Thromboinflammation

in a Model of Hepatocyte Transplantation

ELISABET GUSTAFSON
Abstract


Hepatocyte transplantation is an attractive method for the treatment of metabolic liver disease and acute liver failure. The clinical application of this method has been hampered by a large initial loss of transplanted cells.

This thesis has identified and characterized an instant blood-mediated inflammatory reaction (IBMIR), which is a thromboinflammatory response from the innate immunity that may partly explain the observed loss of cells. In vitro perifusion experiments were performed and established that hepatocytes in contact with blood activate the complement and coagulation systems and induce clot formation in conjunction with the recruitment of neutrophils. Within an hour, the hepatocytes were surrounded by platelets and entrapped in a clot infiltrated by neutrophils. Furthermore, hepatocytes expressed tissue factor (TF), and the reactions were shown to be initiated through the TF pathway. Monitoring of hepatocyte transplantation in vivo revealed activation of the same parameters as were noted in vitro.

For the first time, von Willebrand factor (vWF) was identified on the hepatocyte surface, being demonstrated by flow cytometry and confocal microscopy. mRNA for vWF was also confirmed in hepatocytes. Complex formation between platelets and hepatocytes was also identified. Addition of antibodies targeting the binding site for vWF on the platelets reduced the complex formation.

Two different strategies, systemic and local intervention, were applied to diminish the thromboinflammation elicited from the hepatocytes in contact with ABO-matched blood. Systemic inhibition with LMW-DS, in a clinically applicable dose, was found to be superior in controlling the IBMIR in vitro when compared to heparin. Cryopreserved hepatocytes elicited the IBMIR to the same extent as did fresh hepatocytes, and the IBMIR was equally well controlled with LMW-DS in both cryopreserved and fresh cells.

Hepatocytes were coated with two layers of immobilized heparin in an attempt to protect the cells from the IBMIR. In vitro perifusion experiments showed heparinized hepatocytes triggered a significantly lower degree of IBMIR.

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Gutta cavat lapidem

To my ♥ family
List of Papers

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


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Abbreviations

ADP  Adenosine diphosphate
ALG  Antilymphocyte globulin
APCs  Antigen-presenting cells
AT  Antithrombin
C1-INH  Complement factor C1-inhibitor
CD  Clusters of definition
CHC  Corline heparin conjugate
CRIg  Complement Receptor of the Immunoglobulin superfamily,
CTI  Corn trypsin inhibitor
DAMPs  Damage-associated molecular patterns
DC  Dendritic cell
EDTA  Ethylenediaminetetraacetic acid
EGTA  Ethylene glycol tetraacetic acid
ELISA  Enzyme linked immunosorbent assay
FXIa-AT  Factor Xla-antithrombin complexes
FXIIa-AT  Factor XIIa-antithrombin complexes
GP  Glycoprotein
HBSS  Hank’s balanced salt solution
HcTx  Hepatocyte transplantation
HGF  Hepatocyte growth factor
HBP  Heparin binding peptide
IBMIR  Instant blood-mediated inflammatory reaction
iFVIIa  Inactivated recombinant factor VII
IL  Interleukin
KC  Kupffer cell
LMW-DS  Low molecular weight dextran sulfate
LSECs  Liver sinusoid endothelial cells
MBL  Mannose-binding lectin
mAb  Monoclonal antibody
MASP  Mannan-binding lectin-associated serine protease
MHC  Major histocompatibility complex
MPS  Mononuclear phagocyte system
MO  Monocytes
NK  Natural killer cells
NKT  NATURAL KILLER T CELLS
OLT  ORTHOTOPIC LIVER TRANSPLANTATION
OTC  ORNITHINE TRANSCARBAMYLASE
PAI-1  PLASMINOGEN-ACTIVATOR INHIBITOR 1
PAMPS  PATHOGEN-ASSOCIATED MOLECULAR PATTERNS
PAR  PROTEASE-ACTIVATED RECEPTOR
PCR  POLYMERASE CHAIN REACTION
PDGF  PLATELET-DERIVED GROWTH FACTOR
PEG  POLYETHYLENE GLYCOL
PLT  PLATELETS
PMNs  POLYMORPHONUCLEAR LEUKOCYTES
PRP  PLATELET-RICH PLASMA
PPP  PLATELET-POOR PLASMA
PRRs  PATTERN RECOGNITION RECEPTORS
PVC  POLYVINYL CHLORIDE
rFVIIa  RECOMBINANT ACTIVE FVII
RT  ROOM TEMPERATURE
sC5b-9  TERMINAL COMPLEMENT COMPLEX (TCC)
SEM  STANDARD ERROR OF THE MEAN
TAFI  THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR
TAT  THROMBIN-ANTITHROMBIN COMPLEXES
TBP  TATA-BINDING PROTEIN
TFPI  TISSUE FACTOR PATHWAY INHIBITOR
TCC  TERMINAL COMPLEMENT COMPLEX (sC5b-9)
TF  TISSUE FACTOR
t-PA  TISSUE-TYPE PLASMINOGEN ACTIVATOR
TRAP  THROMBIN RECEPTOR ACTIVATING PEPTIDE-6
TxA2  THROMBOXAN A2
u-PA  UROKINASE-TYPE PLASMINOGEN ACTIVATOR
UW solution  UNIVERSITY OF WISCONSIN SOLUTION
vWF  VON WILLEBRAND FACTOR
WCC  WHITE CELL COUNT
WME  WILLIAM’S MEDIUM E
Introduction

The liver
Liver function is essential for human life, and more than 500 different and complex processes in the body take place in this organ [1]. In the liver, vital proteins are synthesized, endo- and exogen products are detoxified, nutrients are metabolized and converted to functional energy, and bile is produced. The liver is the largest exocrine, endocrine, and paracrine gland in the body. It weighs ~1.5 kg and consists of ~4 x 10^9 cells/kg, which in an adult corresponds to ~2.8 x 10^11 cells. About 80% of the mass of the liver consists of hepatocytes, which constitute 60% of the total number of cells [2]. These are large parenchymal, highly differentiated, epithelial cells of 15-40 µm that act like small efficient factories capable of carrying out all these essential processes. Hepatocytes origin from the hepatoblasts, which are also the precursors of the cholangiocytes. The cholangiocytes are a numerically smaller subset of cells that line the three-dimensional bile tree [3].

The unique microanatomy of the liver is a prerequisite for its extensive function [4]. The hepatocytes are polygonal and arranged in one cell-thick rows/layers in small repetitive units called lobules, which are hexagonal in shape and approximately 1 mm in diameter. This structure maximizes the surface area having contact with the sinusoidal blood on all four sides of the hepatocytes and facilitates the exchange of substances between the hepatocytes and the blood. In the intercellular lateral surfaces between the hepatocytes, the bile canaliculus is formed for further secretion of bile. This polarity is maintained by tight junctions between adjacent hepatocytes. Bile canaliculi further merge into progressively larger bile ducts lined by cholangiocytes [2].

Other non-parenchymal cells in the liver are the Kupffer cells (KCs), stellate cells, and liver sinusoidal endothelial cells (LSECs). The sinusoids also contain all the usual types of blood-borne cells.

KCs are resident macrophages and constitute 15% of the total liver cell population; they are the largest population of macrophages in the body and are located in the sinusoidal vasculature. Stellate cells are located in the space of Disse and are normally quiescent; their main role is to store fat and vitamin A, along with controlling the amount of extracellular matrix.

The blood supply to the liver comes from two sources: two-thirds of it from the portal vein and one-third from the hepatic artery. The supply consists of one to two litres of blood [5] derived from the gastrointestinal tract that is
enriched with nutrients but may also contain potentially harmful substances. The arterial blood supplying the liver mixes with the portal venous blood in a low-pressure capillary system called the sinusoids. This capillary system is unique in many ways in addition to containing a mixture of venous and arterial blood. This is the only place in the body where collected venous blood is forwarded to a secondary capillary system. Also, the LSECs are fenestrated (150-175 nm in diameter) and lack a basement membrane. LSECs may therefore act as a dynamic filter and regulate the exchange of substances and fluids between the blood and the parenchymal hepatocytes [6]. Only a narrow space of Disse separates the endothelial cells from the parenchymal liver cells. Moreover, in the hepatic lobules, the blood flows from the periportal area toward the central vein, which gives rise to a zonal heterogeneity in hepatocyte function as a result of the gradient in oxygen tension, load of nutritional substrates and hormones, and the increase in CO₂ tension and metabolites along this axis. Because of this heterogeneity, hepatocytes can roughly be divided into three zones: 1) periporal, 2) transitional, and 3) perivenous [7]. Hence, the periportal zone 1 hepatocytes are preferentially involved in oxidative processes and protein synthesis, and the zone 3 perivenous hepatocytes are more involved in glycolysis and xenobiotic metabolism [8]. This zonal heterogeneity also leads to a zonal susceptibility to toxic substances and ischemia [5].

This structure as a whole provides the optimal conditions for the exchange of substances between the blood and the liver cells.

In addition, the liver also has an exceptional capacity to regenerate following partial hepatectomy. Hepatocyte growth factor (HGF) is initially increased after partial hepatectomy, followed by other growth factors. The liver regenerative process is strictly controlled and stops when the appropriate mass and function for the body size are restored [2].

Liver disease

There are more than hundred different liver-related diseases, which can be divided into acute or chronic conditions. Liver diseases can be initiated through one of a number of different mechanisms. Specifically, the most common causes of liver disease are alcohol, obesity and viral hepatitis [9].

Untreated, liver diseases generally develop slowly over a long time and proceed through different stages, beginning with inflammation. The healing process causes fibrosis, mainly through the activation of stellate cells. The fibrotic tissue progressively replaces the liver tissue and may further lead to cirrhosis, which is the common final pathway of most chronic liver diseases [5]. In the cirrhotic liver, blood flow is impaired and through shunting, many of the hepatocytes are shut down. The number of remaining functioning hepatocytes is diminished, and all of the processes taking place in the liver decline. The progression of chronic liver disease will eventually also lead to
large extrahepatic portosystemic shunts, which most often predict the final decompensation of the disease [2].

Thanks to the innate overcapacity of liver function, symptoms such as a loss of appetite, loss of weight, jaundice, ascites, fatigue, and bruising of liver failure usually arise only in the later stages of disease when a substantial percentage of the liver cells have been lost. Finally, fulminant liver failure, a life-threatening condition, can occur. Liver failure is defined as reduced hepatocellular function of a clinical grade that is not compatible with prolonged life.

In rare cases, liver failure may also occur with an acute onset in an otherwise healthy person, generally without obvious etiology [10]. It can also occur as a result of drug exposure, viral infection, an ischemic insult, or anything that damages a significant portion of the hepatocytes (>90%). The most common cause in Sweden is an overdosage of paracetamol. Acute fulminant liver failure is an emergency condition that generally leads to rapid development of severe mental disturbances and coagulopathy and, if untreated, is associated with very high mortality. These patients often become candidates for liver transplantation [10].

The most common diseases affecting the bile ducts are biliary atresia in neonates and primary biliary cirrhosis in adults, in which progressive inflammation destroys the bile ducts, leading to cholestasis and further liver failure.

There is also a subgroup of conditions called in-born errors of metabolism. Individuals with this form of liver disease may have only a single enzyme deficiency, resulting from a genetic defect, that produces a blockage in a particular synthetic or metabolic pathway and leads to the accumulation of toxic substances or defective energy production [2]. There are many different types of these deficiencies, which generally cause rare, congenital liver diseases. These diseases are mainly diagnosed in the neonatal period, but they may also initially appear later in life.

