
Egr-1 Early-growth-response gene product 1
F Coagulation factor
F 1+2 Fragments 1+2
FXIa-AT Factor Xla-antithrombin complex
FGF Fibroblast growth factor
Fq Frequency
IBMIR Instant blood-mediated inflammatory reaction
ICAM-1 Intracellular adhesion molecule 1
IEQ Islet equivalent
iFVIIa Inactivated recombinant coagulation factor VIIa
IL Interleukin
LPS Lipopolysaccharide
mAb Monoclonal antibody
MCP-1 Monocyte chemotactic protein 1
mRNA Messenger ribonucleic acid
NF-κβ Nuclear factor κβ
NOD mouse Non-obese diabetic mouse
PAF Platelet activating factor
PAR Protease-activated receptor
PDGF Platelet derived growth factor
PF 4 Platelet factor 4
PFC Perfluorocarbon
PVC Polyvinyl chloride
PP cells Polypeptide cells
sCR1 Soluble complement receptor 1
TAFI Thrombin activatable fibrinolysis inhibitor
TAT Thrombin antithrombin complex
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>Th</td>
<td>T-helper cell</td>
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<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelium growth factor</td>
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INTRODUCTION

In recent years, pancreatic islet transplantation has become an attractive alternative for curing Type 1 diabetes. However, an obstacle to establishing this procedure as standard clinical practice remains: the need for islets from multiple donors in order to achieve normoglycemia.

Previously, an inflammatory reaction termed the instant blood-mediated inflammatory reaction (IBMIR) was proposed to occur when islets are injected into the portal vein. From *in vitro* data, the IBMIR was described as a fast acting reaction culminating in islet disruption. The IBMIR provides an explanation for the tissue loss associated with this procedure and consequently the requirement of large amounts of islets for each patient to become normoglycemic.

This thesis elucidates the underlying mechanism(s) for this injurious response and suggests strategies for circumventing the IBMIR in order to improve islet engraftment and thus the outcome of islet transplantation.

Type 1 diabetes

Diabetes is a metabolic disorder in which the body has lost the ability to utilize glucose, its main source of “fuel.” For glucose to enter the cells of the body, insulin must be present. Insulin is a hormone produced by the pancreatic β-cells, which normally automatically produce the exact amount needed to move glucose into the cells. There are two major forms of diabetes: type 1 diabetes, in which the pancreas produces little or no insulin, and type 2 diabetes, in which the body’s cells do not respond appropriately to the insulin produced.

Type 1 diabetes is an organ-specific autoimmune disease in which the immune system attacks the insulin-producing β-cells. The onset of the disease typically occurs before adulthood, and both genetic and environmental factors such as infectious agents can initiate or trigger the process that leads to destruction of the β-cells.

Type 1 diabetes occurs worldwide and affects approximately 4 million people in North America and Europe. The number of diabetics is expected to increase to approximately 5 million by the year 2010 and to 30 million by 2025. It is a lifelong condition that seriously affects a person’s quality of life. Individuals with the disease have to make major lifestyle changes and...
learn to live with blood glucose measurements as well as multiple drugs and injections and to deal with the complications of the disease. Long-term consequences of type 1 diabetes include blindness, kidney failure, nerve damage and increased susceptibility to heart disease.

Treatment and cure of type 1 diabetes

Today, type 1 diabetes is successfully treated with life-long daily exogenous insulin injections and monitoring of blood glucose levels. However, even careful glucose control cannot adequately substitute for the finely tuned normal balance of the glucose levels found in a healthy body, and many patients will therefore develop secondary complications. It has been clearly demonstrated that good glucose control is important for delaying or preventing the onset of late complications. Despite marked improvements in diabetes care in recent years, insulin-dependent diabetes is one of the leading causes of end-stage renal disease, blindness and amputation.

The only currently available method for restoring endogenous insulin production is to replace the patient’s destroyed insulin-producing β-cells (islets), either by whole pancreas transplantation or by transplanting the islets, which constitute only 1% of the whole organ. Replacement of the whole gland re-establishes long-term normoglycemia, with a success rate of 80% ⁸, and is especially successful for those that undergo simultaneous pancreas and kidney transplantation. However, because of the risk of surgical complications, this procedure will never be a viable option for most type 1 diabetic patients. Those offered this treatment are patients who have already developed many of the secondary complications, including end-stage renal failure, and still have a quality of life that is adequate for undergoing such a difficult treatment.

Since the breakthrough made by Shapiro et al. ⁹, islet transplantation has emerged as an attractive alternative to whole pancreas transplantation. The surgery is minimal, and the procedure can be performed on an almost outpatient basis. During transplantation, isolated islets are infused into the portal vein and allowed to lodge in the distal portal venules, where they eventually engraft. This implantation site has become the common route for islet transplantation, even though other sites have been tested in humans or in rodents (for example, underneath the kidney capsule, in the spleen, or under the skin). In favor of intraportal transplantation is its presumed association with a faster revascularization, with more islets surviving and becoming self-supporting ¹⁰. However, until recently the results of this approach have been unexpectedly poor since, in contrast to the results obtained with transplantation of the whole organ, prolonged insulin independence has been obtained in only approximately 10% of the patients ¹¹.

These differences in success rates are puzzling, since the underlying autoimmune disease, major histocompatibility barriers and immunosuppressive
therapy are the same for both procedures. One major difference, though, is that while the islets are protected from the recipient’s blood by the endothelial lining in the case of whole-pancreas transplantation, they are exposed to the host’s blood when embolized into the portal vein during islet transplantation. This procedure exposes the isolated islets to the various constituents of the blood, i.e., the different cells types and plasma proteins of the cascade systems of the blood.

Blood

Blood performs many functions in the body. In addition to providing a transport system for respiratory gases, nutritive molecules, metabolic wastes and hormones, it also protects the body against invaders (viruses, bacteria and other pathogens). It contains specialized cells and chemicals that recognize pathogens, in both a specific and a non-specific manner, to combat and eliminate these foreign agents.

The blood also has the ability to clot, preventing the body from losing large amounts of blood as the result of an injury. An adult human has 3-5 L of blood, which is composed of many different components. Of this volume, 55% is plasma, and the remaining 45% consists of erythrocytes (red blood cells), white blood cells and platelets.

Blood clotting

To maintain hemostasis (the cessation of blood loss), the coagulation cascade system is activated upon vessel injury. As part of this process, a complex series of interactions between protease zymogens, enzymes and cofactors is activated, generating thrombin and a fibrin clot to stop the bleeding at the site of injury. Simultaneously, platelets are activated and adhere to molecules exposed in the damaged subendothelial layer, then aggregate to form the primary hemostatic plug.

Activated platelets provide an appropriate surface for the assembly of the plasma coagulation factors, thereby generating more thrombin. Thrombin cleaves fibrinogen to fibrin, which in combination with platelets forms an insoluble thrombus to stabilize the formed plug and stop the bleeding. Mechanisms that restrict the formation of platelet aggregates and fibrin clots to the sites of injury are necessary to maintain the fluidity of the blood.

Coagulation is activated via either of two pathways (Fig 1A): the intrinsic (contact activation) or the extrinsic (tissue factor-dependent) pathways, both of which converge in the activation of coagulation factor (F) X to FXa. FXa generates thrombin in a common pathway.
Contact activation

The intrinsic pathway is initiated by foreign negatively charged surfaces, such as collagen, which are exposed at the site of a wound. The initial step in contact activation is binding and activation of the FXII to FXIIa as it binds to the exposed negative surface. FXIIa in turn initiates a cascade of proteolytic reactions involving FXIa and FIXa, which ultimately result in activation of FX (Fig 1A).

The role for FXII in initiating coagulation in vivo is however questioned, since deficiency of FXII is not linked to bleeding but rather associated with thrombosis. There is evidence that the physiological role of FXII may be related to fibrinolysis as is discussed below.

The main function of the contact activation and the activation of FXII may be to amplify the thrombin generation triggered by the tissue factor pathway.

