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Vascular Interactions in Innate Immunity and Immunothrombosis:

Models of Endothelial Protection

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Abstract

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The phenomenon known as immunothrombosis has garnered increased attention over the last few years. Much work has been done to characterize the cross talk between hemostasis and the innate immune system. This thesis outlines the role of the vascular endothelial cells during immunothrombotic events as regulators of coagulation, platelet-, and leukocyte recruitment.

A newly developed method for investigating the interaction between endothelial cells and the blood compartment illustrated the procoagulant and proinflammatory effects elicited by tumor necrosis factor α activated endothelial cells upon exposure to whole blood. The method was utilized in evaluating treatment of endothelial dysfunction and disruption with a heparin conjugate. Damaged or hypoxic endothelial cells, in addition to basement membrane collagen, that were pretreated with the heparin conjugate prior to contact with blood were found to have reduced activation of coagulation, platelet-, and leukocyte recruitment; in contrast to unfractionated heparin, which had no effect on the aforementioned parameters. The treatment was then investigated in the setting of ischemia reperfusion injury during kidney transplantation and the heparin conjugate was found to bind cultured endothelial cells with high avidity under cold storage conditions. Furthermore, it was found to bind to the renal vasculature during static cold storage and was subsequently found to be beneficial with regard to early graft function in an experimental mouse model of syngeneic kidney transplantation. Recipients of kidneys treated with the heparin conjugate had reduced serum creatinine compared to controls 24 hours after transplantation. Lastly, the anticoagulant properties of the heparin conjugate were investigated in comparison to unfractionated heparin. While the conjugate exerted reduced capacity with regard to thrombin inhibition, it rapidly inhibited the binding of platelets to exposed collagen. The conjugate was furthermore found to preferentially locate to sites of endothelial cell activation at early stage during endotoxic shock in mice.

In conclusion, this thesis demonstrates that disrupted functioning of the vascular endothelial cells actively contributes to immunothrombosis, and that it is possible to model endothelial cell function using whole blood assays. Furthermore, this thesis presents a treatment that enhances the hemocompatibility of damaged endothelial cells and subsequently improves the early renal function after kidney transplantation.

Keywords: Endothelial cells, Thrombosis, Innate immunity, Immunothrombosis, Thromboinflammation

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Till Emil

It's not stupid; it's advanced!

- Almighty Tallest Purple

List of Papers

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

I **Nordling, S.**, Nilsson, B., Magnusson, P.U. (2014) A Novel *In vitro* Model for Studying the Interactions Between Human Whole Blood and Endothelium. *Journal of Visualized Experiments: JoVE*, (93):52112

II **Nordling, S.**, Hong, J., Fromell, K., Edin, F., Brännström, J., Larsson, R., Nilsson, B., Magnusson, P.U. (2015) Vascular repair utilising immobilised heparin conjugate for protection against early activation of inflammation and coagulation. *Thrombosis and Haemostasis*, 113(6):1312-22

III **Nordling, S.**, Brännström, J., Carlsson, F., Lu, B., Salvaris, E., Wanders, A., Buijs, J., Estrada, S., Tolmachev, V., Cowan, P., Lorant, T., Magnusson, P.U. (2016) Enhanced protection of the vascular endothelium in porcine and murine kidneys against ischemia reperfusion injury prior to transplantation. *Manuscript.*

IV **Nordling, S.**, Folkesson, J., Brännström, J., Larsson, R., Magnusson, P.U. (2016) A novel anticoagulant targeting vascular wall damage. *Manuscript.*

Contents

Abbreviations

^{111}In	Indium-111
ADAMTS13	A disintegrin and metalloprotease with thrombospondin motifs
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Activated protein C
APTT	Activated partial thromboplastin time
AT	Antithrombin
ATP	Adenosine triphosphate
C	Complement component
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CHC	Corline heparin conjugate
CS	Chondroitin sulfate
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Danger-associated molecular patterns
DGF	Delayed graft function
DS	Dermatan sulfate
EC	Endothelial cell
EPCR	Endothelial protein C receptor
F	Coagulation factor
F1+2	Prothrombin fragment 1+2
FITC	Fluorescein isothiocyanate
GP	Glycoprotein
GPCR	G-protein coupled receptor
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
HUVEC	Human umbilical vein endothelial cell
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IRI	Ischemia reperfusion injury
IU	International units
K_D	Dissociation coefficient