**Liver transplantation**

Today, liver transplantation is the gold-standard method for managing life-threatening liver diseases. The first liver transplantation was performed 1963 by Dr. Thomas Starzl in the USA. Three transplantations were reported, and the longest-living patient survived for 22 days [11]. In 1967, the first patient reached a 1-year survival and had received immunosuppression with azathioprine, prednisolone, and antilymphocyte globulin (ALG) [12]. The results of transplantation improved slowly, and 1-year survival was ~30% in 1969 [13]. Most recipients died from rejection or infection [14]. Over time, the results have improved dramatically with the advent of improved immunosuppressive regimens. The first liver transplantation in Sweden was performed by Dr. C.G. Groth in 1984 [15]. To date, more than 3000 transplantations have been done in this country, and annually, about 160 liver transplantations are performed.
in Sweden [16]. Today, over 90% 1-year survival and 88% 3-year survival can be reached, according to the European Transplant Registry [17]. The most common indications for liver transplantation in Sweden are primary biliary cirrhosis in adults and biliary atresia in children under 5 years of age [18]. Because of the shortage of available organs for transplantation, other methods have been developed to address these indications. A split liver transplantation, in which one organ is transplanted into two patients, was first performed in 1989 [19]. The first successful living-donor procedure was also performed in 1989, with a graft from a mother to her 17-month-old child with biliary atresia [20] and the first similar procedure in Sweden was performed in 1996 [18]. Today, both of these methods are well established, and annually, about five living donor liver transplantations are done in Sweden [16].

Liver cell transplantation

The concept of cell transplantation is far from new. Bone marrow/hematopoietic stem cell transplantations have been used for decades and are established treatments [21]. However, since liver transplantation is a major, irreversible surgical procedure, hepatocyte transplantation emerged as a theoretically attractive concept only during the 1970s. An enzymatic and gentle method for the isolation of hepatocytes was developed by Berry and Friend in 1969 [22], and in 1977 the first experimental hepatocyte transplantation (HcTx) with enzymatically isolated cells infused into the portal vein was performed by C.G. Groth [23]. Since then, several experimental models have proved capable of conferring liver function through transplanted cells [23, 24]. Principally, there are three different types of liver failure for which HcTx has been explored: metabolic liver disease and acute and chronic liver failure. Metabolic liver disease has been regarded as the ideal indication for HcTx [25]. Because it involves a single missing enzyme, a low degree of effect is needed from the transplanted cells, estimated at 5-15%, meaning that the whole liver does not need to be replaced [25]. Chronic liver disease with fibroses is the least suitable for HcTx due to disrupted architecture of the liver parenchyma.

HcTx has been applied clinically in small series of patients and isolated selected cases of all three types of liver failure. The first human cases were reported in 1992 [26], and the procedure has since been shown to be safe and easy [27]. However, after a review of the first 100 clinical transplantations reported to date, it was concluded that no patient had been fully cured with HcTx [28]. One of the main reasons for the lack of sufficient effect is a reported low degree of engraftment [27, 29-31]. The reason for this is loss of transplanted cells due to responses from both the innate and adaptive immune systems [32, 33]. The extensive early destruction of transplanted hepatocytes has been addressed as one obstacle (among others) to be overcome in human HcTx in order to achieve a useful clinical application of this method [31].
Immunosuppression

In the early era of liver transplantation, azathioprine, prednisolone, and ALG were the drugs of choice (with only limited adjustments), and they remained so for almost 20 years. In the early 1980s, the calcineurin inhibitor cyclosporin A [34] became available and was soon adopted for liver transplantation in combination with low-dose prednisolone. Calcineurin inhibition prevents interleukin (IL)-2 production in activated T cells and hence inhibits T-cell proliferation, but it also exerts an effect on B- and natural killer (NK)-cell functions. The second-generation calcineurin inhibitor tacrolimus (introduced in 1990) has produced superior results when compared to cyclosporine and is now preferred in most immunosuppression protocols [35]. Recently, it was reported that prolonged-release tacrolimus could further improve the survival rates [17].

Today, all immunosuppressive treatment is individually customized but is mainly built on the same concept, induction therapy with steroids and maintenance treatment with a calcineurin inhibitor. Adjustments are made according to the patient’s underlying disease, with the goal of minimizing immunosuppression [35].

Centres performing clinical HcTx have usually applied the same immunosuppressive regime as for orthotopic liver transplantation (OLT) [25]. Since there are no reliable markers for graft rejection in HcTx, tailoring the immunosuppressive level is difficult [25].

Innate immunity

Innate immunity is the first line of defense in response to danger and is alerted within seconds, with the aim of eliminating the threat. Innate immunity is a constant, and mostly unnoticed, ongoing process. However, sometimes all of the body’s defense mechanisms are alerted, leading to the final step: inflammation.

The innate immune system is not antigen-specific but instead detects missing “self” and structures that are “unfamiliar” to the host. It is a phylogenetically old defense system and exists to some degree in all multicellular organisms. It includes both a cellular portion and a humoral portion [36].

The cells of the system, phagocytes, dendritic cells, endothelial cells, and NK cells, express different repertoires of pattern recognition receptors (PRRs). These receptors recognize intra- or extracellular “unfamiliar structures” called pathogen-associated molecular patterns (PAMPs) [37] and damage-associated molecular patterns (DAMPs) [38]. In response to stimuli, PRRs trigger a complex course of action. Depending on which specific cell type and receptor has recognized the PAMP/DAMP, a specific pathway is activated in the cell, usually mediated by NF-κB, with the transcription of genes
encoding proteins involved in the effector functions. These proteins are mainly proinflammatory cytokines and chemokines, which further mediate and amplify the inflammation, for example, through the local recruitment of leukocytes and plasma proteins [36].

The humoral arm of the innate immune system includes soluble proteins such as cytokines, soluble recognition molecules, acute-phase proteins, natural antibodies, and the complement system. The soluble recognition molecules recognize extracellular soluble PAMPs/DAMPs. Included here are also the molecules that can activate the complement system: pentraxins, collectins, and ficolins. The complement system is composed of numerous proteins that work as a proteolytic cascade and can be activated through three pathways that all converge in the formation of two C3 convertases [39]. Activation of the complement system further leads to three major effector functions: opsonization of microbes, lysis of microbes, and enhanced inflammation (through the production of the anaphylatoxins C3a and C5a). The complement system also influences the adaptive immune responses.

The liver and immunology

The liver is heavily loaded with immune cells, particularly those associated with the innate immune system, and in healthy individuals, these cells maintain a balance between tolerance and immune activation. Most acute-phase proteins and complement factors are also synthesized in the liver. Generally, the hepatic microcirculation is regarded as a tolerogenic environment, with only a restricted activation of adaptive immunity despite a huge inflow of blood-borne, potentially immunogenic elements from the gastrointestinal tract [40]. This tolerogenic environment is made possible by a highly efficient and tightly regulated local mononuclear phagocyte system (the MPS), with a unique capacity to clear most potential pathogenic elements while inducing tolerance [41]. For example, endotoxin levels are 100-fold higher in portal blood than in peripheral venous blood [42]. The MPS includes three cell types: KCs, dendritic cells (DCs), and monocytes. Most of the cells are KCs and DCs that exhibit a liver-specific tolerogenic profile.

KCs are located in the vasculature, tightly attached to the sinusoidal wall, primarily in the periportal areas. KCs express various PRRs: scavenger receptors, Toll-like receptors, CR1gl receptors binding C3-opsonized pathogens, and Fc receptors for IgA [43-45]. Under normal conditions, KCs do not effectively act as antigen-presenting cells (APCs) to elicit T-cell activation; rather, they induce T-cell tolerance because of the lack of co-stimulating cytokines [46]. However, during inflammation and in the presence of the right combination of cytokines and co-stimulating factors, the KCs can switch to become efficient APCs and T-cell activators [46].
DCs in the liver consist of many subpopulations and may be located in the sinusoids, the space of Disse, and to a lesser extent the liver parenchyma. They generally have an immature phenotype and express a low level of major histocompatibility complex (MHC) class II antigens [47]. As is true for KCs, DCs can ingest antigens, but to a lesser extent, and they are less prone to migrate and act as efficient APCs under basal conditions than are DCs from other tissues [41, 44]. A factor contributing to this low level of APC activity is the local cytokine profile in the liver vasculature, which features high IL-10 levels (which are also produced by the DCs) [48], and low IL-12 levels [47, 49, 50].

LSECs express a variety of PRRs in addition to both MHC class I and II molecules. They can ingest cell debris and macromolecules up to ~1 mm and act as efficient scavenger cells [51]. As such, they may modify the immune response; however, they do not produce IL-12, which is one of the reasons that they are not efficient APCs [41].

In case of inflammation, the normally quiescent stellate cells in the space of Disse may differentiate to become efficient producers of collagen and contribute to the resulting liver fibrosis [52]. Hepatocytes are also reported to act as “non-professional” APCs when they come in contact with CD4+ T cells and may induce the expansion of regulatory T cells [53].

Even the lymphocyte population in the liver differs from that in the systemic circulation. About half of the lymphocytes in the liver carry T-cell receptors, and most of these cells are of the CD8+ type, as opposed to the normal condition in which CD4+ cells predominate. Furthermore, the T-cell receptors also have a lower level of expression than do those on lymphocytes in the blood, and they are more often of the γδ-type. About 30-50% of the lymphocytes are NK cells, which participate in innate immunity and can rapidly detect cells with missing MHC and kill them via the secretion of perforin and granzyme, in addition to substantial release of cytokines.

In addition, a large proportion of natural killer T cells (NK-T), ~20-30%, is found in the liver. These cells detect hostile cells in the same way that NK cells do, but they also display a regulatory anti-inflammatory repertoire [44].

Hemostasis

The body’s ability to achieve hemostasis is essential for life. Hemostasis is a complex process depending on platelets, vascular factors, and a variety of fine-tuned plasma protein systems that act in a coordinated manner to enable clot formation directed to a specific site, while preventing widespread coagulation. Furthermore, regulatory mechanisms counterbalance the clotting so that it comes to a halt when the bleeding is under control and a healing process can be initiated.
Hemostasis goes through different stages that to some extent take place simultaneously: vasoconstriction, platelet plug formation, coagulation, and fibrinolysis.

Platelets

Platelets are small, 2-4 µm, cell fragments containing many organelles but no nucleus. They are derived from the megakaryocytes in the bone-marrow and have a life-span of 8-12 days in circulation. Normal human platelet counts range from 150-300 x10^9/L blood [54]. In circulation, platelets roll close to the vessel wall when in the resting state [55]. The platelets are covered with several different receptors for activation, adhesion and aggregation [56], (Table 1).

Table 1. Major platelet receptors involved in hemostasis

<table>
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<th>Receptor</th>
<th>Ligand</th>
<th>Major function</th>
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<tr>
<td>PAR1</td>
<td>Thrombin</td>
<td>Activation</td>
</tr>
<tr>
<td>PAR4</td>
<td>Thrombin</td>
<td>Activation</td>
</tr>
<tr>
<td>GP1bα</td>
<td>A1 domain on vWF, Thrombin</td>
<td>Adhesion, Activation</td>
</tr>
<tr>
<td>GPIIb-IIIa</td>
<td>Fibrinogen, vWF</td>
<td>Aggregation</td>
</tr>
<tr>
<td>GPVI</td>
<td>Collagen</td>
<td>Adhesion, Activation</td>
</tr>
<tr>
<td>P2Y1</td>
<td>ADP</td>
<td>Activation, † GPIIb-IIIa, † Ca²⁺</td>
</tr>
<tr>
<td>P2Y12</td>
<td>ADP</td>
<td>Activation, † GPIIb-IIIa, † Ca²⁺</td>
</tr>
<tr>
<td>TP</td>
<td>TxA₂</td>
<td>Activation, † GPIIb-IIIa</td>
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In response to agonistic stimuli or high shear force, platelets are rapidly activated. Thrombin, collagen, and thromboxanA₂ (TxA₂) are strong platelet activators [56]. The activation causes a cytoskeletal rearrangement and alters the shape of the platelets, tremendously increasing their surface area and the release of dense granules. This is followed by secretion of α granules [55] (Table 2). The activated platelets then express P-selectin and CD40L on their surfaces and expose negatively charged phospholipids, which constitute an optimal surface for coagulation. P-selectin also attracts leukocytes to the area.

During the activation, the GPIIb-IIIa receptor undergoes conformational changes that increase the affinity of the receptor and enable binding to fibrinogen and von Willebrand factor (vWF) to enhance the formation of bridges between platelets, which is essential for platelet aggregation [56].
Table 2. Platelet granular content

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<th>Granula</th>
<th>Content</th>
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<tr>
<td>Dense granule</td>
<td>ADP, thromboxane A₂, serotonin</td>
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<tr>
<td>α−granule</td>
<td>β-thromboglobulin, PDGF, P-selectin, fibronectin, fibrinogen, thrombospondin</td>
</tr>
</tbody>
</table>

Primary hemostasis – formation of a platelet plug

Primary hemostasis consists of two initial steps: vasoconstriction and the formation of a platelet plug.