TF pathway

The primary physiological initiator of the coagulation system is tissue factor (TF), a 47-kDa transmembrane glycoprotein. Under normal conditions TF is not exposed to blood but is found on a variety of extravascular cells (i.e., cells in the adventitia of the blood vessels) and also in richly vascularized tissues such as the cerebral cortex, placenta, renal glomeruli and lungs. However, TF expression can be induced in cells that are in contact with blood (such as endothelial cells and monocytes) by certain inflammatory stimuli such as lipopolysaccharide (LPS), immune complexes, and cytokines. Moreover, TF has been shown to exist in plasma in the form of circulating microparticles derived from monocytes and platelets. Increased levels of plasma TF have been noted in patients with myocardial infarction, unstable angina or diabetes.

The initiating event upon a break in the vessel wall is exposure of blood to TF-bearing extravascular cells (Fig 1A). FVII/FVIIa present in blood binds to exposed TF, followed by a proteolytic activation of FVII to FVIIa. This active complex in turn activates FX to FXa and FIX to FIXa. Activated FX (FXa) in complex with FVas produces small amounts of thrombin, the main enzyme in the clotting cascade. These minute amounts of thrombin generated are sufficient for complete activation of platelets adhering to structures exposed in the subendothelium. It also activates FXI and the co-factors FVIII and FV, connecting the TF pathway initiation with the intrinsic amplification loop (Fig 1B).

Thrombin, a potent activator of platelets, acting through protease-activated receptors (PAR) 1-4, triggers shape changes in platelets, mobilizes the adhesion molecule P-selectin to the platelet surface and increases the affinity of the integrin αIIb/β3 for fibrin, which mediates platelet aggregation. These activated platelets, together with thrombin activated FXI, FVIII and
Figure 1. The coagulation system. The coagulation activation occurs in three overlapping stages. A. Initiation. Coagulation is activated by either of two pathways: the contact activation pathway or the TF-pathway. The physiological relevant initiation occurs on a TF-bearing cell generating small amounts of thrombin, sufficient to activate platelets. B. Amplification. Occurs as the “action” moves from the TF-bearing surface to the platelet. Platelets offer an optimal surface for generation of large amounts of thrombin. This burst in thrombin generation results in fibrin polymerization that stabilizes the formed plug. C. Natural inhibition. Restricts the clot to the site of injury. The plasma protease inhibitors, TFPI and AT, localize the reaction to cell surfaces by inhibiting active coagulation factors that diffuses into the blood. In addition, intact endothelium possesses antithrombotic features, i.e., expression of TM that prevents coagulation from being initiated in the intact endothelium by activating APC. APC in turn, attenuates further thrombin generation by blocking FVIIIa and FVa. FXIIa, a member of the contact pathway, acts on the fibrinolytic system, to dissolve the formed fibrin plug.
FV, support the second burst of thrombin production by expressing phosphatidylserine-rich membrane surfaces that promote the assembly of coagulation enzyme-cofactor complexes. This explosion of thrombin formation is mainly formed inside the clot, produced via the intrinsic pathway amplification loop, and is necessary for an adequate hemostatic response (Fig 1B).

Mechanisms that restrict the formation of platelet aggregates and fibrin clots to sites of injury are necessary to maintain the fluidity of the blood (Fig 1C). Tissue factor pathway inhibitor (TFPI) regulates the extrinsic pathway of coagulation by blocking intravascular coagulation induced by TF. TFPI is mainly produced in endothelial cells and accumulated in the endothelium, plasma and platelets. TFPI released upon activation of coagulation forms a complex with FXa. TFPI/FXa binds to TF-FVIIa, resulting in a quaternary complex that no longer can sustain coagulation.

The thrombus extension is also prevented by vascular endothelial cells that have specialized anti-coagulant features to restrict clot formation to the site of injury. Thrombin that escapes into the circulation is either inhibited by antithrombin (AT) complexed with endothelial heparin-like glycosaminoglycans or binds to thrombomodulin (TM) on intact endothelial cells. Upon binding to TM, the specificity of thrombin is changed. It no longer clots fibrinogen or activates platelets but instead activates protein C (APC). APC inactivates FVIIIa and FVa: that is, thrombin attenuates its own generation. This inactivation prevents the formation of additional procoagulant enzymes at sites where a healthy intact endothelial lining layer is presented.

In contrast, the high concentrations of thrombin generated inside a clot activate thrombin activatable fibrinolysis inhibitor (TAFI), which inhibits fibrinolysis and hence clot lysis. TAFI has to be considered a very important link between coagulation and fibrinolysis. Inhibition of thrombin generation by APC thus attenuates activation of TAFI, and thereby links the pro-fibrinolytic effect of APC to its anticoagulant activity.

Fibrinolysis is the final control mechanism that limits clot formation and works in opposition to the coagulation. The fibrinolytic system involves a series of enzymes. Plasmin, the final protease formed by fibrinolysis, catalyzes the cleavage of fibrin to produce soluble fibrin degradation products, thereby removing the fibrin clot. The major activator of plasminogen in vivo is tissue plasminogen activator (tPA), a serine protease produced by endothelial cells. In the absence of fibrin, it is an inefficient activator of plasminogen but once bound to fibrin, activation is greatly enhanced. In addition, studies have demonstrated that FXIIa (part of the contact activation) can also activate plasminogen to plasmin, a process that is potentiated by negatively charged surfaces.

To avoid bleeding and thrombus formation, a balance between the two cascade systems (i.e., coagulation and fibrinolysis) is imperative.
Blood as a part of the body’s defense

Blood is part of the immune system. This system has evolved with the sophisticated biologic capacity to distinguish self from nonself and to protect the body from potentially harmful pathogens. As soon as foreign substances, viruses, or bacteria enter the bloodstream, they encounter mechanisms specialized in recognizing foreign elements and eliminating them. There are two main branches of immunity, termed innate and adaptive immunity.

Innate immune system

The innate immune system is present since birth and exists in some form in most organisms. This line of defense operates fast to pathogens unlike the adaptive immune system, which may take days to mobilize. Innate immunity does not require repeated exposure to pathogens. Instead, it is capable of differentiation between self and nonself by recognizing highly conserved structures that are common to groups of related microbes, i.e., nucleic acids that are unique to microbes, such as double stranded RNA found in replicating viruses or unmethylated DNA sequences found in bacteria; features of proteins which are typical of bacterial proteins; complex lipids and carbohydrates and carbohydrate-like molecules that are found in microbial but not in mammalian glycoproteins. The microbial targets of innate immunity have been described as “molecular patterns” and the receptors that bind these conserved structures are called “pattern recognition receptors”. Because of this specificity for microbial structures the innate system, like the adaptive immune system, is able to distinguish between self and nonself.

Recognition of foreign structures in innate immunity involves blood clotting to prevent the spread of microbes by entrapping them in fibrin formations, and recruitment of phagocytic cells (neutrophilic granulocytes and macrophages) that have the capacity to engulf (phagocytose) foreign molecules. First to accumulate around the invaders and initiate the phagocytic processes are the neutrophilic granulocytes. Their cytoplasm is filled with granules that contain various agents for killing microbes. Monocytes/macrophages are also attracted to the area; although slower to arrive, they have another indispensable function in host defense: they “process” the antigenic components of the foreign invaders and present them to the adaptive immune system, the lymphocytes (described below).

Pathogen-associated molecules can also be recognized by a series of pre-existing antibodies in the blood that function as opsonins and activate the complement pathways. Complement, acting through several cascade systems, consists of a large number of proteins that are found in the blood in an inactive proenzyme state. These proenzymes can be activated by a number of specific or nonspecific immunological mechanisms. Once activated complement proteins carry out three major functions (Fig 2). First, they can de
Figure 2. The complement system. Complement activation has three major functions: (1) Recruitment of inflammatory cells, i.e., neutrophilic granulocytes, by the released split products (C5a and C3a). (2) Lysis of foreign cell membranes to eliminate the intruders, by the formation of membrane attack complexes. (3) Enhancement of phagocytic activities by coating (opsonizing) microbes so that they are recognized by phagocytic cells expressing complement receptors.

Adaptive immunity
Adaptive (specific) immunity involves specific recognition and selective elimination of microorganisms and foreign molecules. Unlike innate immunity, adaptive immunity displays specificity, diversity and memory. If the
innate defenses are not sufficient to eliminate the intruders, the adaptive system is alerted and uses its specific actions to combat the intruders until they are eliminated. The constituents of the adaptive system are B and T lymphocytes. The B cells mediate antibody production to combat bacterial infections. T cells are divided in two major subtypes, cytotoxic T lymphocytes (CTLs) that attack host cells that have become infected with viruses or fungi, and T helper cells (Th). Th cells are divided into Th1 cells that activate more CTLs and Th2 cells that enhance antibody production by B cells.