LPS	Lipopolysaccharide
NFκB	Nuclear factor κB
NET	Neutrophil extracellular trap
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PAV	Polyallylamine
PC	Protein C
PG	Proteoglycan
PGI_2	Prostacyclin
PVC	Polyvinyl chloride
QCM-D	Quartz crystal microbalance with dissipation
ROS	Reactive oxygen species
SPECT/CT	Single-photon emission computed tomography/ computed tomography
TAT	Thrombin-antithrombin
TAFI	Thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TNFα	Tumor necrosis factor α
tPA	Tissue plasminogen activator
TSP-1	Thrombospondin-1
TXA2	Thromboxane A2
UFH	Unfractionated heparin
uPA	Urokinase plasminogen activator
UW	University of Wisconsin
VCAM-1	Vascular adhesion molecule-1
VEGF-A	Vascular endothelial growth factor-A
VPB	Veibel-Palade body
VWF	von Willebrand factor

Introduction

Our understanding of the vast crosstalk between innate immunity and hemostasis has increased substantially over the last few decades. To date, multiple points of convergence between inflammation and hemostasis have been recognized and described in the literature as either immunothrombosis (1) or thromboinflammation (2).

There are several evolutionary benefits of the coincidental activation of hemostasis and innate immunity. The entrapment of invading pathogens by thrombus formation may indeed limit the spread of infection (1) and the activation of multiple signaling cascades may enhance any subsequent activation of the adaptive immune system (3). Nonetheless, during conditions of sterile inflammation, immunothrombosis may serve to exacerbate the inflammatory environment, reducing vessel function and, ultimately, organ function (1, 4).

The focus of this thesis is on the role of the vascular endothelial cells (EC) as regulators of immunothrombosis. The overall aim of the included papers was to study the effects of damaged or dysfunctional EC with regard to immunothrombosis, and to evaluate a novel treatment for subduing the effects of functionally perturbed EC.

Blood vessel endothelium

The initial discoveries of circulatory system functioning, which form the basis of our modern comprehension, were made in the 1600s. Wilhelm His was first to suggest the existence of a blood vessel *endothelium* in 1865, and our understanding of endothelial structure was ultimately made possible during the mid-twentieth century, upon invention of the electron microscope (5). Research into the functional role of the endothelium was advanced further by the successful isolation and culture of EC during the 1960s and 1970s (6, 7), which greatly contributed to our increased knowledge of EC biology.

The endothelium has multiple functions, including the transportation of oxygen, nutrients, hormones, and blood cells between the blood compartment and the tissue parenchyma. EC also facilitate blood circulation by participating in the control of vascular resistance and the regulation of hemostasis and inflammation (5). Furthermore, EC exhibit substantial heterogeneity

throughout the vascular tree with regard to their various functions. Differences between EC can be attributed to vessel type (i.e. arterial/ venous/ microvascular EC) as well as to organ specific characteristics. Indeed, the phenotypes of EC vary depending on the function of the organ in question and the microenvironmental cues originating from the surrounding tissue (5). All healthy EC do, however, share a common attribute—they are the only truly hemocompatible surface recognized by modern science (5, 8).

Hemostasis

A disruption of the blood vessel endothelial lining initiates hemostasis, a process involving both platelet adhesion, i.e. primary hemostasis, and the activation of coagulation, secondary hemostasis. Hemostasis is of vital importance to the upholding of normal physiological function and is heavily regulated to avoid uncontrolled events. Uninhibited hemostasis is referred to as thrombosis, a pathological state that may have severe consequences on organ function (4).

Platelets

Platelets are small anuclear cells, or cell fragments, produced by megakaryocytes residing in the bone marrow. Under physiological conditions, platelets circulate in the blood at a density of approximately 200,000/μL. A reduction in the platelet count is referred to as thrombocytopenia, which may indicate a major thrombotic event, or autoimmune disease, or may be a side effect of ongoing medical treatment (9). Platelets are rapidly recruited in the instance of vascular damage, whereupon they secrete multiple prothrombogenic, pro-inflammatory, and tissue remodeling signals that participate in the process of hemostasis, leukocyte recruitment, and wound healing (10).