Local spasming of vascular smooth muscle cells occurs as an immediate response to an injury to a vessel in order to diminish blood loss. This spasmotic behaviour is mediated by neurogenic pain reflexes, endothelin released from the injured endothelial cells, and thromboxane A₂ and serotonin from dense granules released by platelets.

**Adhesion:** In the classical model of thrombus formation, subendothelial collagen is exposed in close association with vasoconstriction, and rolling platelets become anchored to the collagen by glycoprotein (GP) VI and by means of von Willebrand factor (vWF) secreted from the endothelial cells. vWF attracts and binds to collagen, enabling platelet adhesion by binding to the GP1bα receptor on the platelets. This binding, in turn, triggers the activation of the platelets, which leads to degranulation and a subsequent up-regulation and increase in affinity of the GPIIb-IIIa receptor. Firm adhesion also requires the involvement of other platelet receptors [57].

**Aggregation:** The secretions rapidly recruit more platelets to the clotting site, resulting in platelet aggregation. Platelets are initially kept together through the binding of vWF and GPIIb/IIIa/fibrinogen. However, these associations still do not form a solid plug until fibrin is formed. Furthermore, the plug formation wanes when intact endothelium is reached, because normal endothelium is covered with a glycocalyx that is studded with bound heparan sulfate molecules that act as anti-thrombotic agents [57].
Secondary hemostasis - coagulation

The secondary hemostasis consists of the coagulation process, resulting in generation of fibrin strands, which leads to the formation of a stable clot. Coagulation preferentially takes place on surfaces and is also dependent of calcium. The negatively charged platelet surface is ideal for coagulation, but the process may also be initiated on other cell surfaces expressing tissue factor (TF). In a cell-based model [58], coagulation can be described in three phases:

Initiation: TF exposed on cells not normally in contact with blood or activated platelets binds to factor VII, creating a surface-bound TF-FVIIa complex that is localized to the site of injury and elicits the “extrinsic pathway” of the coagulation cascade. The TF-FVIIa complex converts FIX and FX to their active forms, FIXα and FXα. FXα and FVα form a prothombinase complex that cleaves prothrombin to thrombin. FXα, when dissociated from the membranes, is rapidly inhibited. Thrombin catalyzes the conversion of fibrinogen to fibrin. However, the fibrin produced this way is not sufficient to stabilize the clot. Thrombin has a wide range of additional effects that catalyze the conversion of fibrinogen to fibrin. Most importantly, thrombin amplifies the coagulation cascade.

Amplification: The initial amount of thrombin forms an amplification loop via the enhanced adhesion and activation of more platelets. Thrombin further activates FV on the surfaces, as well as FIX and FXI. FVIII is also released from its carrier, vWF, activated, and further localized to the procoagulant surface. FVIII, FIX, and FXI are traditionally included in the “intrinsic pathway.”

Propagation: The activation of the intrinsic pathway, recruitment, activation and degranulation of platelets strongly contributes to the formation of large amounts of thrombin, which are the driving force in these reactions.[59]. This massive generation of thrombin leads to formation of fibrin strands cross-linked by activated FXIII that create a stable meshwork with the activated platelets at the site of injury.
Figure 1. Schematic overview of a cell-based model of coagulation. The coagulation is initiated on the TF-bearing cells and is amplified and propagated on the platelets.

Inhibition of coagulation

There are three major endogenous inhibitors of coagulation [60]. Antithrombin (AT) is the main serine protease inhibitor (serpin) and works through covalent binding to the target, whereupon the serine protease is irreversibly inactivated. In presence of a specific pentasaccharide sequence in heparin, the AT effect increases 500-fold. Other inhibitors are the tissue factor pathway inhibitor (TFPI) and protein C. TFPI is a reversible inhibitor of FXa and the TF/FVIIa complex and is particularly efficient in complex with FXa [60]. Activated protein C, with the co-factor protein S, cleaves and inactivates FVa and FVIIIa efficiently on endothelial cells, limiting the coagulation to the pro-coagulant surface.
Fibrinolysis

The clot that is formed is subsequently degraded by enzymatic lysis of the fibrin strands. Plasmin is the main fibrinolytic enzyme that cleaves fibrin, leading to the formation of degradation fragments that can be further cleared from the body. Plasmin is formed from plasminogen by two activators, tissue-type and urokinase-type plasminogen activator (t-PA and u-PA). The fibrinolytic system is tightly regulated by plasminogen activator inhibitor (PAI) and thrombin-activatable fibrinolysis inhibitor (TAFI) [61].

Crosstalk between coagulation and inflammation

Conditions that signal danger, such as infection, tissue damage, or bleeding, can trigger both coagulation and complement activation simultaneously [62]. These cascade systems are constructed similarly, with inactive zymogens that can be proteolytically cleaved to yield downstream-acting serine proteases. Several mutual interactions also occur between these systems. Complement can contribute to a local enhancement of coagulation through the action of C5a, which can induce TF expression on leukocytes [63] and endothelial cells [64] and an up-regulation of PAI-1 [62]. Mannan-binding lectin-associated serine protease (MASP) 2 converts the prothrombin to thrombin [65]. In the other direction, FIXa, FXa, FXIa, and thrombin can cleave and activate complement factors C3 and C5, as has been confirmed in vitro [66, 67]. The complement factor C1 inhibitor inhibits the classical and MBL pathways of complement and inactivates FXIa [68].

A major part of the crosstalk between coagulation and inflammation is mediated through cell activation. C3a activates platelets, and sC5b-9 complexes (TCCs) become incorporated into the platelet membranes, causing (in addition to activation) surface conformational changes and inducing the release of microparticles. Activated platelets release chondroitin sulfate, which in turn activates complement [69].
Hepatocytes in contact with blood

In an experimental model, it was observed that 80% of the transplanted liver cells were lost within 24-48 h [70]. An indication of the fate of the hepatocytes comes from the observation that within 2-3 hours after transplantation, the cells are already surrounded by neutrophils and KCs in the sinusoids [71, 72], and after 6 h, major pro-inflammatory genes are up-regulated [73]. Thus, the conclusion is that the recipient’s innate immunity clears a large number of the transplanted liver cells. Later, the engrafted hepatocytes are rejected as a result of both CD4+ and CD8+ T-cell responses, outlined by Bumgardner, that have not been curbed by ordinary immunosuppressive treatment [74].

This thesis is focuses solely on this issue and will address the initial phase when isolated hepatocytes are infused into the bloodstream and will explore the interactions that occur between the transplanted cells and the innate immune system.

The IBMIR

In islet transplantation, an instant blood-mediated inflammatory reaction (the IBMIR) has been identified, and this reaction provides an explanation for the early loss of transplanted islets that occurs during the transplantation procedure [75, 76].

The IBMIR is the result of an innate immune response that includes a rapid activation of the coagulation cascade through the TF pathway, with the generation of thrombin, which in turn further activates platelets and amplifies the coagulation cascade through the intrinsic pathway. Concomitantly, the complement system is activated, with accumulation of the anaphylotoxin C3a and recruitment of PMNs to the conglomerate of platelets and islets buried within the clot [75, 76]. This thesis focuses on the similar reactions elicited by hepatocytes in direct contact with ABO-matched blood. Many articles have been published with reports of observations in HcTx that relate to this field and have been reviewed by Lee et al [77].
Surface and cell-surface modification

Biomaterials and therapeutic cells introduced into the blood provoke various adverse incompatibility reactions from the innate immunity [78]. Modification of different biomaterials in direct contact with blood has been applied in various settings to increase hemocompatibility. Most widely used is the heparin coating of medical devices, for example in cardiovascular surgery and hemodialysis [79]. This approach, is an attempt to imitate the only truly blood-compatible surface, the vascular endothelial wall, whose glycocalyx contains an abundance of the heparin analog heparan sulfate (50-90% of the total amount of the proteoglycans in the glycocalyx) [80].

Modification of cell surfaces can also be performed in order to protect cells or establish new functions for the cell surface. Principally, three different methods are used to connect carriers/substances to the cell membrane: (1) interaction between the negatively charged cell surface and cationic polymers, (2) covalent conjunction of polymers to the amino groups of the membrane proteins, and (3) incorporation of lipid chains into the cell membrane as the result of a hydrophobic interaction in the solution [81]. Polyethylene glycol (PEG) is a commonly used carrier in this context and is regularly employed in both the second and third methods. Bioactive substances can be further immobilized on the cell surface by linkage to the bound PEG chain.

PEG chains are hydrophilic, non-toxic, inert synthetic polymers that are used in a great diversity of applications (including toothpastes and biopharmaceuticals) [82]. By conjugating a lipid to the PEG-chain, a polarized amphiphilic molecule is created that is particularly useful for surface modification of cells and liposomes when the lipids are incorporated into membranes and the hydrophilic PEG-chain is conjugated with the desired component.
Aims of the studies

General aims
The overall aim of this thesis was to study the early interactions between hepatocytes in blood and the innate immunity in order to identify the mechanisms involved in the early clearance of hepatocytes in the context of hepatocyte transplantation.

Specific aims were:
- To outline the thromboinflammatory/IBMIR reactions evoked by hepatocytes in blood.
- To explore the pro-coagulative phenotype of hepatocytes.
- To explore potential systemic and local strategies to prevent destruction of hepatocytes mediated by the IBMIR.

Paper I
To study the early interactions between hepatocytes and the blood and outline the basic mechanisms for the IBMIR.

Paper II
To explore the procoagulative phenotype of isolated hepatocytes and study the interactions between hepatocytes and platelets.

Paper III
To evaluate systemic inhibition of the IBMIR by use of low molecular weight dextran sulfate.

Paper IV
To study the possibility of surface modification of hepatocytes with heparin to protect cells from destruction by the IBMIR.
Study design and methods

Ethical considerations

All experimental procedures were carried out in compliance with Swedish law and regulations and approved by the regional Ethics Committee. Informed consent was obtained from each patient donating tissue.

Hepatocytes (Papers I-IV)

In papers I and II, freshly isolated hepatocytes were used. In some experiments (in paper II), these cells were further cryopreserved for comparison. In papers II and IV, commercially prepared hepatocytes were used.

Isolation and culture of human hepatocytes (Papers I and III)

The hepatocytes used were isolated from wedge biopsies taken from patients who had undergone liver surgery for secondary malignancy (mainly colorectal metastases). Samples were obtained from the non-tumour margin. Human hepatocytes were isolated by a three-step perfusion technique [83, 84]: in brief, the specimen was initially rinsed and transported in saline solution at 4°C. The cold ischemic time was less than 90 min. Two large veins were identified at the cut surface; smaller visible vessels were sutured to prevent leakage, and the Glisson’s capsule was restored with tissue glue. The perfusion was performed through the existing vasculature at 37°C. The first washing buffer consisted of Ca^{2+}- and Mg^{2+}-free Hank’s balanced salt solution (HBSS) with 0.5 mM ethylene glycol tetraacetic acid (EGTA) and 50 mM HEPES. The perfusion lasted for 20 min without recirculation, and a flow rate of 20 mL/min per cannula was used. A short interperfusion (2 min) was performed with HBSS to rinse the EGTA from the specimen. The perfusion was continued using HBSS containing 0.05% collagenase type IV and 5 mM CaCl₂. The perfusion was stopped when the liver sample was determined to be soft. After the perfusion was completed, the capsule was opened, and the hepatocytes were dissociated by gently chopping the tissue with a pair of scissors. This procedure was performed in the warmed collagenase solution. The cell suspension was then filtered through a 100-μm mesh into ice-cold William’s medium E (WME) and then purified by three centrifugations in ice-cold WME.
(5 min, 50xg, 4°C). The hepatocyte suspension was (when necessary) finally enriched through a Percoll gradient.

Freshly isolated hepatocytes were cultured on collagen-coated (rat-tail type 1 collagen) plates (1x10⁵ viable cells per cm²) in WME supplemented with 10% FBS, 50 µg/mL gentamicin, 25 IU/L insulin, 2 mM L-glutamine, 0.1 µM dexamethasone, penicillin G 100 U/mL and streptomycin 100 µg/mL.