The adaptive system does not function independently of innate immunity. For example, cells of the phagocytic system play an important role in activation of the adaptive immune system. Likewise, soluble factors produced by the adaptive immune system have been shown to enhance phagocytic activity. Bacteria that are coated with antibodies are better targets for phagocytosis by neutrophilic granulocytes and macrophages. Therefore, interplay between these two systems is necessary for the effective elimination of the intruders.

Cross-talk between coagulation and the blood cells

A tight interplay exists between coagulation and inflammation (Fig 3). Inflammatory mediators released from stimulated endothelial cells, i.e., interleukin (IL)-6 and IL-8 at the site of inflammation, have been shown to promote coagulation by elevating TF on monocytes/macrophages in vitro. IL-6 and IL-8 may also induce expression of other monocyte-derived cytokines, exerting autocrine effects on TF expression. Among monocyte-derived cytokines, IL-1β and tumor necrosis factor α (TNFα) are known to induce TF expression.

In addition, IL-6 increases the thrombogenic reactivity of platelets, further linking inflammation and thrombosis. IL-8, is one of the most potent chemotactants for neutrophilic granulocytes. Besides being released from activated endothelium, neutrophilic granulocytes themselves release IL-8 upon activation, thereby attracting more of their own “kind” to the site of inflammation.

Also platelets induce inflammation by releasing their granule contents upon activation, promoting the recruitment of various leukocytes. Platelet factor 4 released from their granule is known to have chemotactic activity for neutrophilic granulocytes and monocytes. In addition, adhesive proteins, i.e., P-selectin and intracellular adhesion molecule 1 (ICAM-1) are exposed on the platelet surface upon activation, giving the platelets a sticky surface to which the recruited cells can adhere.

The primary receptor on platelets for interaction with neutrophilic granulocytes and monocytes is P-selectin. Monocytes adhering to P-selectin on activated platelets are stimulated to secrete chemokines (monocyte chemo-
tactic protein-1 [MCP-1] and IL-8) for further recruitment of leukocytes. Also, the expression of TF on monocytes is markedly stimulated by the presence of platelets in a P-selectin-dependent reaction. This effect may be caused by the activation of the nuclear factor κB (NF-κB) transcription factor induced by binding of activated platelets to neutrophilic granulocytes and monocytes/macrophages. These cellular interactions also enhance the production of TF, MCP-1, IL-1β, IL-8, and TNFα, primarily in monocytes/macrophages.

Figure 3. Crosstalk between coagulation and inflammation. (1) As long as the endothelium is intact it possesses antithrombotic properties, but is lost when a vessel is damaged. Thrombin generated via exposed TF stimulates endothelial cells to upregulate adhesion molecules (i.e., P-selectin and ICAM-1) on their surface, to which recruited cells can adhere, and to secrete various cytokines (i.e., IL-6, IL-8 and PAF) to attract blood cells to the site of inflammation. IL-6 and IL-8 acts on monocytes/macrophages and IL-8 and PAF on neutrophilic granulocytes. (2) At the same time, at the site of injury, platelets become activated and upregulate adhesion molecules which render them sticky. In addition, they release their α-granule contents (PF 4), also affecting the recruitment of leukocytes. (3) Activated monocytes/macrophages release a broad range of cytokines acting in an autocrine manner to further amplify the inflammatory response and their expression of TF, thereby supporting more thrombin generation. (4) Activated neutrophilic granulocytes potentiate their own activation by producing more IL-8.
In addition to thrombin’s self-amplifying capacity and its role in activating platelets, it also acts on other cell types through specific protease-activated receptors (PAR 1-4) involved in inflammation. The thrombin-activated PAR-1 receptor triggers intracellular signal transducing systems, with the subsequent activation of transcription factors that induce early response genes. Factors such as NF-κB, activator protein 1 (AP-1) and early-growth-response gene product (Egr-1) are activated. These factors are known to regulate the transcription of TF, ICAM-1 and IL-8 in monocytes. Thrombin activation of endothelium results in high levels of platelet activating factor (PAF) formation, which works as a potent neutrophil agonist, especially when neutrophilic granulocytes are tethered to selectins on the activated endothelium. Thrombin also induces IL-6 and IL-8 production in endothelial cells. These effects of thrombin are probably mediated by PARs 1, 3 and 4.

This inflammatory-coagulation interaction is dampened by three natural anticoagulant mechanisms: TFPI, AT, and APC (Fig 1C).

- TFPI complexed with FXa blocks the activity of TF/FVIIa. In an E. coli sepsis model in baboons, the addition of TFPI substantially attenuated inflammation, as indicated by decreased plasma levels of IL-6 and IL-8. In phase II clinical trials of TFPI in patients with severe sepsis, there was a trend toward improved outcome.

- AT inactivates thrombin and other serine proteases in the coagulation system. These reactions are accelerated by heparin-like proteoglycans on the endothelium. High levels of AT prevent the proteins of the coagulation cascade from activating cells and thereby limit the expression of adhesion molecules, cytokines and other mediators such as PAF. In baboons challenged with E. coli, IL-6 and IL-8 levels were reduced about 4-fold by treatment with AT when compared to controls.

- APC has been shown to inhibit a variety of cellular responses in vitro, including the nuclear translocation of NF-κB, a key step in the generation of the inflammatory response. Since increases in the levels of adhesion molecules and generation of inflammatory cytokines frequently require NF-κB translocation, the ability of APC to block this process would account for the observed inhibition of TNFα and the decrease in leukocyte activation that is seen when APC is administered to endotoxin-treated animals. APC has also been shown to prevent endotoxin-induced expression of TF in monocytic cell lines.

Islets in contact with blood

Intraportal infusion of islets is the common route for clinical islet transplantation. However, whether this is the best implantation site remains contro-
versial. A feared complication is portal thrombosis, and fatal cases were reported in the 1990s\textsuperscript{79,80}.

Today, intraportal islet transplantation offers patients with type 1 diabetes improved glycemic control and insulin independence, although the most successful transplantation protocol requires islets from at least two donors to render the recipient insulin-independent\textsuperscript{8}. Moreover, the functional capacity of the transplanted islets corresponds to only about 20\% of that in a non-diabetic\textsuperscript{81}, indicating suboptimal engraftment in the liver. A contributing factor may be that the glucose levels in the portal blood are higher than those in other sites, since the portal vein is derived from small intestine capillaries. This situation probably exerts a greater stress on the islets and exhausts them before they have readily engrafted, leading to loss of tissue\textsuperscript{82}. Despite these drawbacks, the intraportal infusion has proved to be superior to the other previously mentioned sites (i.e., beneath the kidney capsule or the spleen) because of safety reasons. Also, there are reports indicating that revascularization and re-innervation in the portal system are more efficient in the portal vein\textsuperscript{10}. Until safer methods are developed or newly generated data contradict the priority of the portal route, this will be the procedure used in the future.

Still, before this procedure can become established in clinical practice, the islet number required for successful transplant must be reduced, and the risk of thrombosis has to be well controlled. Although the portal vein is believed to be the best choice of implantation site, the infusion of islets also involves interaction with all the various components of the blood.

The IBMIR

Previous studies by Bennet et al.\textsuperscript{83,84} have described a thrombotic reaction elicited when islets come in direct contact with ABO-compatible blood, termed the instant blood-mediated inflammatory reaction (IBMIR) (Fig 4). The IBMIR is characterized by activation of the coagulation and complement systems and a rapid binding of activated platelets to the islet surface. The effects of this reaction culminate rapidly (within 15 min) with the islets being entrapped in clot formations and the islet morphology is disrupted by infiltrating leukocytes\textsuperscript{83}. This reaction was observed in both an experimental \textit{in vitro} model and in an allogeneic \textit{in vivo} pig model\textsuperscript{83,84}. The IBMIR has been described as a likely cause of both islet loss in islet transplantation and the intraportal thrombosis associated with this procedure.
Figure 4. The IBMIR (Instant blood-mediated inflammatory reaction). An injurious blood-mediated inflammatory response is elicited when islets are exposed to ABO-compatible blood. This reaction is characterized by activation of the cascade systems in blood, i.e., coagulation and complement, and activation of the platelets. The IBMIR culminates in islet destruction by infiltrating CD11b\(^+\) blood leukocytes.
AIMS OF THIS THESIS

General aims
Before bringing islet transplantation into routine clinical use, the consistently observed need for islets from multiple donors to achieve normoglycemia must be eliminated. The injurious actions of the IBMIR that culminates in damaged islet morphology, provides a possible explanation for the tissue loss observed in islet transplantation.