Platelet granules

The majority of the platelet intracellular space is occupied by secretory granules, from which the platelet contents are secreted upon activation (10). To date, three major types of granules have been recognized:

Dense granules

Dense granules are rapidly secreted following the stimulation of platelets. The factors of dense granules are mainly proaggregating in the sense that they facilitate paracrine stimulation of other platelets in close proximity. Factors stored in these granules include adenosine tri- or diphosphate (ATP or ADP), serotonin, and calcium ions [listed in Table 1 (11)].

Alpha granules

The α-granule is the most abundant form of vesicle present in platelets. Its contents include a wide variety of proteins, some of which are produced already in the megakaryocyte and others that are absorbed from the plasma during the platelet's roughly weeklong life span (12). Estimates regarding the number of proteins stored in α-granules range in the hundreds, of which a partial list is presented in Table 1. The proteins contained within the α-granule are either in a secreted form [e.g. thrombospondin-1 (TSP-1)] or in the form of membrane associated receptors that are presented on the platelet surface upon activation (P-selectin). The majority of these proteins may be viewed either as procoagulants or as factors involved in tissue remodeling or inflammation (11-13).

Lysosomes

Lysosomes are (to date) the least characterized of the platelet granules. It has been suggested that lysosome contents are released late during platelet recruitment and that the glycosidases, proteases, and bactericidal proteins are mainly involved in the clearing of formed thrombi [Table 1 (11)].

Table 1. An abbreviated list of platelet granule contents.

Granule	Contents	Function
Dense granules	ATP and ADP	Platelet activation
	Serotonin	
	Cations; Ca^{2+}, Mg^{2+}	
	Polyphosphate	
Alpha granules	P-selectin	Platelet - EC, Platelet - Leukocyte interactions
	von Willebrand factor	Platelet - Platelet interactions
	Fibrinogen	Coagulation
	FV, FVII, FIX, FXIII, Kininogen	
	TSP-1	Tissue remodeling
	Platelet-derived growth factor	
	Transforming growth factor-β	
	Vascular endothelial growth factor-A	
	CD40L	Inflammation
	CCL3, CCL5, CCL7, CCL22	
	CXCL1, CXCL4, CXCL5, CXCL7, CXCL8, CXCL12, CXCL12	
Lysosomes	Cathepsins	Degradation
	Carboxypeptidases	
	Collagenase	
	Acid phosphatase	
	Heparinase	

Platelet activation

Platelets bound to exposed collagen via the glycoprotein (GP) VI receptor are rapidly activated by intracellular signals triggered by receptor cross-linking (14). Platelets are furthermore recruited to activated EC by binding to von Willebrand factor (VWF) or P-selectin via the receptor complex GPIb-IX-V (15). The initial tethering interaction between platelets and EC is reversible and allows platelets to translocate over the EC surface in a sliding fashion (16). In order to become wholly activated, platelets require a secondary stimulus in the form of soluble agonists such as thrombin, ADP, or thromboxane A2 [TXA2; (14)]. Activated platelets undergo a shape change and secrete granule contents, resulting in the exposure of additional receptors that facilitate aggregation and soluble factors that promote thrombosis and inflammation (17). Platelet aggregation is suggested to differ with variations in shear stress; platelets tend to aggregate via fibrinogen/fibrin in venous shear, and via VWF in higher shear (16).

Coagulation

The activation of coagulation is initiated upon exposure of tissue factor (TF) to the blood stream, triggering the stepwise activation of the serine proteases that constitute the extrinsic coagulation cascade (see Figure 1). TF is constitutively expressed by the surrounding tissue parenchymal cells and some leukocyte subsets, in addition to circulating in the blood stream incorporated in microparticles (18). TF binds serine protease factor VII (FVII); moreover, upon activation, the TF-FVIIa complex is capable of activating FIX and FX. FXa, in turn, binds and activates FV, forming the prothrombinase complex, which catalyzes the activation of thrombin from its precursory form known as prothrombin. Thrombin catalyzes the activation of FV and FVIII, and the complex formation between FVIIIa and FIXa (the tenase complex) results in a positive feedback loop brought about by the activation of additional FX (19).