Viability, plating, and functional analyses (Papers I-IV)

Viability was measured using the trypan blue exclusion test. The plating efficiency was calculated at 16 to 20 h after seeding, by determining the number of non-adherent cells in the culture medium. Plating efficiency was expressed as a percentage of the viable cells initially seeded. Functional assays were performed on intact cell monolayers after a change into serum-free culture medium. Samples were collected from the incubation medium at 24-h intervals, centrifuged (10 min, 1000g), and then frozen at -20°C until assayed. The amount of human albumin secreted into the culture was determined using the Aeroset ci8000 system (Abbott, Albumin Detection Kit, 7D54-20). Urea production was determined by incubating the hepatocytes in 10 mM NH₄Cl for 24 h. The urea in the supernatant was quantified using the Aeroset c8000 system from Abbott (Urea Detection Kit, 7D75-20). In experiments studying effects of LMW-DS on hepatocytes in culture, the generated urea was analyzed with the colorimetric Urea Assay Kit (MAK006, Sigma-Aldrich, St. Louis, MO, USA), and the albumin produced was quantified with the Albumin Human ELISA kit (ab108788, Abcam, Cambridge, UK). CYP3A4 function was analyzed by incubating cultures with 10 µM of midazolam, and samples from the supernatant were assayed at the given time points. The 1-hydroxymidazolam that was formed was detected with high-performance liquid chromatography.

Cryopreservation of hepatocytes (Paper III)

Hepatocytes (1.5 x 10⁷) were centrifuged at 50xg for 5 min at 4°C. The supernatant was removed, and ice-cold University of Wisconsin (UW) solution was added to the pellet to yield a total volume of 4.5 mL; the sample was further distributed into ice-cold cryo-vials. DMSO was added drop-wise to a volume of 5 mL in each vial, and the samples were cryopreserved using a computer-controlled rate freezer (Planer, Sunbury, UK) according to a freezing protocol described in [85, 86]. The cells were kept overnight in liquid nitrogen.

Thawing of cryopreserved cells (Papers II, III and IV)

The cells were rapidly thawed in a 37°C water bath while gentle agitating of the cryo-vial, and then further transferred to an ice-cold tube. Dilution of the
cryoprotectant was carried out immediately by drop-wise addition of WME according to Steinberg et al. [86, 87]. Cells were washed twice, dissolved in WME, and kept at 4°C until used.

Commercial hepatocyte preparations (Paper III)
Commercial hepatocytes were all cryopreserved, and batches were selected with care to include only those with documented high post-thaw viability. Equal numbers of female and male donors were used. The cells were stored at -190°C until use and thawed and handled in the same manner as locally isolated and cryopreserved cells.

Human blood, platelet-poor and -rich plasma (Papers I-IV)
All human blood was obtained from healthy volunteers who had received no medication for at least 10 days prior to the experiments. Blood was collected in an open system in which all surfaces that came into contact with blood were coated with immobilized corline heparin (CHC™, Corline, Uppsala, Sweden). Platelet-poor plasma (PPP) was obtained from lepirudin-anticoagulated blood (final concentration, 50µg/mL) that had been centrifuged twice at 3400×g for 15 min at room temperature (RT). Platelet-rich plasma (PRP) was prepared from anticoagulated (lepirudin 50 µg/mL) whole blood by centrifugation at 150×g for 15 min at RT [69].

Platelet handling and activation (Paper II)
Platelets in PRP were diluted to a physiological concentration (200 × 10^9/L) with autologous plasma and then protease-activated receptor (PAR)-1-activated by the addition of thrombin activating peptide-6 (TRAP; 25 µg/mL) and incubated for 15 min at 37°C. EDTA (10 mM) was added to stop the activation process. The platelets were precipitated by centrifugation at 1100×g for 10 min at RT and diluted to physiologic concentration.

In experiments with platelets in medium free from plasma, platelets were pelleted from PRP by centrifugation at 1100×g for 10 min and washed three times as described above to remove plasma proteins. After being washed, the platelets were pelleted and resuspended in Tyrode’s solution. The platelets were then activated by the addition of TRAP and incubated as described above. After activation, platelets were washed once and resuspended in Tyrode’s solution. Activated and non-activated platelets were diluted to a concentration of 200 × 10^9/L with Tyrode’s solution.
Experimental *in vitro* loop models (Papers I-IV)

Two different loop models, the whole-blood loop and the shear force loop model, were used in order to study the immediate thromboinflammatory response evoked from hepatocytes in direct contact with blood and platelets. Both models were constructed by use of polyvinyl chloride (PVC) tubing furnished with immobilized heparin. Tubing loops were closed with custom-designed heparinized connectors of the appropriate size. The loops were then held at 37°C and kept in motion at a predetermined speed during the experiment.

Heparinization of tubings and materials (Papers I-IV)

The CHC™ was used to provide all material in contact with blood with two layers of immobilized heparin in order to minimize material induced platelet activation. The CHC™ consists of heparin conjugates (Mₜₐₜ =13 kDa) in which heparin is covalently bound to a polyamine carrier, approximately 70 moles of heparin per mole carrier of protein [88].

The coating procedure was carried out according to the manufacturer’s recommendations. Before use of the material, the efficacy of the heparinization was controlled with toluidine blue.

Whole-blood loop model (Papers I, III, and IV)

A previously described *in vitro* tubing loop model was used [76, 89] in two set-ups, large and small. In brief, the model consists of loops made from heparinized PVC tubing, with an inner diameter of 6.3 mm and length of 390 mm used (the larger used in Papers I and III) or with an inner diameter of 4 mm and a length of 300 mm (the smaller tubing in Paper 4). The closed pieces of tubing were placed on a rocking device (vertically rotated at 30 rpm) to generate a blood flow of 45 mL/min, in an attempt to mimic the portal venous flow, and placed within a 37°C incubator.

A volume of 7 mL of ABO-matched blood and 1x10⁵ hepatocytes in 100 µL WME were added to each loop in the large model. In the smaller model (Paper IV), 3mL of blood and 1.7 x10⁴ hepatocytes were used.

In every experiment, at least one loop containing only blood and medium served as a negative control. A series of experiments was conducted with the addition of different inhibitors targeting different steps in the coagulation and complement cascades. Before and at 5, 15, 30, and 60 min after the start of the experiments, 1 mL of blood was collected from each loop for analysis. The collected samples were immediately added to tubes containing 10 mM EDTA to stop further reactions. After the perfusion, macroscopic clots were retrieved for section analysis.
4.0-mm shear force loop model (Paper II)

To study the specific interaction between hepatocytes and platelets, an anti-coagulated system was used. Loops were made from heparinized PVC tubing (inner diameter, 4 mm and length, 95 mm). The closed loops were connected to a fast-rotating wheel to generate shear forces, and the wheel was placed in a 37°C water bath. One mL of plasma or medium was added to every loop. To avoid interference with thrombin, the major coagulation serine protease, the thrombin inhibitor lepirudin (50 µg/mL) was added to each loop. Hepatocytes were used in the same concentration as in previous loop experiments [90], and platelets were used at physiologic concentrations in order to mimic the situation in clinical HcTx.

A series of experiments were performed to study the interaction between hepatocytes and platelets. To some loops, vWF or inhibitory antibody was added to supplement the control loop. Each series of experiments was performed with matched controls. The incubation time was 15 min, after which the contents of the loop were retrieved into EDTA-containing tubes (10 mM final concentration). The samples were centrifuged at 200xg for 5 min in order to collect the cell complexes, and the supernatant was removed. The cell complexes were washed twice and marked with antibodies for flow cytometric analysis.

Blood and plasma analysis (Papers I, III, and IV)

The blood samples retrieved during the loop experiments were analyzed for changes in platelet and leukocyte count as well as blood activation markers. The samples were immediately analyzed by cell counting on a Coulter AcT differential analyzer (Beckman Coulter, Miami, FL, USA).

The remaining samples were centrifuged at 3000xg for 20 min at 4°C. Plasma was collected and stored at -80°C until analyzed.

Enzyme immunoassays (Papers I, III, and IV)

Generation of blood activation markers and coagulation and complement parameters was measured by ELISA. The rationale behind the analysis of serine proteases in complex with protease inhibitors (serpins) was that the serine proteases can only form stable complexes with the serpins after they have been activated and thus are able of cleaving the bait region of the serpins (antithrombin [AT] and C1-INH). Thrombin/AT complexes (TAT) were measured with commercial EIA kits (Beheringswerke, Marburg Germany), and FXIa/AT, FXIIa/AT and FXIIa/C1-INH were all analyzed according to Sanchez et al. [91], and kallikrein/AT, and kallikrein/C1-INH acccording to
Bäck et al. [92]. MASP-1/AT, MASP-2/AT, MASP-1/ C1-INH and MASP-2/C1-INH were analyzed as described by Kozarcanin et al. [93].

C3a and the sC5b-9 (the TCC) were analyzed as described by KN Ekdahl [94].

Fibrin-activated serine proteases (Paper III)

Initially, fibrin was prepared by the addition of 0.05 IU of thrombin (Hoffman-La Roche, Basel, Switzerland) to 400 µg of fibrinogen (Haemochrom Diagnostica, Mölndal, Sweden) at a ratio of 1:2650 (mole/mole). Clots were formed in polypropylene Eppendorf tubes for 30 min at 37°C and then sonicated.

To study the fibrin-mediated activation of serine proteases, PPP was further incubated for 30 min at 37°C with 20 µg/mL fibrin (positive control). To analyze the effects of LMW-DS, parallel experiments were performed in which samples were pre-incubated for 30 min at 37°C with 100 µg/mL LMW-DS before the addition of fibrin. PPP without fibrin and LMW-DS were used as negative controls. Activation of the samples was stopped by the addition of EDTA (10 mM). The samples were then centrifuged at 3400xg for 15 min at 4°C, and the collected PPP was used for the detection assays.

Immunohistochemical staining (Papers I, II, and IV)

To visualize cell complexes and cell-surface structures, cells were marked with fluorochrome-conjugated antibodies and subsequently analyzed by confocal microscopy or flow cytometry.

In paper I, clots retrieved from loop experiments were used. Clots were embedded in Tissue-Tek, snap-frozen in liquid nitrogen, and stored at -70°C until analyzed. Cryostat sections of 5-µm thickness were fixed with 4% paraformaldehyde and 50% ethanol before analysis, and then stained with the antibodies and fluorophores listed in Table 3 and analyzed by confocal microscopy. In Paper II, thawed, cryopreserved single cells and retrieved cellular complexes were fixed with 1% paraformaldehyde and stained with the fluorophore-conjugated antibodies or cell tracer listed in Table 4 and then analyzed by confocal microscopy and flow cytometry.

Confocal microscopy (Paper I, II, and IV)

The fixed cells and cellular complexes were visualized in a LSM700 laser scanning microscope with ZEN 2011 software. The antibodies used are listed in Table 3.
Flow cytometric analysis (Paper II)

Retrieved cells and cellular complexes from the loop experiments were analyzed in flow cytometry. Hepatocytes were analyzed for the expression of vWF on their cell surfaces. The complexes formed between activated platelets and hepatocytes in connection with the presence of vWF or inhibitory antibodies were also investigated. In each experiment, the activation status of the platelets was controlled by monitoring the expression of P-selectin. Cells were treated with antibodies, which were visualized according to Table 4.

Table 4. Details for markers used for flow cytometric analysis

<table>
<thead>
<tr>
<th>Paper</th>
<th>Target</th>
<th>Fluorophore</th>
<th>Marker for</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Hepatocyte</td>
<td>Cell Trace far red</td>
<td>Hepatocytes</td>
</tr>
<tr>
<td></td>
<td>CD41</td>
<td>RPE</td>
<td>Platelets</td>
</tr>
<tr>
<td></td>
<td>vWF</td>
<td>FITC</td>
<td>vWF</td>
</tr>
<tr>
<td></td>
<td>P-selectin</td>
<td>PE</td>
<td>Platelet activation</td>
</tr>
</tbody>
</table>

Semi-quantitative RT-PCR (Paper II)

RNA was isolated using an RNeasy Plus Micro kit™ (Qiagen, Hilden, Germany) according to the manufacturer’s manual. The RNA was eluted with RNase-free water and stored at −70°C.
cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR was then performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green JumpStart Taq ReadyMix™ (Sigma-Aldrich, St. Louis, MO, USA). Specific primers for the target genes were purchased from Invitrogen and were designed to span from one exon to another to avoid amplification of the genomic DNA. Primers for the TATA-binding protein (TBP) were used as internal standards. The products were analyzed using melting curve analysis.