The general aim of this work was to further characterize the mechanism(s) behind the IBMIR, to establish its existence in vivo and to find strategies to prevent this reaction in clinical islet transplantation.

Specific aims

Paper I
The initial event of the IBMIR was previously characterized to involve the rapid activation of platelets and activation of the cascade systems, coagulation and complement. A potent activator of platelets and a key molecule in clotting is thrombin.

To elucidate the role of thrombin in the IBMIR, melagatran, a low molecular weight thrombin inhibitor, was tested in the in vitro loop model system.

Paper II
Thrombin is generated by either of two pathways, the contact activation pathway or the TF pathway. This study was undertaken to identify the trigger of the IBMIR and to find a strategy to inhibit this initiation. The presence of this inflammatory process in clinical islet transplantation was also investigated.

Paper III
Islets are able to escape the injurious effects of the IBMIR when antithrombotic agents are used to block TF activity in an in vitro system. An elegant way of reducing the adverse effects of the IBMIR and eliminate the risk of
systemic administration of antithrombotics would be to down-regulate TF expression in culture prior to transplantation. In this study, we asked whether compounds previously shown to affect TF and MCP-1 expression in monocytes and endothelial cells would have the same effect in human islet cells.

Paper IV
IBMIR terminates in clot formation, entrapping the islets. In this study we developed an in vitro system in which islets stuck in clots could be easily recovered for further morphological analysis. Applying this system, we were able to evaluate the types of blood cells involved in the IBMIR at defined time points (up to 6h after the beginning of the reaction).
STUDY DESIGN AND METHODS

Essentially all the methods used in this work have been designed to characterize the reaction(s) elicited when isolated human islets are exposed to ABO-compatible blood. Additional experimental models and analysis were used to characterize the underlying mechanisms of the IBMIR. More detailed information is given in Papers I-IV.

Ethical considerations
Human islets were isolated after informed consent was given for multiorgan donation and research. The protocol used for islet preparation was approved by the local ethical committee of Uppsala University.

Isolation of human islets (Papers I-IV)
Islets used in this study were isolated from human cadaver donors using a modification of previously described semiautomated digestion-filtration methods. This isolation procedure was followed by further purification on a continuous density Ficoll® gradient in a refrigerated COBE 2991 centrifuge. The quality and purity of the isolated islets were equal to that of islets released for clinical transplantation, but the total islet yield was too low, and therefore these samples were available for experimental studies. The islets were placed in untreated culture flasks and maintained in suspension culture at 37°C (5% CO₂) for 1 to 7 days.

In vitro blood experimental models (Papers I-III)
In order to create a surface resembling the inner surface of our vessels (i.e., the portal vein), all materials that came in contact with blood were coated with heparin.
Preparation of blood

Fresh human blood was obtained from healthy volunteers who had received no medication for at least 14 days. Care was taken not to activate the components of the blood and especially the platelets, which are easily activated. Therefore, blood was collected in an “open system” using a 60-mL syringe with no anticoagulants added, using an 18-G cannula connected to silicon tubing. During sampling, the syringe was gently rotated continuously. For the clotting time analyses, blood was collected in tubes containing citrate. Before the experiment was begun, the blood was re-calcified to normalize the concentration of Ca²⁺ and recovered its coagulative function.

Tubing loops as a model (Papers I-III)

To allow us to study the interaction between blood and islets in a manner similar to that seen in the portal vein during transplantation, we used a previously described model. In brief, this system consisted of loops made of PVC tubing (inner diameter=6.3 mm, length=390 mm) with heparin attached to the inner surface, designed to resemble a blood vessel. The tubing was held together with specially designed heparinized connectors that allowed circular tubing loops to be formed. Fresh human blood was added to each loop, and then placed on a rocking device at 37°C to generate a blood flow of approximately 45 mL/min (corresponding to portal flow) (Fig 5).

Figure 5. The in vitro tubing blood loop system.

In the studies comprising this thesis, the loop system was used in two ways: either the blood was pre-treated with anticoagulants prior to the addition of
islets. (i.e., with melagatran [Paper I]), or the islets were pre-treated before perifusing them in the loops containing fresh human blood (i.e., with site-inactivated FVIIa, iFVIIa [Paper II], mAbs against TF [Paper II] and nicotinamide [Paper III]). The test tubes with or without islets and blood were incubated on the rocking device at 37 °C for 30 min (monoclonal antibody experiments) or 60 min (melagatran, iFVIIa and nicotinamide experiments). At 5, 15, 30 and 60 min after perifusion, 1 mL of blood was collected from each loop into EDTA-containing tubes for further hematologic analysis (described below). Samples taken at 0 min, in which blood was not added to the tubing but was instead immediately transferred to the EDTA, were also included.

Considerations of generated data

The values obtained in an \textit{in vitro} blood loop tubing system are of a higher magnitude than would be expected \textit{in vivo}. This difference can be explained by the fact that the same aliquot of blood is re-circulated in the tubing during the whole observation period, which contributes to the accumulation of coagulation activation products. The artificial inner surface of the tubing covered with heparin does not behave precisely like the vascular endothelium, which displays other anticoagulant activities related to expression of thrombomodulin and the activation of fibrinolysis. Also, the inevitable air interfaces created in the tubing contribute to pronounced activation of the blood. Therefore, interpretation of data retrieved from \textit{in vitro} systems should be focused on differences between experimental groups rather than absolute values.

Clotting time (Paper I)

Clotting time in plasma was measured in a four-channel free oscillating rheometer, the ReoRox 4. In essence, this technique is based on the damping (D) and frequency (Fq) registered, when a sample cup is set to nearly free oscillation (11 Hz). D and Fq are dependent on the viscosity and elasticity of the sample. When clotting occurs, the rheological properties of the sample change, the frequency is decreased and damping is increased. The software determines the time at which the sum of the changes in these two variables reaches a preset value, defining a clot.

Tube system (Paper IV)

This experimental system was designed to mimic the first hours after islet infusion, when islets are lodged in portal microvenules and surrounded by clots. Of special concern was the need to design a system that was suitable for immunohistochemical evaluation. By using lower blood volumes, the
islets in the clots were easily recovered, and the IBMIR could be studied for a longer period of time. Heparinized 2.5-mL Ellerman tubes were used to mix 1.5 µL of islets (corresponding to ~1500 islet equivalents, [IEQ]) with 250 µL of fresh human ABO-compatible blood (no anticoagulant added). The tubes were incubated on a rocking device from 5 min up to 6 hr at 37°C. Samples were collected at 5, 15, 30, 60, 120, 180, 240, 300 and 360 min. For each time point, one tube with islets and blood was used. In order to stop the ongoing clotting reaction, EDTA was added to the tubes. Immediately after the reaction was stopped, islets and macroscopic clots from each tube were recovered on filters, collected in embedding medium (Tissue-Tek) and snap-frozen in liquid nitrogen for further immunohistochemical analyses. All experiments included a control tube that had no islets but contained 1.5µL of serum-free culture medium (the buffer in which the islets were resuspended). The reaction in the control tube was stopped at the first time point at which a clot appeared, to ascertain that the formed clots were triggered by the islets.

Clinical islet transplantation (Paper II)

The major inclusion criteria for the patients in this study were long-standing type 1 diabetes mellitus, frequent uncontrollable hypoglycemic attacks, lack of awareness of these problems, and a previous transplantation with a cadaver kidney graft. The patients were already on immunosuppression because of the previous kidney graft. At the time of the islet transplantation, immunosuppression was switched to the steroid-free protocol applied in Edmonton, including dacluzimab, sirolimus and tacrolimus. The islets were implanted by infusion into the portal vein, which is reached through a transhepatic percutaneous approach under angiographic guidance. The mean number of islets in each graft was 270,000 IEQ, with a purity of 70% or more. The IBMIR was monitored in patients receiving the islet transplant, by analyzing blood samples obtained either from a central venous catheter or from a peripheral vein.

Blood and plasma analysis (Papers I-III)

After incubation in the tubing loop model, the EDTA blood samples were analyzed for various parameters. Platelet and differential leukocyte counts were obtained using a cell counter. Plasma retrieved from the blood was analyzed for coagulation parameters (prothrombin fragments 1+2 [F1+2], thrombin-antithrombin complexes [TAT], FXIa-AT complexes), complement activation markers (C3a and sC5b-9) and platelet activation (β-thromboglobulin [β- TG]) either by using
commercially available EIA kits or methods previously described (for detailed information, see the papers).

Blood collected from patients undergoing an islet transplant was analyzed for TAT (as a measure of the coagulation activation of the IBMIR) and C-peptide (as a measure of islet function) using commercially available EIA kits.

Immunohistochemical staining

Papers I-II

Islets and macroscopic clots recovered on filters after perifusion with blood were collected in Tissue-Tek embedding medium and snap-frozen in liquid nitrogen. Islets were sectioned and subsequently stained for anti-human CD41a (a platelet marker) and anti-CD11b (a leukocyte marker).

Paper IV

Islets and clots harvested from the tubes at various time points (5, 15, 30, 60, 120, 240, 300 and 360 min) and stored at -70°C in Tissue Tek were sectioned and stained for markers specific for various blood cell types. Depending on the origin of the primary antibody, three different sets of reagents and protocols were used: Mouse-Envision for monoclonal antibodies (i.e., TF, CD41a [platelets], CD11b [leukocytes], CD20 [B-cells], CD68 [macrophages], CD16 [granulocytes] and neutrophil elastase [neutrophilic granulocytes]); Rabbit-Envision for polyclonal antibodies (lysozyme); and the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique for the CD209/DC-SIGN antibody. Eosinophilic granulocytes were detected by histochemical visualization of cyanide-resistant endogenous peroxidase activity.

Statistics (Papers I-III)

All results are expressed as mean ± standard error of the mean (SEM). In Papers I and II, the non-parametric Friedman ANOVA was used to compare mean values between several groups for paired data. In Paper III, the ratio t-test was used to compare paired data between two groups. In this paper, the correlation between MCP-1 and TF expressed by the islets and their TF content in regard to coagulation activation (as measured by TAT) was calculated by linear regression.

Individual variations in blood characteristics and the degree of proinflammatory mediators expressed by islets isolated from different donors made it
difficult to present data from in vitro studies as absolute numbers. To be able to interpret and compile the data collected, we have instead expressed them as a percentage of the value for a control tubing loop included in each experiment. This tubing consisted of whole blood, medium in which islets were resuspended (but with no islets) and any drugs being tested.
RESULTS AND DISCUSSION

IBMIR in clinical islet transplantation (Paper II)

In Paper II, the IBMIR was shown for the first time to consistently occur in patients receiving islets intraportally. This reaction was demonstrated by analyzing blood samples taken from patients before the islet infusion and at 15 min, 60 min and 1 day after infusion. These retrieved samples were analyzed for coagulation activation (TAT, as a measure of the IBMIR) and C-peptide, reflecting insulin dumping in damaged islets.

The TAT levels peaked after 15 min of infusion and thereafter tapered off, reaching normal values within 1 day. The following increase in C-peptide suggests damage to the graft (Fig 6). No clinical signs of portal thrombosis occurred in association with any of the islet transplantations, a finding that is not very surprising, since the thrombus probably originated from the infused islets and was therefore not occlusive.

Figure 6. The IBMIR in vivo. The IBMIR occurs in patients receiving an islet transplant. Analyzed blood samples taken from patients during islet transplantation show an increase in TAT formation, which indicates coagulation activation. A peak is reached after 15 min after the start of infusion. C-peptide measurements followed a similar but somewhat delayed pattern. This point to leakage of insulin from the transplanted graft, which occurs in damaged islets.
Values for the clotting parameter TAT in these patients were much lower than those obtained in the closed tubing blood loop model. Nevertheless, they were of the same order of magnitude as those found in patients undergoing orthopedic surgery and in patients with sepsis and thromboembolism.

Our demonstration that the IBMIR is present in clinical islet transplantation in the absence of clear clinical signs of intraportal thrombosis indicates that inhibition of the process might increase the success rate of islet transplantation and reduce the number of donors needed for each patient. This finding provided the rationale for the remaining work in this thesis, which was focused on understanding the underlying mechanisms of the IBMIR and assessing potential reagents and approaches to intervene with this reaction.

Mechanisms of the IBMIR (Papers I, II and IV)

Thrombin, a driving force in the IBMIR (Paper I)

The IBMIR has repeatedly been defined as a clotting reaction. The key molecule and driving force in the coagulation system \textit{in vivo} is thrombin. In this study we therefore examined the role of thrombin in the IBMIR (Fig 7A). Melagatran, a low molecular weight thrombin inhibitor, was used in the \textit{in vitro} loop model to successfully block thrombin and its effects.

In tubing loops without melagatran, macroscopic clotting with cell consumption (platelets, granulocytes and monocytes) was seen after 60 min and was accompanied by a significant rise in coagulation activation markers (TAT, F1+2 and FXIa-AT). Marked increase in the complement activation product C3a was also observed.

The addition of melagatran in various concentrations (0.4, 1 and 10 µM) diminished cell consumption in a dose-dependent manner. An effect on most parameters was observed at concentrations as low as 0.4 µM. The cell counts were fully restored at ≥ 4µM. The addition of melagatran also inhibited the complement system (decreased C3a). To rule out the possibility that melagatran exerted nonspecific actions on other serine proteinases (i.e., complement); we used human serum in which complement was activated on a polystyrene surface. Melagatran had no effect on the complement activation, in terms of C3 binding to the surface and the generation of C3a. Therefore, we concluded that complement activity was dependent on the thrombin generation as a trigger pushing the IBMIR reaction forward (Fig 7B).

Complement is a component of innate immunity and plays a pivotal role in inflammation by recruiting leukocytes to the site of inflammation. Our immunohistochemical staining showed a marked decrease in CD 11b+ leukocyte infiltration into the islets with the highest dose of melagatran (10 µM).
Thus, inhibition of the clotting reaction and complement system decreased but did not completely abrogate the infiltration into the islets. Taken together, these results suggest that thrombin is the main driving force in platelet, coagulation, and complement activation during the injurious actions of the IBMIR. However, since the infiltration was not completely blocked, some other mechanism(s) are probably involved (discussed below).

**Figure 7. Thrombin, a driving force in the IBMIR.** A. Thrombin is the key molecule in the coagulation system. To elucidate the role of thrombin in the inflammatory reactions of the IBMIR, a thrombin inhibitor (melagatran) was used in the blood loop system. B. Addition of melagatran to the blood prior to perfusion of the islets resulted in blockade of the IBMIR.

The presence of tissue factor in islets (Paper II)

TF is the physiological activator of coagulation and is expressed in tissues that are not normally in direct contact with blood but which are exposed to blood when a vessel is damaged. Unexpectedly, TF was found to be expressed by the endocrine cells in the pancreatic islets. This finding suggests that islets infused intraportally in a transplantation setting are pro-coagulative by exposing the initiator of coagulation. Hence, the islet surface provides an appropriate surface on which the clotting cascade can take place and further sustain the amplification of the reaction. This observation explains the paradigm that no clinical signs of thrombosis are detected in most patients receiving an islet transplant. The thrombus originates on the islet surface and is therefore not occlusive.
To further confirm the presence of TF in the islets, various techniques were applied. Immunohistochemical analysis using an anti-TF mAb revealed the presence of TF in the isolated islets of Langerhans (Fig 8A). Electron microscopic findings pointed to the expression of TF in both α- and β-cells but not in δ- or pancreatic polypeptide (PP) cells. Surprisingly, the exocrine portion of the pancreas, i.e., the major part of the pancreas, was devoid of TF expression (Fig 8B).

The TF protein was further immunoprecipitated from lysates of pure islets and analyzed by SDS-PAGE and Western blotting. The polypeptide had a molecular mass of 47 kDa, identical to that of full-length TF.

**Figure 8. The presence of tissue factor in islets.** A. Immunohistochemical stainings demonstrate the expression of TF in isolated islets. B. Staining for TF was performed on pancreas biopsies to rule out the possibility that TF expression is induced by the isolation procedure. Interestingly, only the islets and an adjacent located vessel stained for TF. No staining was seen in the exocrine parts of the pancreas.