The primer sequences are shown below:

vWF_forward: 5’- TGC AAC ACT TGT GTC TGT CG - 3’
vWF_reverse: 5’ - GGG TGG CTG CAT CCC TTA TT -3’
TF_forward: 5’ - AGC AGT GAT TCC CTC CCG AA - 3’
TF_reverse: 5’ - GTA GCT CCA ACA GTG CTT CCT - 3’
TBP_forward: 5’ - GTG GGG AGC TGT GAT GTG AA - 3’
TBP_reverse: 5’- TGC TCT GAC TTT AGC ACC TGT -3’

Heparinization of the hepatocyte cell surface (Paper IV)

Heparinization of the hepatocyte surfaces was accomplished by using a PEG-phospholipid derivate (see Figure 2A for details) connected to a maleimide group (Mal-PEG-DPPE). Mal-PEG-DPPE was synthesized as previously described [95]. This substance was used because hydrophobic interaction between the cell membrane and the lipid (DPPE) would spontaneously anchor the PEG group to the bilayer of cell membrane, while the hydrophilic PEG-chain would be linked through a binding peptide to the heparin conjugates (Figure 2B).
Two different heparin-binding peptides were each conjugated to the PEG derivative and tested for efficacy in heparinizing hepatocytes. Heparin-binding peptide (HBP) II consisted of a sequence of 7 arginine residues [96], and HBPIII was a sequence of 13 amino acid residues identified by random screening of a combinatorial phage display [97]. A cysteine residue was added to the N- or C-terminus of the peptides to allow conjugation to the maleimide molecule. The HBPs were purchased from Sigma-Aldrich.

The Mal-PEG-lipid (3.4 \times 10^{-7} \text{ mole} \text{ and } 5.1 \times 10^{-7} \text{ mole}, respectively) was then mixed with HBPII and III (3.4 \times 10^{-7} \text{ mole} \text{ and } 5.4 \times 10^{-7} \text{ mole}, respectively) in Dulbecco’s phosphate-buffered saline without calcium and magnesium (PBS, pH 7.4, GIBCO) (200, and 300 µL, respectively). After a thorough mixing, the solution was incubated at RT for 24 h. The mixture was purified on a spin column equilibrated with PBS to produce the two forms of heparin-binding peptide-PEG-lipid.

A volume of 50 µL of HBP-PEG-lipid solution (0.15 mM in PBS) was added to the cell pellet with 5 \times 10^4 hepatocytes, followed by a 30-min incubation at RT with gentle agitation. The cells were then washed with PBS and centrifuged (180xg, 3 min, RT) to remove unreacted heparin-binding peptide-

\[ \text{Figure 2. A. Schematic structure of the heparin-peptide-PEG-lipid. B. Incorporation of the lipid chain into the cell membrane enables heparinization of the cell surface.} \]
PEG-lipid. Next, a solution of CHC\textsuperscript{TM} conjugate (100 µg/mL in PBS) was added and incubated at RT for 10 min. The cells were further washed twice before use. Cell viability was determined using the Alamar Blue\textsuperscript{®} reagent.

**Stability of the heparinization of hepatocytes (Paper IV)**

To examine the stability of the heparin conjugates immobilized on the hepatocyte surfaces, FXa activity was measured in order to measure the release of the heparin conjugates. This assay is based on the fact that AT (present in plasma) in complex with heparin inhibits exogenously added FXa [39]. In brief, after the heparinization procedure, hepatocytes were incubated in human plasma ($1.7 \times 10^4$ cells/mL) for 24 h at 37 °C. The cells were collected by centrifugation at 180xg for 3 min in RT after a specified time (0, 1, 6, or 24 h), and the FXa activity of the supernatant was measured according to the manufacturer’s instructions (Chromogenic Activity Assay Kit; Chromogenix, Bedford, MA, USA). The supernatant was mixed with FXa and incubated for 5 min. Then, after the substrate had reacted, the resulting solution was read at 405 nm.

**Statistics**

All results are presented as means ± standard error of the mean (SEM) in Papers I-III and means ± standard deviation (SD) in Paper IV. For all statistical calculations, Prism, version 5.0 for Macintosh was used (GraphPad Software, Inc., La Jolla, CA, USA). The loop experiments with more than two groups were evaluated using repeated measures analysis of variance in Papers I and IV. Differences between two groups were analyzed with the Wilcoxon non-parametric two-tailed matched-pairs test in Papers I (Table 2, part B), Paper II, and Paper III, and by a paired Student’s t test in paper IV. P values less than or equal to 0.05 were considered significant.
Experiments and results

Thromboinflammation and basic mechanisms of the IBMIR triggered by isolated hepatocytes (Paper I)

Loop experiments

Experiments studied the early responses from the innate immunity when freshly isolated hepatocytes came into contact with ABO-matched blood. Before the experiments a dose-response curve was performed to establish the amount of hepatocytes that should be used in each experiments.

The larger whole blood tubing loop model was used, and the activation of the cascade systems and changes in cell-count were monitored through repeated blood-sampling throughout the experiment. Fresh hepatocytes in contact with blood elicited a substantial activation of the coagulation system, with consumption of platelets and PMNs. Likewise, a significant activation of the complement systems occurred, reflected by accumulation of C3a. These reactions had already begun within 5 min of hepatocyte/blood contact. Values are presented for the results obtained after 1 hour of perfusion (Table 5). Macroscopic clotting was also regularly observed in loops containing hepatocytes.

Table 5. Blood cell counts and complement and coagulation parameters before and after 60 min of perfusion in the blood loop model. Loops with hepatocytes are compared with loops containing blood alone with the same amount of medium but without hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>Control</th>
<th>Hepatocytes</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood cell count (x10^9/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>265 ± 8.4</td>
<td>132 ± 6.13</td>
<td>70.4 ± 14.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>3.76 ± 0.23</td>
<td>3.67 ± 0.20</td>
<td>2.39 ± 0.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.46 ± 0.04</td>
<td>0.5 ± 0.1</td>
<td>0.45 ± 0.08</td>
<td>ns</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.3 ± 0.09</td>
<td>2.24 ± 0.09</td>
<td>2.16 ± 0.09</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Coagulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT (μg/L)</td>
<td>3.9 ± 0.51</td>
<td>416 ± 104</td>
<td>2433 ± 514</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FXIIIa/AT (μM)</td>
<td>0.08 ± 0.02</td>
<td>0.30 ± 0.03</td>
<td>2.84 ± 0.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FXIIa/AT (μM)</td>
<td>0.07 ± 0.01</td>
<td>0.25 ± 0.05</td>
<td>0.74 ± 0.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Complement</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3a (ng/mL)</td>
<td>95.1 ± 5.54</td>
<td>752 ± 68</td>
<td>1183 ± 110</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>sCSB-6 (AU/mL)</td>
<td>23.0 ± 4.89</td>
<td>73 ± 4.6</td>
<td>84.7 ± 7.7</td>
<td>ns</td>
</tr>
</tbody>
</table>
In order to further dissect the hepatocyte-elicited thromboinflammatory reactions, various substances targeting different steps in the coagulation system were tested (Figure 3).

Figure 3. Schematic presentation of targets for inhibition. A: Inhibition of thrombin with melagatran. B: Contact activation was inhibited by CTI. C: The TF pathway was inhibited with iFVIIa and inhibitory mAb, both targeting the active binding site for FVII/FVIIa on TF.

Initially, inhibition by the specific thrombin inhibitor melagatran was tested. Melagatran totally blocked both the platelet depletion and activation of the coagulation system; the generation of TAT, FXIIa/AT, and FXIa/AT complexes was lower in all cases than in the negative control tubing. In addition, there was no consumption of PMNs and no generation of C3a in the loops containing melagatran, nor was macroscopic clotting observed.

To investigate whether the reactions were initiated through the contact activation pathway or the TF pathway, inhibition of both these pathways was undertaken. Corn trypsin inhibitor (CTI), a specific human FXIIa inhibitor, had no detectable effect at all on the reactions. For the TF pathway, two substances were tested: inactivated FVIIa(iFVIIa) and anti-TF mAb; both of these substances occupy the binding site for FVII/FVIIa on TF. Hence, in the experiments, the hepatocytes were pre-incubated with each of these substances.

Both of these inhibitors had an obvious initial effect, in that the cascade systems were significantly depressed during the first 30 min of perifusion. Neither of the inhibitors could fully control the activation of the cascade systems over time. After 60 min of perifusion, the platelets and PMNs were still significantly restored to a greater extent when compared to the positive control. TAT generation was also significantly lower in loops containing inhibitor, and the amount of TAT generated was ~40% of the amount generated in the positive control. The corresponding results for melagatran was 1%.
**TF and hepatocytes**

Stained hepatocytes were examined by confocal microscopy and an obvious expression of TF was observed. RT-PCR also confirmed the occurrence of mRNA for TF in the isolated hepatocytes.

**Hepatocytes entrapped in clots**

Examination by confocal microscopy of the clots retrieved after loop experiments revealed that the hepatocytes were entrapped in the clots and were surrounded by platelets. The clots were also infiltrated by CD11b⁺ leukocytes.

**IBMIR in clinical hepatocyte transplantation (Paper I)**

In order to demonstrate the occurrence of a hepatocyte-triggered IBMIR in vivo, we obtained samples from a recipient who had been diagnosed antenatally with ornithine transcarbamylase (OTC) deficiency. In connection with the first hepatocyte infusion, the recipient was treated with prednisolone and tacrolimus according to a current protocol [98] and received 8.2 x 10⁷ ABO-matched cryopreserved hepatocytes (72% viability) into the portal vein. Samples were drawn before transplantation, at time 0, and at 30, 60, and 180 min and 1 and 3 days after transplantation (Figure 4). Immediately after infusion of the cells, a drop in the platelet and leukocyte counts occurred, together with complement activation (formation of C3a). These responses were followed by the generation of TAT, FXIIa/AT, and FXIa/AT complexes, which peaked at 60 min after the cell infusion was stopped and disappeared by 180 min.
Figure 4. A recipient monitored for parameters relevant to the IBMIR during a clinical hepatocyte transplantation procedure. Time point 0 was the beginning of the infusion of cells.
Hepatocytes with a procoagulative phenotype (Paper II)

When clots from loop experiments were examined, a corona of platelets was observed around the hepatocytes (Paper I). This phenomenon was further explored in paper II. During clot formation, platelets with GP1bα receptors anchor to subendothelial collagen by means of vWF; therefore, this factor was evaluated as a potential candidate to act as a link between these cells. vWF is a large multimeric protein that has been reported to be synthesized exclusively in endothelial cells and megakaryocytes and further transferred to mature platelets.

vWF on the hepatocytes

Eight different liver batches were tested by flow cytometry for the presence of vWF (FITC-conjugated). All batches included hepatocytes that expressed vWF. On average, 82% (range, 52-96%) of the hepatocytes expressed vWF on their surface. A control series with lymphocytes and a non-binding antibody was also performed, from which no FITC-signal could be detected.

Binding of vWF to the hepatocytes

Hepatocytes also bound soluble vWF/FVIII. When hepatocytes were incubated with a physiological concentration of vWF/FVIII, the mean fluorescence intensity (MFI) for the FITC-conjugated vWF bound to hepatocytes increased by 10-fold (1840=>21100; n=3, data not shown). Since collagen is a preferred binding target for vWF, hepatocytes were also analyzed for the possible presence of collagen on the surface, but in our hands, no collagen could be detected.

mRNA for vWF and TF

Three different liver batches were analyzed by RT-PCR to detect the potential occurrence of mRNA for vWF and for comparison to corresponding levels for TF as a positive control. mRNA for both proteins was detected, and the mRNA levels for vWF were 4-12 times higher than those for TF.

vWF on the hepatocytes

Confocal microscopy also revealed a strong signal from the FITC-conjugated anti-vWF mAbs, detecting vFW on the surfaces of the hepatocytes.
Figure 5. FITC-conjugated vWF (green) on the surfaces of hepatocytes stained with celltrace Far Red™ (violet).
Platelet-hepatocyte complexes

To study the direct interaction between hepatocytes and platelets, the two cell types were incubated together. Two different set-ups were tested in the shear force loop model. Hepatocytes and activated platelets were incubated in medium and in lepirudin-anti-coagulated plasma for 15 min. In the second set-up, the aggregation of platelets was inhibited by blocking the GPIIb/IIIa receptor on the platelets with Abciximab (ReoPro, Eli Lilly, Sweden AB, Solna, Sweden). After the experiments, the cells were collected, stained, fixed, and analyzed in flow cytometry. Formation of complexes between the two types of cells was revealed. A median of 60% of the hepatocytes incubated with platelets showed platelets bound to their surfaces; for hepatocytes incubated with platelets in plasma, the corresponding number was 35%.