We also calculated the amount of TF per cultured islet after quantification of TF in the lysates by EIA. Directly after and on days 2 and 7 after isolation, the TF levels were 2.4, 7.1 and 3.7 nmol/µg DNA, respectively (no significant difference, p=0.08). This result indicates that the expression is probably induced by some stimulus and may be reduced by culture prior to transplantation.

**Tissue factor, the trigger of the IBMIR (Paper II)**

To establish that TF, expressed and synthesized by islets, is the trigger of the IBMIR, we perifused human islets with fresh human ABO-compatible blood in the tubing blood loop model for 30 min (Fig 9A). Islets were pretreated with active site-directed inhibitory (4509) or non-inhibitory (4503) anti-TF mAb for 10 min and washed before being added to the tubing loops. In the control samples (blood with islets alone or treated with the non-inhibitory
anti-TF), clotting occurred within 15 min, but with the inhibitory anti-TF, clotting was inhibited over the whole observation period. This difference was reflected in the consumption of platelets (platelet count), in the release of platelet α-granule content (β-TG), and in the generation of TAT, F1+2 and FXIa-AT, all of which were decreased by inhibitory mAb 4509 but not by non-inhibitory mAb 4503. An even more pronounced inhibition of the IBMIR was obtained with inactivated recombinant FVIIa (iFVIIa), an efficient inhibitor of TF activity. Blood containing 40 pmol/L iFVIIa completely inhibited the drop in platelet count and the increase in TAT, FXIa-AT, and C3a. The effect of these two inhibitors strongly indicates that TF is the trigger of the IBMIR (Fig 9B).

![Figure 9](image)

**Figure 9. Tissue factor, the trigger of the IBMIR.** A. Islets were shown to express TF, which is the physiological trigger of the coagulation. Consequently, islets expressing TF may initiate the IBMIR. The role of this expression was investigated in the blood loop system. B. When islets were pretreated with site specific inhibitors for TF, either an anti-TF mAb or iFVIIa, before exposing them to blood the IBMIR was inhibited.

Procoagulant activity was also found in the culture medium of isolated human islets when the medium was mixed with human plasma. The clotting time in the presence of culture medium was less than 5 min, and the TF activity was dependent on the dilution factor. The clotting activity was blocked by inhibitory mAb 4509, but control mAb 4503 had no effect. After ultracentrifugation of the medium at 100,000 x g, the resulting supernatants

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showed no clotting activity, but the pellet had twice the activity of the untreated culture medium. Thus, the TF activity is apparently associated with a high molecular weight fraction, and since TF is a membrane-bound protein with a transmembrane region, the protein is most likely associated with microparticles.

Neutrophilic granulocytes: the predominant blood cells infiltrating islets (Paper IV)

The IBMIR culminates in disruption of islet morphology by infiltrating CD11b⁺ leukocytes. The contact between islets and blood has previously been studied only in the short-time perspective, and no detailed characterization has yet been reported concerning blood cells infiltrating the islets after exposure to blood. In this study we used the tube system, the design of which is particularly suitable for immunohistochemical evaluation of islets in contact with blood. Samples were collected at various time points and characterized for infiltration of blood cells during the early phase of the IBMIR up to 6h. The neutrophilic granulocyte was found to be the predominant cell type infiltrating the islets (Fig 10). These cells appeared already after 15 min, with massive infiltration occurring within an hour and peaked at 2h.

**Figure 10. Neutrophilic granulocytes, the main actors in the IBMIR.** Islets were harvested after 2h in contact with blood, sectioned and stained for leukocyte markers. **A.** Islets unexposed to blood (negative control). **B.** Staining for CD11b⁺ cells, a broad marker for leukocytes. **C.** Staining for neutrophil elastase, a specific marker for neutrophilic granulocytes.

Macrophages were also found to infiltrate the islets, although the number of infiltrating cells increased slightly over time. B and T cells were not detected at all in the islets during the 6h observation period, suggesting that the specific immune response is not involved in the early phase of the IBMIR. Neutrophilic granulocytes fulfill their role by killing pathogens via enzymatic digestion and phagocytosis. Macrophages also act as phagocytic cells,
but simultaneously function as antigen-presenting cells communicating with lymphocytes in the activation phase of specific immune responses. These findings points to the need to adjust the immunosuppression for islet transplantation to also target the IBMIR. The protocols applied at present (rapamune, rapamycin and daclizumab) are directed against the specific immune response, but the IBMIR is most likely not affected by this medication.

The mechanisms by which the islets stimulate neutrophilic granulocytes and macrophage recruitment are unknown. Results from Paper I, as well as from the study by Bennet et al. 83, suggest that complement activation enhances the recruitment of CD11b+ cells to the islets. The anaphylatoxins C3a and C5a, released upon complement activation, are known to be major mediators of neutrophilic granulocyte migration 97. However, even when complement was blocked with melagatran, infiltration of CD11b+ leukocytes was still found in the islets, although to a lesser extent. Taken together, these observations indicate that complement activation alone is not the only mechanism by which neutrophilic granulocytes are recruited to the graft; one or more additional mechanisms are also involved in recruiting blood cells to the graft.

Recent reports have revealed the presence of various inflammatory mediators in isolated islets that could have implications for the infiltration pattern observed in this study 98. Piemonti and coworkers 99 reported a correlation between the number of infiltrating macrophages and the amount of MCP-1 produced by the islet. Islets have also been shown to express IL-8, a known chemotactic agent for neutrophilic granulocytes 98.

The “primed” proinflammatory activities exerted by the islets themselves are most probably induced by some stimulus present during the process of organ retrieval and islet isolation. This conclusion is supported by the individual variations that have been observed in TF (Paper II) and MCP-1 99, at both the mRNA and protein levels, among islet preparations from different donors. Just prior to death, abnormal physiological events are known to occur in brain-dead patients, such as a stress-related catecholamine influx. Also, prolonged cold ischemia as well as the isolation procedure and culture conditions can induce the expression of a variety of pro-inflammatory genes.

Strategies to prevent the IBMIR (Papers I-III)

The observation that the IBMIR also occurs in the clinical setting (Paper II) points to the apparent need to control this reaction. Several steps in the IBMIR process could serve as targets for pharmacological intervention, as a means of preventing the reaction and thereby promoting successful engraftment of the islets.

In this thesis, the in vitro blood loop model system was used to evaluate the effects of various drugs targeting various aspects of the IBMIR. The compounds were either added to the blood prior to exposure to the islets, simu-
lating systemic anticoagulant therapies (melagatran targeting thrombin or iFVIIa interfering with TF, Papers I and II) or supplemented in the culture medium of the islets prior to the blood loop experiments as a model for pretreating the islets prior to transplantation (nicotinamide, Paper III).

Inhibition of thrombin propagation (Paper I)

Because of its pivotal role in hemostasis, thrombin is a key target in the therapeutic prevention of thrombosis. The current treatment for thromboembolic disorders is administration of heparin, which exerts its effects via indirect inhibition of thrombin by AT. It has previously been shown that treatment with heparin, in combination with soluble complement receptor 1 (sCR1), can reduce leukocyte infiltration into islets 83. However, heparin alone, even in high doses that are not clinically applicable, is unable to affect complement activation and leukocyte infiltration. In particular, despite combined treatment with sCR1 and heparin in high concentration, extensive platelet accumulation and fibrin formation around the islets are observed. This result could be attributed to the poor ability of heparin to penetrate into growing thrombi 100. Also, heparin is limited in terms of its efficacy and safety because of its narrow therapeutic window. In light of these shortcomings, efforts have been made to develop direct thrombin inhibitors.

Melagatran is a low molecular weight inhibitor of thrombin that exhibits several of the characteristics of an “ideal” anticoagulant agent 101-105. It is highly selective for thrombin, with no cross-interactions with other enzymes. Because of its small size (430 Da) and high affinity for its substrate, melagatran has the ability to rapidly inhibit thrombin within the developing clot 106. It has been extensively used in clinical studies and has been shown to have a wide therapeutic window, with minimal bleeding complications 104,106.

The results from Paper I clearly demonstrate the benefit of administering melagatran in the in vitro loop model system prior to islet perfusion, in terms of blocking the IBMIR. In addition to its inhibitory effect on platelet activation and inhibition of both coagulation and complement, immunohistochemical staining showed a marked decrease in CD11b+ leukocyte infiltration with the highest dose of melagatran (10 µM) (Fig 11).

However, to block coagulation and complement activation, lower concentrations were sufficient. Despite the inhibition of complement, an absolute blockade of infiltrating immune cells was not attained, suggesting that other mechanisms are involved in the infiltration associated with the IBMIR (as discussed in Paper IV). Also, even though no clot was observed after 60 min, not all platelets were prevented from adhering to the islet surface in the presence of the highest dose of melagatran (10 µM).
The effect of these remaining platelets is, however, not necessarily a disadvantage. Animal studies have shown that after transplantation, at least one week elapses before revascularization of the islets is completed\textsuperscript{10}. Platelets contain a number of important growth factors, such as PDGF, VEGF and FGF\textsuperscript{107,108}, that may support revascularization and islet engraftment in the liver. Hence, a complete blockade of the IBMIR is not necessarily the optimal strategy for intraportal islet transplantation; instead, a fine-tuned balance between successful islet engraftment and thrombosis might be preferred.

In contrast to heparin, melagatran had pronounced inhibitory effects on all the known components of the IBMIR, i.e., complement and coagulation activation as well as the adherence of platelets to the islet surface and the infiltration of leukocytes. Melagatran may therefore be a suitable candidate drug for use in the effort to develop optimal tolerance-based therapies for islet engraftment. One major advantage of melagatran is that it also can be administered orally as a prodrug\textsuperscript{106}, Ximelagatran.

Inhibition of TF initiation (Papers II-III)

\textbf{iFVIIa (Paper II)}

Since the findings in Paper II implicate TF as the trigger of the IBMIR, it appears to be a suitable target for preventing this inflammatory reaction in clinical islet transplantation. \textit{In vivo}, TF is crucial for the initiation of the coagulation cascade and thrombus formation\textsuperscript{109-111}. It acts as a co-factor for FVII/FVIIa\textsuperscript{109,111} present in blood and elicits the cascade reaction by activating FX (FXa) and FIX (FIXa), resulting in the generation of thrombin and fibrin formation at the site of a vascular lesion.

iFVIIa has been developed as an inhibitor for TF-initiated coagulation and is a modified form of the FVIIa normally present in blood. Since the active site has been irreversibly inactivated, iFVII is unable to trigger TF/FVIIa-
induced thrombus formation. A comparably higher affinity for TF (compare
to native FVIIa) has made iFVIIa a candidate drug in TF-induced
thrombosis. iFVIIa has been used extensively in animal models and does not
induce surgical bleeding, neither when applied locally at high concentra-
tions nor when given intravenously in large doses.

From phase I and II trials it has been reported that the administration of
iFVIIa does not affect safety in terms of bleeding complications in the sub-
jects.

In Paper II we used iFVIIa to test the inhibitory effects on the IBMIR in
experiments using the *in vitro* blood loop model system. Islets pre-treated
with iFVIIa did not trigger the IBMIR in this *in vitro* system. Perfusion of
islets with this inhibitor completely blocked the drop in platelet count and
the generation of TAT, FXIa-AT and C3a. These results indicate that iFVIIa
is also a potentially suitable drug for the stated purposes and offers another
approach to preventing the IBMIR after clinical islet transplantation.

**Nicotinamide (Paper III)**

Strategies to remove or prevent the synthesis of TF in islets in culture, prior
to the transplantation, would offer a valuable complement to systemic anti-
coagulant treatments, which have obvious limitations due to the potentially
adverse effects on hemostasis in the recipient. In Paper III, we tested nicoti-
namide, a B vitamin known to have protective effects on islets in culture.

In the presence of nicotinamide, pancreatic β-cells exhibit increased resis-
tance to toxic chemicals, inflammatory macrophages and their products.
It has also been shown that this compound prevents or delays disease
development in the spontaneously non-obese diabetic (NOD) mouse, a
model of human type 1 diabetes.

Nicotinamide is believed to interfere with a mechanism involving NF-κβ,
the transcription factor for proinflammatory cytokines as well as for TF and
MCP-1.

In this study we evaluated the potential down-regulating effects of nicotina-
mide on TF and MCP-1 in isolated islets. Freshly isolated islets were cul-
tured in various concentrations of nicotinamide, and the levels of both TF
did not affect safety in terms of bleeding complications in the sub-
jects. When used in experiments using the *in vitro* blood loop model system.
Islets pre-treated with iFVIIa did not trigger the IBMIR. Perfusion of
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tured in various concentrations of nicotinamide, and the levels of both TF
did not affect safety in terms of bleeding complications in the presence of
this antioxidant. With the highest dose of nicotinamide used (50 mM), the base-
line levels (i.e., islets not exposed to nicotinamide) of TF were reduced by
85 %.

To test whether this down-regulation of TF had any effect on the islets’ in-
herent pro-coagulative state, islets that were cultured in the presence of
nicotinamide were tested in the *in vitro* loop model system. Their ability to
induce the IBMIR, when exposed to fresh ABO-identical human blood, was
assessed in terms of TAT generation. The level of TF expression showed a
strong correlation with the relative potency of the islets in initiating IBMIR.
Figure 12. The islets potency in triggering the IBMIR relates to their expression of TF.
Islets were pre-treated with various concentrations of nicotinamide prior to perifusion in the blood loop system. The TF content of the islets was assessed and correlated to their potency in initiating the IBMIR, measured as TAT generation in the blood.

in this blood system ($r^2=0.94$; Fig 12).

Islets cultured in the presence of higher doses of nicotinamide lost the capacity to trigger the coagulation system.

The culture conditions described here, based on the islet protective properties of nicotinamide, may be an effective way of reducing the adverse effects of the IBMIR in clinical islet transplantation.

Importantly, the effect of nicotinamide has implications not only for the procoagulative activity of islets but also for the inflammatory response, which is likely to be significant not only for the acute recruitment of immune cells but also for the activation of the specific immune response at later stages after transplantation.

This study also demonstrates the possibility of manipulating the islets prior to transplantation, thereby circumventing the effects of the IBMIR without using systemic anti-thrombotic or anti-inflammatory drugs.
CONCLUSIONS

- From *in vitro* data, the IBMIR was previously characterized by activation of coagulation and complement system as well as activation of the platelets. Consequently, islets were damaged by infiltrating leukocytes.

- We show that the IBMIR consistently occurs in patients receiving islets intraportally. This may explain the poor outcome reported for this procedure, including tissue loss and the need of islets from multiple donors to render patients normoglycemic.

- The underlying mechanisms are investigated to assess tools for intervention with the ultimate goal to improve the outcome of islet transplantation and reduce the number of islets needed for each recipient.

  Thrombin is shown to be a driving force in the IBMIR reaction, pointing to a platelet amplified reaction.

  TF produced and secreted by the endocrine cells of the pancreatic islets is the trigger of this innate response.

  The neutrophilic granulocytes are the main blood cells infiltrating the islets in the IBMIR. Also, monocytes are detected, although to a lesser extent, that could act as antigen presenting cells directing the specific immunoresponse in later phase of the rejection.

  Complement exert chemotactic activities for leukocytes. However, inhibition of complement, does not completely block cell infiltration, pointing to other processes being involved.

  Islets are “primed” to express various inflammatory mediators which could influence the composition of infiltrating cells. This expression is most probably induced by stimuli related to events prior to the death of the donor, the organ retrieval, ischemia during islet isolation and the culture of the islets.

- The consequences of the IBMIR points to the need of controlling this reaction. A complete blockade is not necessarily the ideal approach; in
stead a balanced IBMIR might be the best. Activation of blood components are known to support revascularization in other systems and might enhance engraftment also of the islets in the liver (Fig 13).

Figure 13. The consequences of the IBMIR. The IBMIR culminates in islet disruption by infiltrating leukocytes. Antigen presentation is enhanced and directs the players of the specific immune response (involving both allo-rejection and autoimmuno-rejection) to the inflammation site. If balanced, the IBMIR is not necessarily a disadvantage. With a restricted IBMIR, in which platelets are allowed to adhere to the islet surface, engraftment might be improved. The stickiness of the platelets in turn may enable islets to adhere to the vessel wall and induce revascularization.