In follow-up experiments, inhibitory antibodies targeting GP1bα on the platelets (h6B4, kindly supplied from Professor H Deckmyn, Laboratory for Thrombosis Research, KU Leuven, Belgium) were added to the loops and resulted in a decrease in the number of complexes by two-thirds (Figure 6). In follow-up experiments, inhibitory antibodies targeting GP1bα on the platelets (h6B4, kindly supplied from Professor H Deckmyn, Laboratory for Thrombosis Research, KU Leuven, Belgium) were added to the loops and resulted in a decrease in the number of complexes by two-thirds (Figure 6).

![Figure 6. Percentage of hepatocytes with platelets bound to their surface after 15 min of incubation in the shear force loop model. The gray box shows the numbers for hepatocytes incubated with platelets diluted in medium, and the white box shows the cells incubated with platelets diluted in lepirudin-anticoagulated plasma with the addition of ReoPro. The dotted box shows the numbers for hepatocytes incubated under the same conditions as in the white box, with the addition of anti-GP1bα mAb.](image-url)
**Activation of vWF on the surface of hepatocytes**

To further confirm the presence of vWF on the surface of the hepatocytes and the possibility of activating the vWF, we examined the binding of rGP1bα to the surface. Hepatocytes were incubated with rGP1bα and then analyzed by flow cytometry to detect binding. No binding of rGP1bα to hepatocytes was found without ristocetin activation of vWF, and there was no significant difference between the MFI in Pacific Blue (rGP1bα) and background levels. In the hepatocyte-samples that were pre-incubated with ristocetin for 5 min, causing activation of vWF, binding of rGP1bα to the hepatocyte surface was observed (Fig 7). MFI-values for Pacific Blue increased an average of 4.3 times in these samples (p<0.05).

![Figure 7](image)

*Figure 7. Binding of recombinant GP1bα to isolated hepatocytes. A Histogram of flow cytometry analysis of hepatocytes incubated with rGP1bα stained with Pacific Blue +/- ristocetin. Gray: hepatocytes incubated with rGP1bα. Red: the batch of cells after pre-incubation with ristocetin. One representative experiment out of five is shown. B. Increase in MFI for Pacific Blue in the five experiments; on average, the MFI increased significantly, by 4.3-fold (p<0.05).*
Control of the IBMIR with low molecular weight dextran sulfate (Paper III)

In order to control the reactions from the innate immunity elicited by hepatocytes in contact with ABO-matched blood, we needed an inhibitor of both the coagulation and complement systems. For this purpose, we tested dextran sulfate (LMW-DS), a highly sulfated synthetic polysaccharide.

Figure 8. LMW-DS: a synthetic polymer of D-glucose that is 17-20% sulfated, corresponding to almost 2 NaSO$_3^-$-groups per glucosyl residue in every dextran molecule. $M_w$ 5000 Da.

Inhibition of hepatocyte-triggered IBMIR

LMW-DS was tested at two different doses, with fresh hepatocytes being perifused for 60 min in ABO-matched blood in the larger tubing loop model. At both 100 and 1000 µg/mL, LMW-DS influenced all the measured parameters. Because of the overall effectiveness of the lower dose, all results are presented for this dose. The level of TAT, reflecting thrombin generation, was only 2% of the level in the positive control tubing. Activation of FXI and FXII was also significantly suppressed. More than 70% of the platelet count was maintained during the whole experiment, and PMNs were kept constant throughout the observation time. Complement activation, reflected by C3a and sC5b-9 formation, was significantly decreased.
Figure 9. Human hepatocytes incubated in the tubing model with ABO-compatible blood. Results are given for pre-experimental values and values after 60 min for loops containing hepatocytes and loops containing hepatocytes with the addition of 100 µg/mL LMW-DS. Panel A-D: Activation of coagulation and inhibition of LMW-DS. All parameters except kallikrein/AT were significantly restored by LMW-DS (p<0.05). E, F: Changes in cell count after inhibition with LMW-DS.
Control of the IBMIR induced by fresh and cryopreserved hepatocytes

Given the complexity of innate immunity and the fact that different types of surfaces elicit different responses, it is not clear that the results from fresh cells are transferable to freeze-thawed cells. Therefore, loop experiments were designed to keep all parameters constant except for the freeze-thawing step to investigate whether fresh and freeze-thawed cells give rise to the same amount of thromboinflammation. Freshly isolated hepatocytes were divided into two portions, one cryopreserved and the other cold-stored in UW solution over night. The subsequent experiments were later performed at the same time with the same cells, handled in two different ways, and tested against blood from the same blood donor, (Figure 10A).

Figure 10. A. Experimental setup using isolated hepatocytes, with one portion cold-stored in UW solution overnight and the other cryopreserved and thawed before the experiments. Loop experiments were conducted at the same time with the same ABO-matched blood donor. B. Blood cell counts and complement and coagulation parameters after 60 min of perifusion in the loop model, using fresh and freeze-thawed hepatocytes with fresh ABO-compatible blood +/- 100 µg/mL LMW-DS. Data are normalized and show the percentage change compared to the level before the experiments for the respective parameters. Data are means (+SEM), n=5. All parameters were significantly restored by LMW-DS (P<0.05).
Both groups showed the same degree of consumption of platelets, along with pronounced activation of the coagulation system and equivalent complement activation. No significant differences between cold-stored and freeze-thawed hepatocytes in terms of the tendency to induce thromboinflammatory reactions were registered. Inhibition studies in which LMW-DS was added to the loops at the concentration 100 µg/mL showed that it controlled the cascade systems and cell consumption equally well in freeze-thawed and cold-stored hepatocytes (Figure 10B).

**Heparin vs LMW-DS in controlling hepatocyte-induced IBMIR**

In order to determine whether LMW-DS was more efficient than conventional anticoagulant therapy in controlling the IBMIR, another series of loop experiments were performed with the addition of heparin to certain loops.

*Figure 11. Inhibition of hepatocyte-triggered thromboinflammation by heparin and LMW-DS. A. Remaining cells and activation of the coagulation and complement systems after 60 min of perfusion of fresh hepatocytes in ABO-compatible blood. All loops contained 1x 10⁵ fresh human hepatocytes. Uninhibited controls were compared with loops to which 1 IU/mL heparin or 100 µg/mL LMW-DS had been added. Because of the wide inter-individual differences, all values are normalized. The remaining cell counts are expressed as a percentage of the initial individual value. Platelets (PLT), monocytes (MO) and lymphocytes (LY). There was no significant difference in cell count between loops containing heparin and those containing LMW-DS. B. Coagulation and complement activation parameters are expressed as a percentage of the generated values for the control loop containing only 1 x 10⁵ fresh hepatocytes and ABO-compatible blood. Data are expressed as means +SEM. *The generation of TAT, FXIIa/AT, C3a, and sC5b-9 was significantly lower in loops treated with LMW-DS. n=7 for MO, FXIIa/AT, and FXIa/AT; n=8 for all other parameters. *The generation of TAT, C3a, and TCC was significantly lower in loops treated with LMW-DS. n=8 for both LMW-DS and heparin.*
Heparin was tested at two different concentrations, 0.5 IU and 1 IU/mL. Heparin had almost no effect at the lower concentration. There was no significant difference between loops containing LMW-DS or heparin in terms of changes in cell counts. LMW-DS was significantly more efficient than heparin in inhibiting the generation of TAT and FXIIa/AT and the activation of the complement system. No significant effect at all was observed with heparin in terms of preventing the activation of the complement system.

Effects of LMW-DS on metabolic function
Fresh hepatocytes in culture were evaluated for changes in metabolic function when 100 µg/mL LMW-DS was added to the medium. There was no difference in metabolic function regarding urea formation, albumin synthesis, or CYP3A4 oxidization between cells cultured in the presence of LMW-DS and those cultured in ordinary medium only.

Surface modification by heparinization (Paper IV)
In order to evaluate whether the biocompatibility of transplanted cells could be increased, a surface modification method was employed to modify the hepatocytes.

The PEG-phospholipid derivative (see Figure 2A for details) Mal-PEG-DPPE (PEG-lipid) was used. In order to connect the heparin conjugate to the PEG-lipid, initial studies were performed with a T lymphoblastoid cell line, CCRF-CEM, to identify an optimal binding peptide. Surface heparinization of CCRF-CEM cells was evaluated by surface plasmon resonance (SPR), which enabled us to monitor events on the surface in real time. Two heparin-binding peptides, HBPII and HBPIII, were identified that demonstrated superior immobilization of heparin to the cell surfaces. These molecules were further evaluated for heparinization of hepatocytes.

Stability of immobilized heparin on the surface of hepatocytes
Hepatocytes were furnished with two layers of immobilized heparin on the surface via the PEG-lipid in order to test both types of binding peptide. Heparinized hepatocytes were incubated in plasma for 24 h. Examination of the FXa activity in aliquots of sampled plasma during the incubation time allowed us to determine the amount of heparin released from the hepatocyte surface into the plasma. In experiments with hepatocytes heparinized with HBPII-PEG-lipid, the heparin conjugates rapidly detached from the cells; in contrast, the HBPIII-PEG-lipid detached only gradually from the surface, and most of the heparin conjugate was retained for 24 h (Figure 12).
Hepatocytes with heparin immobilized on the surface by the use of PEG-lipids with two different binding peptides, HBPII and HBPIII. Prepared hepatocytes were incubated in human plasma at 37 °C, and the supernatants were subjected to the FXa assay after various times. The FXa activity of the supernatant was measured to examine the stability of the heparin conjugates on hepatocytes after coating. Error bars indicate standard deviation; \( n = 3 \).

**Blood compatibility of hepatocytes with surface-immobilized heparin**

Experiments were performed in the smaller whole-blood loop model. When unmodified control hepatocytes were exposed to human whole blood, the expected platelet aggregation, coagulation activation, and complement activation were observed (Figure 13). In the case of hepatocytes coated with PEG-lipid and control peptide-PEG-lipid (without heparin) there was some platelet aggregation and increase in TAT, C3a, and sC5b-9 levels, but these increases were lower than those in the unmodified control group. On the other hand, platelet aggregation and coagulation activation were significantly suppressed when the hepatocytes were treated with HBPII-PEG-lipid or HBPIII-PEG-lipid. Also, the markers of complement activation (C3a, sC5b-9) were significantly reduced in both these groups.
Figure 13. Effect on blood compatibility of the immobilization of heparin conjugates on human hepatocytes. (A–D) Whole-blood experiments with hepatocytes after modification with heparin conjugates. After the hepatocytes were added to human whole blood without any additives, they were incubated for 60 min at 37°C. In order to immobilize the heparin conjugates, the cells were treated with PEG-lipid, control peptide-PEG-lipid, HBPI-PEG-lipid, HBPII-PEG-lipid, or 70% HBPIII-PEG-lipid. As a control, non-modified hepatocytes were used. Error bars indicate standard deviation; n = 5. The figures show (A) relative platelet consumption and generation of (B) TAT, (C) C3a, and (D) sC5b-9.