- Several strategies for interference targeting different levels of the IBMIR are discussed and may be suitable:
  - Blocking thrombin, a driving force in the IBMIR:
    - with systemic administration of melagatran
  - Blocking of the initiator, TF:
    - with systemic inhibition using iFVIIa
  - Down regulation of TF:
    - pre-treating islets in culture with nicotinamide prior to transplantation
  - Adjusting the immunosuppressive protocols to target the infiltration seen in the IBMIR.

A working hypothesis for the IBMIR

Based on the observations presented in this thesis, a working hypothesis for the IBMIR has been constructed (Fig 14). TF expressed and synthesized by the islets triggers the clotting cascade system in the IBMIR. In vivo, only minute amounts of TF are needed to generate thrombin. Thrombin is one of the most potent activators of platelets. It triggers shape changes and mobilizes the adhesion molecule P-selectin to the
surface, rendering the platelets sticky. The ligand(s) to which the platelets bind on the islet surface is (are) still unidentified, but collagen, which surrounds human islets, is one likely candidate. The platelet receptor for collagen is αIIβ1 integrin and is also upregulated upon platelet activation. The adhering activated platelets provide a suitable negatively charged surface on which coagulation factors can assemble on and move the reaction forward. Thrombin generation is accelerated by the “amplification loop” acting in a TF-independent manner. In addition to activating more platelets, thrombin converts circulating fibrinogen to fibrin surrounding the islets, further stabilizing the clot with aggregated platelets entrapping the islets. Secondary to coagulation activation, the complement system is activated. Split products from the complement system, C5a and C3a, are known to exert chemotactic effects on leukocytes, probably affecting the morphological damage to the islets. The IBMIR culminates in clot-bound islets being disrupted by infiltrating neutrophilic granulocytes and monocytes.

Figure 14. A working hypothesis of the IBMIR. A. Islets express TF, MCP-1 and IL-8. When injected into the portal vein they come in direct contact with the different blood components, such as coagulation and complement proteins, platelets and leukocytes. B. TF-bearing islets trigger the coagulation system and generate enough thrombin, to activate the platelets. Islets are known to express different collagen structures to which the platelets may adhere. Activated platelets provide an appropriate surface for the second burst in thrombin generation. MCP-1, synthesized by the islets, attracts monocytes and IL-8 present in islets might influence the recruitment of neutrophilic granulocytes. C. Concomitantly to the clotting reaction the complement system is activated. In addition, complement split products, C5a and C3a, act chemotactically on neutrophilic granulocytes and monocytes. Other chemotactic factors (IL-8 and IL-1β) for leukocytes are also released from the α-granule of activated platelets. Thus, the IBMIR as well as the islets themselves influence the composition of infiltrating leukocytes. D. The IBMIR results in a formed fibrin clot encapsulating the islets with infiltrating leukocytes. Within an hour islet morphology is disrupted.
Other processes influencing infiltration of the islets are probably also involved, since complete abrogation of complement does not fully block infiltration. One such mechanism could be exerted by the islets themselves. They have been shown to be “primed” by expressing inflammatory proteins such as MCP-1 and IL-8 that attract monocytes and neutrophilic granulocytes to the islets.

In addition to the direct damage caused by the IBMIR, the ongoing inflammation also enhances antigen presentation, evoking a specific immune response involving B and T cells.
FUTURE PERSPECTIVES

In Papers I and II we used melagatran and iFVIIa, which had pronounced inhibitory effects on the IBMIR when tested in the in vitro loop model. However, it has not yet been demonstrated that these drugs are effective in limiting the IBMIR in clinical islet transplantation. Both iFVIIa and melagatran have been extensively used with success in clinical trials in patients suffering from deep vein thrombosis. For the purpose of islet transplantation, these components must be evaluated in relevant non-human primate models before they can be used in clinical trials. In our department, the IBMIR has been established in a small animal model that could be used for preliminary testing before proceeding to experiments in larger animals. The advantage of using melagatran is that it can be administered orally as a prodrug (Ximelagatran). Further studies have yet to be performed to clarify what concentrations are appropriate, so that it can be safely used in patients undergoing an islet transplant. Other candidate drugs targeting coagulation include TFPI and Arixtra, a novel compound that targets FXa. Both these drugs have been extensively tested in other hyper-coagulative states. Still, systemic administration of anticoagulants will always be associated with an increased risk of bleeding, a consideration of particular importance in islet transplantation, since the procedure involves puncture of a large vessel. From this perspective, pre-treatment of the islets in culture prior to transplantation would offer a far safer alternative that would also potentially allow for reduced, and therefore better tolerated, doses of systemic anticoagulants. In Paper III we added nicotinamide to the culture medium. Nicotinamide is believed to act through inhibition of NF-κB activity, a transcription factor known to regulate the expression of many pro-inflammatory mediators. We are currently also investigating the possibility of using anti-sense RNA for NF-κB, since a variety of inflammatory mediators that are dependent on NF-κB are believed to be induced in the islets by stress-related incidents (e.g., in the donor in the intensive care unit, by hypoxia during organ retrieval, by the islet isolation procedure and during the time in culture). Other strategies to intervene early in these processes are of course of interest. Oxygenation of the organ during transportation by the perfluorocarbon (PFC)-based two-layer method is one reasonable alternative. Transportation of the pancreas in PFC has been shown to result in greater islet yields. Efforts to shorten the isolation procedure may help to down-regulate the stress-induced expression of inflammatory mediators by the islets. Nicoti-
Namide has been shown to have an inhibitory effect on TF and MCP-1 in culture; however, it remains unclear whether this compound affects islets during isolation. Clotting time analysis using the in vitro systems presented in this thesis, i.e., the loop model system and the ReoRox system will be very valuable for choosing among the various novel candidate drugs and different culture conditions.

In Paper IV we described the natural course of the immune cell infiltration into islets after contact with blood in vitro. Since the use of melagatran resulted in complete blockade of both coagulation and complement activation, but still had only a partial effect on leukocyte consumption, other mechanism(s) are likely to be involved in the infiltration of neutrophils and monocytes into the islets. To investigate the events underlying this infiltration, a variety of immunosuppressive protocols targeting all levels of the IBMIR will need to be evaluated in the in vitro tube system. Targets of this intervention may include C5a, a split product of complement activation known to induce leukocyte chemotaxis and expression of adhesion molecules and integrins such as P-selectin, ICAM and VCAM.

The fact that the IBMIR occurs in patients receiving an islet transplant via the portal vein raises the question of whether the same reaction would be triggered during other potential future cell therapies such as hepatocyte transplantation.

As has been reported on rare occasions after islet transplantations, the most severe complication after intraportal islet transplantation has been portal vein thrombosis resulting in severe hypertension and, in some cases, liver failure. Also, infusion of larger amounts of hepatocytes has been reported to result in poor engraftment in the liver.

Whether mechanisms like those that govern the IBMIR after islet transplantation are also operative after the infusion of other types of cells remains to be confirmed. However, initial experiments in which hepatocytes were added to the blood loop system also resulted in blood clot formation, following the same dynamics as seen for islets exposed to blood (not published data).

The association between cancer and thrombosis is well established; however, the exact mechanisms of activation in different kinds of tumors are still unknown. Also, formation of blood-borne metastases is poorly understood and would be interesting to explore further. Intraportal injection in rats has often been used as a model for studying liver metastases. It has been demonstrated that only 0.68% of the infused cell mass forms the liver metastasis. In a study by Graf et al., a critical number of cancer cells had to be injected to form a metastasis (5 x 10⁶), whereas 0.5 x 10⁶ cells failed to metastasize. Injection of 10⁷ cells caused occlusions. Many tumor cells are known to express TF; therefore, determining the role of TF in the activation of coagulation and spreading could be of wide-ranging interest.
To achieve the best results in preventing the IBMIR, combination therapies will have to be developed, since none of the approaches mentioned above are likely to single-handedly produce an ideal effect on all levels of the IBMIR. Also, completely blocking the activities of the IBMIR may not be the ideal strategy for obtaining optimal engraftment. Activated platelets may support the revascularization of the islets, since their granules contain multiple mediators affecting angiogenesis and also activate endothelial cells in close proximity. Therefore, a balanced protocol, in which the harmful effects of the IBMIR are limited but allowance is made for beneficial platelet deposition on the islet surface, may prove to be the ideal strategy for optimizing graft survival and improving the outcome of future clinical islet transplantations.
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