In loops with hepatocytes covered with HPBII and HPBIII platelets were significantly restored compared to control loops with untreated hepatocytes. Generation of TAT, C3a and sC5b-9 was also significantly lower in loops with hepatocytes covered with HPBII and HPBIII than in loops with uncovered hepatocytes.
Discussion

Thromboinflammation and basic mechanisms for the IBMIR triggered by isolated hepatocytes (Paper I)

The experiments performed in this paper revealed that hepatocytes in direct contact with blood regularly elicit thromboinflammatory reactions. The most striking findings from the experiments were the extensive generation of TAT and depletion of platelets along with the accumulation of the anaphylatoxin C3a. These results indicate that all the hallmarks of the IBMIR are induced, and the studies with inhibitors targeting different steps in the coagulation cascade also indicated that the IBMIR was initiated through the TF pathway. TF has also been confirmed on the surface of the hepatocytes by us and others [99, 100]. However, blocking the initiating factor, the TF/FVIIa complex, in these studies, delayed the cascade reactions, but the IBMIR could not be abolished, as it was when the blocking occurred at the level of thrombin. The inhibition achieved with iFVIIa was also less efficient than that observed in islet-triggered IBMIR [75]. We interpreted the lack of sustained effect after inhibition of the TF pathway to both the metabolic effect of hepatocytes, which rapidly internalize and clear FVIIa [101] (and therefore also iFVII), and the short half-life of iFVII. A recent publication has also revealed that isolated hepatocytes are even preloaded with endogenously produced FVII/FVIIa [100], which further complicates the picture. An additional potential explanation for the lack of sustained effect is that when the TF pathway is inhibited, small amounts of thrombin may still be generated by the activation of platelets, and this thrombin production enables the amplification and propagation of the coagulation. Because of the extensive cross-talk between the coagulation and complement systems, the reactions are further self-amplifying. For example, released chondroitin sulfate from the platelets also triggers complement activation [69]. Complement activation may also be triggered independently of the coagulation system, most likely through natural antibodies recognizing structures on the hepatocyte surfaces and initiating the classical pathway; at the same time, the lectin pathway is triggered by MBL or ficolins recognizing surface-bound carbohydrates and debris from damaged cells [102].

Examination by confocal microscopy of retrieved clots has revealed an infiltration of CD11b-positive leukocytes and platelets surrounding the liver cells. This finding also verifies the activation of platelets. Furthermore, it may
explain why KCs are rapidly surround the transplanted cells after HTx [71]. However, if most of the transplanted hepatocytes are cleared as a result of innate immunity, there is an obvious link to the triggering of the adaptive immune system. Activated KCs can rapidly switch to become efficient APCs and T-cell activators [46] and to form a link to an adaptive immune response. Given this risk, it seems reasonable to optimize initial cell survival in order minimize the triggering of innate and subsequent adaptive immune responses and to avoid repeated HcTx procedures.

The crucial question with respect to the IBMIR is whether it is relevant in vivo. The report of successful HcTx in siblings with FVII deficiency [103], which decreased the requirement for exogenous recombinant factor VII (rFVIIa) to approximately 20% of that before cell transplantation, is extremely interesting in this context. This case report suggests that FVII (and consequently the TF pathway) is involved in the clearance of the transplanted cells. In this thesis, we also observed a case in which relevant parameters for IBMIR were registered during the cell infusion. A strong correlation between the infusion of hepatocytes and systemic changes in complement and coagulation activation markers was observed, and this suggest that the IBMIR is also triggered in vivo.

Hepatocytes with a procoagulative phenotype (Paper II)

Hepatocytes are highly metabolic cells that raise several issues in HcTx situations. The expression of TF on the surface of isolated hepatocytes is already a complicating factor. In addition, hepatocytes might be preloaded with endogenously produced FVII/FVIIa [100]. During the work outlining the basic mechanism for the IBMIR, it was also observed that platelets surrounded the surfaces of the hepatocytes.

These studies aimed to investigate the interaction between isolated hepatocytes and platelets and to identify the link between these cell types. The experiments revealed that platelets and hepatocytes regularly formed complexes independently of the coagulation system. The amount of complexes varied between different batches and our results might also be underestimating the degree of complex formation since the experiments were performed in anticoagulated systems and with the addition of Reopro. The GP1bα-A1 binding is not firm unless it is further stabilized, and in the absence of coagulation, the cells may disconnect after a time representing a dynamic process in which the cells connect loosely awaiting stabilization. Moreover, analysis using flow cytometry might even have missed larger conglomerates of cells. We also demonstrated that it was possible to influence the complex formation by using inhibitory antibodies targeting the GP1bα receptor on the platelets.

Furthermore, hepatocytes expressed vWF on their surface. All tested batches of isolated hepatocytes expressed vWF to some degree, and the fact
that a single batch of hepatocytes constitutes a heterogeneous mixture of perivenous and periportal cells whose proportions may vary between batches may explain the varying degree of vWF expression observed between different batches.

Hepatocytes also effectively bound soluble vWF, and our observation of almost five times as much mRNA for vWF as compared to TF in the hepatocytes indicates that de facto synthesis of vWF occurs in isolated hepatocytes. To further evaluate the activation status of vWF on the hepatocytes, the binding of rGP1α was also tested. Only when vWF was activated with ristocetin could an obvious binding of rGP1α to the hepatocytes be confirmed.

To our knowledge, vWF synthesis has not been reported thus far in cell lines other than endothelial cells and megakaryocytes [104], and in the Human Protein Atlas, hepatocytes in liver tissue (before isolation) are stained negative for vWF [105]. It is reasonable to assume that the procurement of cells, including events before the organ donation and isolation procedures, causes cellular stress and an ensuing up-regulation of inflammatory genes, leading to the transcription and expression of new surface molecules. It is reported that hypoxia up-regulates the transcription of vWF in cultured lung endothelial cells, likely as a result of NF-1B signalling [106].

The clinical relevance of vWF on hepatocytes is still undecided, but it is clear that isolated hepatocytes express a more complex procoagulative phenotype than had been previously known. This fact might also have implications for the interpolation to hepatocytes of data from studying IBMIR in other cell lines. It is likely that the high metabolic potential of hepatocytes, which interferes with certain processes, will need attention in the future.

For example, it has been observed that during the transplantation procedure, hepatocytes are exposed to shear stress in the intravenous lines during cell infusion [107, 108], putting them at risk of the unfolding and activation of vWF and the triggering of unwanted reactions.

Control of the IBMIR with low molecular weight dextran sulfate (Paper III)

Although heparin is regularly added to the cell suspension during the HcTx procedure, a massive cell loss still occurs [28, 70]. When the hepatocyte-induced IBMIR was characterized, we observed that an obvious activation of the complement system occurred, with an accumulation of the anaphylatoxin C3a. Hence, it would be desirable to find ways to control the inflammatory reactions without extensive anticoagulation. For this reason, we tested the usefulness of adding a highly sulfated synthetic polysaccharide, LMW-DS, which has demonstrated numerous positive qualities in other contexts [109-111].
Its anticoagulatory effect is achieved through a potentiation of the C1-INH that inactivates FXIa (and FXIIa), thus preventing the amplification loop in the coagulation cascade [112]. All three complement activation pathways are also suppressed [113, 114].

The present in vitro study demonstrated that LMW-DS at 100 µg/mL effectively inhibited the activation of the coagulation system, and the complement activation was also controlled. In experiments comparing LMW-DS and heparin, LMW-DS was found to be superior in terms of controlling the activation of the cascade systems triggered by hepatocytes when compared to heparin. Significant inhibition of the complement system was not achieved with heparin. The LMW-DS concentration tested in this thesis is in the same range as the levels previously used in a phase I study in which LMW-DS was shown to be safe when infused intravenously, with no adverse effects; furthermore, the tested dose kept the APTT under 150 sec [109-111].

Encouraged by the positive results on fresh hepatocytes, it was logical to go on and evaluate the effect on cryopreserved hepatocytes. Fresh hepatocytes are generally considered to be preferred for transplantation, with a better metabolic profile and superior viability [31]. Nevertheless, cryopreserved hepatocytes are regularly used in HcTx [28, 31], since they offer the advantage of being readily available and better characterized before transplantation. In addition to the initial enzymatic digestion, cryopreserved hepatocytes have undergone two stressful critical procedures, freezing and thawing [115], and it is not clear what impact this handling (with its associated increased stress and potential changes in the cell surface) has on the tendency to elicit the IBMIR. Experiments addressing this question were performed in which all parameters were kept constant except for the storage procedure. The same cells were matched to the same blood donor, and the subsequent loop experiments were performed at the same time by the same persons. Our results indicated that the cryopreserved hepatocytes induced the IBMIR in contact with ABO-compatible blood to the same extent as did fresh hepatocytes, and that LMW-DS was equally effective in preventing the IBMIR in both types of hepatocytes.

The current study also showed that LMW-DS has the potential to interact with and reduce fibrin activation of the serine proteases FXIIa and kallikrein in the contact activation system, as well as the important amplifying molecules in the lectin pathway, MASP-1 and MASP-2 of the complement system.

There is also abundant cross-talk between the coagulation and complement systems [62], and therefore the optimal strategy for prohibiting the instant triggering of thromboinflammation is to target both the complement and coagulation systems during the transplantation procedure [102]. The current study was a limited in vitro study, but the results were positive, and the upstream inhibition of the cascade systems achieved with LMW-DS makes it potentially attractive for clinical applications. LMW-DS inhibits coagulation at the level of FXIa, and since one molecule of FXa generates up to 1000 molecules of
thrombin [116], a broad downstream effect is generated. The complement system also has a large in-born amplification potential, and therefore upstream inhibition is desirable. With respect to the complexity and redundancy of the immune system, there are probably numerous factors to consider before the detrimental effects of innate immunity can be completely controlled. The environment into which the hepatocytes are infused is a minefield: The sinusoids are heavily loaded with immune cells, particularly innate immune cells that maintain the balance between tolerance and activation [41]. Furthermore, the liver produces numerous acute-phase proteins, complement factors, and soluble pathogen-recognition receptors and is therefore predominantly an innate immune organ [117]. Given these conditions, it is essential to evoke as little reaction as possible from the innate immunity in the initial phase, in order to improve the initial percentage of engrafted cells and also to diminish the linkage to adaptive immunity.

Heparinization of the hepatocyte surface (Paper IV)

Hepatocytes are immediately recognized by the innate immune system after infusion into the portal vein. The hepatocyte surface not normally in contact with blood may express many potential immunogenic epitopes. Therefore, even under optimal conditions, 100% viability of the transplanted cells is not very likely, so the occurrence of potential DAMPS is probably unavoidable.

To modify the cell surface and protect the hepatocytes from recognition and destruction is an attractive approach to increasing the biocompatibility. An additional advantage is that the need for systemic administration of anticoagulants or complement inhibitors is diminished, which is particularly advantageously in patients with liver disease and impaired coagulation. Therefore, immobilizing heparin conjugates to the cell surface by use of PEG-lipid derivatives was employed and evaluated.

The PEG-lipid derivative intercalates into the cell membrane through hydrophobic interactions, and the hydrophilic PEG molecule can be connected to the heparin conjugate via a carrier molecule (heparin binding peptide/HBP).

Two potential PEG-lipid derivatives, HBPII- and HBPIII-PEG-lipid, were evaluated. In the case of the HBPII derivative, the heparin conjugates detached from the surface within the first few hours. About 50% remained after 5 h. The HBPIII-PEG-lipid showed a higher degree of stability, with gradually detachment of the heparin and ~75% still remaining after 24 h.

When tested in the loop model, the HBPIII derivative significantly attenuated all aspects of the IBMIR, whereas HBPII showed a lesser capacity to control the thromboinflammation. The reason for this difference probably reflects the low proportion of the heparin remaining on the hepatocytes treated with the HBPII-PEG-lipid derivative.
This study demonstrated that it is possible to protect the hepatocytes from an innate immune attack in the blood over a limited period of time. The optimal time for detachment of the heparin coating needs to be determined. Since hepatocytes break the endothelial wall to be further incorporated into the liver parenchyma within 16-20 h [72], by then the heparinization needs to be gone to enable establishment of interactions with the hepatocytes in recipient’s liver.

Summary

- Hepatocytes in contact with ABO-matched blood regularly induce innate immune reactions. The coagulation cascade is instantly activated, along with the complement system. At the same time, platelets and PMNs are activated and consumed. This thromboinflammatory phenomenon has been demonstrated both in vitro and in vivo.

- Thromboinflammation elicited by hepatocytes in contact with blood exhibits all the hallmarks of the IBMIR reported for the transplantation of islet and mesenchymal stem cells. Even though there are similarities between the reactions elicited from different cells, this thesis reports a more complex and distinctive picture of the IBMIR triggered by hepatocytes.

- Hepatocytes express TF and vWF on their surfaces, which means that isolated hepatocytes have a strong prothrombotic phenotype.

- Systemic inhibition with LMW-DS abrogates all components of the IBMIR.

- Heparinization of the hepatocyte cell surfaces protects the cells from the IBMIR when they come into contact with blood.

Concluding remarks

HcTx differs from other types of cell transplantation in that the cells being transplanted are parenchymal cells that have lost their intercellular connection through enzymatic digestion (isolation with collagenase). During the isolation process, the cells are also undergoing stress related to being placed in a Ca\(^{2+}\)-depleted environment. During this procurement process, it is likely that up-
and-down regulation of transcription factors for surface proteins occurs. The hepatocyte surface may also be damaged during the enzymatic digestion procedure. Pancreatic islets have certainly also undergone an enzymatic isolation procedure, but, unlike hepatocytes, they are protected by a collagen layer. Hepatocytes are also highly metabolic cells, with an exceptional capacity to produce proteins that also might interact with the local environment.

The tendency of hepatocytes to activate innate immunity in a limited *in vitro* milieu is, of course, a greatly simplified version of the *in vivo* situation. However, if contact with ABO-matched blood alone can trigger these reactions, it is reasonable to assume that downstream activation of the local MPS will occur in the sinusoids, with further clotting and destruction of the transplanted hepatocytes. In addition, clotting in the sinusoids will further trigger local ischemia and reperfusion mechanisms.

Ultimately, it will be important to take all possible measures to evoke as little reaction as possible from the innate immunity in the initial phase, in order to improve the initial percentage of engrafted cells and diminish the linkage to adaptive immunity and the need for repeated transplantation.
Future perspectives

Cell transplantation procedures have many theoretical advantages over full organ transplantation. As regards to HcTx, the procedure has been shown to be safe and cost-effective, but sustained benefits have not yet been achieved because of insufficient engraftment [28, 31]. There are many hurdles to overcome in order to establish this method clinically. The main focus of research in this field has explored various methods to enhance engraftment by using different approaches to manipulate the recipient by partial hepatectomy and/or systemic treatment. However, this thesis has mainly focused on gaining knowledge regarding the immediate recognition of hepatocytes in blood. Based on the insights I have received in this field; I would like to share my thoughts on possible future areas of development. First, a brief summary of a number of issues raised during this work:

What is the hepatocyte cell surface like after isolation?
The finding in this thesis that isolated hepatocytes express both TF and vWF, leading to a procoagulative phenotype with implications for IBMIR, is not surprising. This raises the question: how many still-unidentified proteins relevant for interactions with the cascade systems and local vasculature are up-regulated? On the other hand, it was also shown that isolated hepatocytes lack CD47 on their surfaces, making them prone to attack from macrophages [118]. Isolated hepatocytes have a weak expression of CD55, but a more pronounced expression of CD59 [119]; whether this expression is sufficient for protection during complement activation in connection with HcTx has not been explored. What has been established is probably only the tip of the iceberg regarding how the cell surface of isolated hepatocytes cell may interact with potential ligands to alert MPS, NK, and NKT cells.

- In this field, more knowledge of the hepatocyte surface would be beneficial, along with studies of how the cells interact with blood and the cascade systems.
- In order to study the interaction with the local MPS and LSEC, an optimal *in vitro* model would be desirable. Since most of the interactions are triggered immediately after the blood/hepatocyte contact, it is difficult to study this rapid course of events in current animal models.
Control of vWF
The presence of vWF on the hepatocyte surface and the potential of binding platelets is a complicating factor in many ways. First, activated platelets on the hepatocytes provide an ideal surface for coagulation, and, second, hepatocytes with bound platelets would probably exhibit an impeded integration in the liver parenchyma. Therefore, vWF is an interesting target for protecting the infused hepatocytes from the binding of platelets, even when a tailored anticoagulation is used in connection with HcTx. Questions yet to be answered:

- Immobilized vWF on the surface may easily be activated during the infusion as a result of shear forces [107]. This activation need to be further evaluated in experimental models.

- Would transplantation of vWF-deficient hepatocytes be superior to transplantation of normal hepatocytes in terms of engraftment?

- At present, there are many ongoing preclinical and clinical studies in which the inhibition of vWF binding to platelets (using inhibitory antibodies, nanoparticles, or aptamers) has prevented thrombus formation without increasing the bleeding risk [120, 121]. To test this possibility in an experimental model would be interesting.

Control of the cascade systems
LMW-DS has the capacity to control the serine protease cascade systems in vitro in connection with hepatocyte-induced IBMIR; is this effect possible to achieve in vivo? If so, does an initial reduction in the involvement of the IBMIR have implications for the outcome of the transplantation?

- Experimental HcTx with concomitant administration of LMW-DS needs to be performed.

Surface modification
Depending on the partially unknown nature of the hepatocyte surface and its potential interaction with the local environment, it would be ideal to cover and protect the cells by surface modification. This thesis presents some positive results in this context, but this field need to be further explored.

- It is necessary to test transplantation of heparinized hepatocytes in an experimental model. To what extent does the immobilization of heparin on the surfaces affect the engraftment?
• What is the ideal time for detachment of heparin from the hepatocyte surface for the optimal engraftment of hepatocytes?

Considerations regarding future studies
I would also like to convey a few final thoughts: In order to meet the regulations concerning animal testing and ethical considerations, it is important to take into account the 3R’s: replace, reduce, and refine. I do believe that a great deal of the basic research in this field can be done in in vitro models before moving into animal experimental models.

Regarding animal experimental models, because there are probably several factors to consider that need to work together before successful Hctx is achieved, it might be difficult to interpret the results of early interventions, and positive findings may even be missed that might be beneficial because they are overshadowed by other problems that occur later in the process and require other specific interventions.

As a clinician, I also believe that it would be beneficial if most manipulation occurs “at the donor side” (e.g. the donor hepatocytes), in order to reduce the need for heavy medication and major medical procedures in the recipient.
Levercells transplantation är ett tilltalande behandlingsalternativ för att behandla olika metabola leversjukdomar och akut leversvikst istället för en full levertransplantation. Proceduren går till så att leverceller som tagits tillvara från donerad levervävnad sprutas in i ett blodkärl. Cellerna följer blodströmmen in i levern där de sedan sprider ut sig i de minsta kärlförgränsningarna. Trots att levercellstransplantation bedöms som en säker och enkel procedur att genomföra för patienten samt kostnadseffektiv för sjukvården har metoden hittills bara genomförts i ett begränsat antal selekterade fall.

Detta beror på att merparten av de transplanterade levercellerna avstöts i direkt anslutning till transplantationstillfället. Dessa avstötningsmekanismer skiljer sig från den typ av avstötning som sker vid transplantation av hel lever.

Tidig avstötning av transplanterade celler är problematisk ur flera aspekter:

- Man uppnår inte tillräcklig behandlingseffekt för patienten.
- Värdefulla celler förloras och just tillgången till leverceller för transplantation är begränsad.
- Upprepade transplantationer riskerar att aktivera kroppens immunförsvar ytterligare som då kan utveckla snabbare och starkare avstötning.

Detta avhandlingsarbete fokuserar på studier kring hur tidiga avstötningsmekanismer medieras av kroppens medfödda immunförsvar. Avhandlingen består av fyra delarbeten där de två första arbeten kartlägger uppkomsten och förloppet av tidig avstötning. De två sista arbetena undersöker möjliga strategier för att hindra uppkomsten av tidiga avstötningsmekanismer riktade mot de transplanterade levercellerna.

Arbete I

Alla försök har genomförts i en så kallad ”loop-modell” som konstruerats för att kunna studera olika interaktioner mellan celler i blod. Modellen är uppbyggd av slangar som har ytbehandlas för att inte störa de naturliga reaktionerna. Till slangarna sätts blod från friska donatorer. Under försöken hålls slangarna i rörelse i 37 för att imitera blodflödet i levern. Leverceller införs i slangarna och genom regelbundna blodprover från slangarna under försöket går kan reaktioner från ämnena i blodet studeras.
Försöken visade att levercellerna inom ett par minuter i kontakt med blod orsakar inflammatoriska reaktioner samtidigt som de stimulerar att blodet levrar sig. Inom 60 min så var levercellerna inbäddade i ett blodkoagel. I detta koagel fanns både blodplättar och vita blodkroppar. I kvarvarande blod sjönk samtidigt också nivåerna av dessa blodceller. Vården för markörer för aktivering av kroppens försvavssystem och blodlevring var förhöjda i de blodprover som togs under försökins gång.

Genom att tillsätta olika substanser som hämman viktiga steg i processen som leder fram till att blodet levrar sig kunde vi analysera hur olika reaktioner uppkommer och samverkar.

Fria leverceller visade sig dessutom ha proteiner (vävnads faktorn = tissue factor) på cellytan som normalt sett inte finns på vävnader i kontakt med blod. När vävnadsfaktorn kommer i kontakt med blod så utlöser den en kedja av reaktioner som leder till att blodet levrar sig.

En klinisk levercells transplantation följs också med provtagningar under tiden cellerna sprutades in i blodet. De värden som då uppmättes följde samma förlopp som tidigare studerats i vår ”loop-modell”.

Utifrån resultaten kunde vi fastställa det reaktionsmönster som leverceller i kontakt med blod initierar.

**Arbete II**

Under arbetet I hade vi noterat att blodplättar fäster på levercellerna. Detta är ogynnamt ur två aspekter:

- Blodplättar utgör en perfekt yta för blodet att levrar sig på och det kan medföra att levercellerna blir omgårdade av ett blodkoagel.
- Fria leverceller kan inrätta sig i mottagarens levervävnad och bör då inte vara omgårdade av blodplättar och koagel för att skapa normala bindningar till omgivande celler.

Vi använde oss av en något annan typ av ”loop-modell” i detta arbete. Mindre slangar som samtidigt roterades snabbt för att efterlikna skjuvkrafter. Sjuvkrafter kan uppkomma i de slangarna som används vid kliniska transplantationer och i blodkärl som delvis täppts till.


Sammantaget så visade våra försök att fria levercellerna har en karaktär som befärmjar blodets levring. För övrigt så har vWF-förekomst i leverceller tidigare inte rapporterats.
Arbete III
I detta arbete testades substansen LMW-DS. Denna substans kan hämma både blodets förmåga att levra sig, samt hindra att inflammatioosskapande reaktioner i blodet aktiveras. Vi användes samma typ av ”loop-modell” som i arbete I. Vid tillsats av LMW-DS i loopen med blod och leverceller uppmättes endast låga värden på aktivering av levringsprocesser och inflammation. LMW-DS testades också på leverceller som varit nedfrysta, vilka skulle kunna förväntas skapa mera inflammation. Resultaten visade att i loopen där LMW-DS var till- satt utvecklades endast marginella nivåer av blodlevring och inflammations- processer.

Vi jämförde också LMW-DS med heparin, en substans som regelmässigt används vid levercells transplantation. LMW-DS visade sig ha en mer skyddande effekt än heparin då både mindre inflammation och blodlevring uppstod i loopen med tillsatt LMW-DS jämfört med loopen med heparin.

Våra slutsatser är att den testade substansen uppvisade positiva önskvärda effekter. Den dos av LMW-DS som testades var i nivå med doser som tidigare använts kliniskt. Utifrån detta skulle det vara värdefullt med ytterligare studier av denna substans.

Arbete IV

Vi använde oss av en PEG-lipid som utgörs av två enheter. Den ena är vattenlöslig och den andra är fettlöslig (vattenavvisande). När PEG-lipiden sätts till cellerna i lösning så kommer den vattenavvisande delen att ”fly undan” vättskan som reaktionen sker i och spontant länkas in i cellväggen (som bl a är uppbyggd av fettkjedjor). Den vattenlösliga enheten kommer att hamna utan- för cellväggen och kan då kopplas till heparin.

Våra försök med olika typer av förankningsgrupper visade att man kan få heparinet att sitta kvar olika länge på levercellerna. Så länge som levercellerna var beklädda med heparin så aktiverade de inte inflammation eller levring av blodet.

Sammanfattning
Avhandlingen har visat hur de reaktioner för tidig avstötning kan uppstå och påvisar också tänkbara alternative för att förhindra att levercellerna utlöser dessa skadliga reaktioner. Dessa strategier kan vara lämpliga för fortsatta stu- dier.
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