Thrombin is a potent platelet agonist that triggers platelet activation resulting in granule secretion. Activated platelets, in turn, enhance coagulation by exposing negatively charged phospholipids that facilitate the activity of the prothrombinase and tenase complexes. Moreover; the negatively charged surface on activated platelets triggers the intrinsic pathway by activating FXII (see Figure 1). Indeed, the intrinsic pathway is activated by a number of negatively charged molecules including exposed basement membrane collagen as well as polyphosphates, which are released from activated platelets (20). Whereas hemostasis may be initiated by either the coagulation cascade or platelets, both pathways are dependent on one another for stable clot formation. The resulting platelet plug is stabilized by the cleavage of

fibrinogen into fibrin by thrombin, a process that is also enhanced by the intrinsic pathway (4).

Hemostasis is counteracted by the plasminogen/plasmin system. Plasminogen circulates in a closed form structure that is opened upon binding to fibrin. The structural change in plasminogen enables its activation by either tissue-, or urokinase plasminogen activator (tPA or uPA) into plasmin. Plasmin thus acts locally in the effective degradation of fibrin in a process termed fibrinolysis. Activated plasmin released into the blood stream is rapidly neutralized by α_2-antiplasmin and, to a lesser extent, by α_2-macroglobulin, thus ensuring the restricted activation of the fibrinolysis pathway (21).

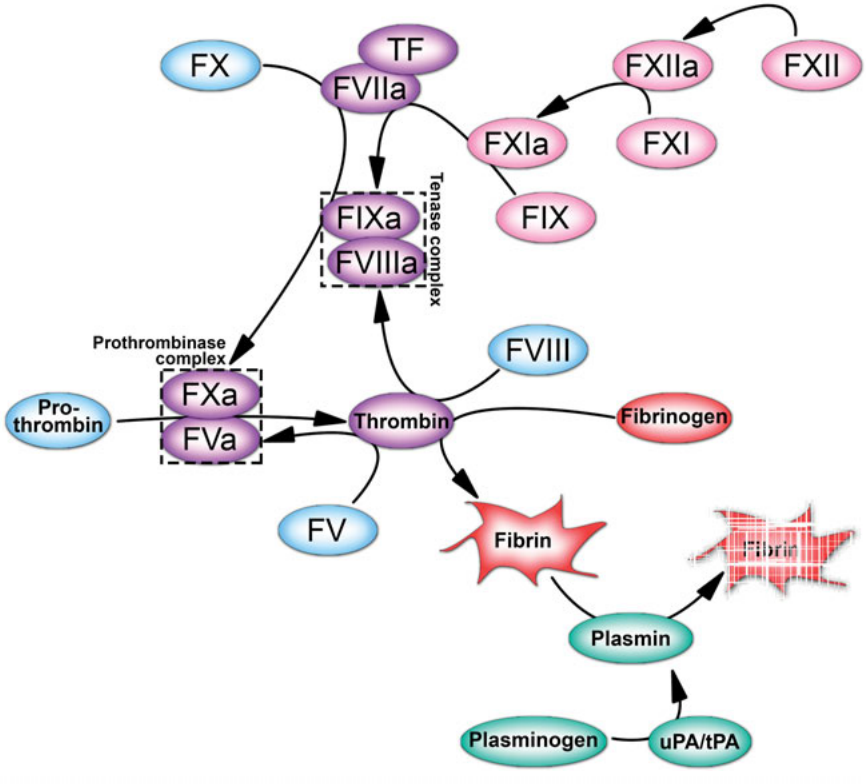

Figure 1. The coagulation cascade is traditionally divided into the intrinsic/contact activation (pink – purple) and the extrinsic/tissue factor (blue – purple) pathways. Both pathways rely on the formation of the tenase complex (FIXa-FVIIIa), which forms a feedback loop for FX activation, and the prothrombinase complex (FXa-FVa), which is responsible for activating thrombin. The final step of both pathways is the conversion of fibrinogen into fibrin (red) by thrombin. Fibrin formation is ultimately counteracted by the plasminogen/plasmin system (green).

Innate immunity

Innate immunity is comprised of a humoral complement system and cellular subsets of granulocytes and monocytes. Of the various granulocytic cells, neutrophils are most commonly associated with immunothrombosis and are thus the only type of granulocyte covered in this thesis.

The complement system

The complement system is traditionally portrayed as three separate pathways of activation converging into one final common pathway. The complement system is thus categorized into the classical, the alternative, and the lectin pathways (22). Substantial cross activation between the initiating pathways, especially through the activation of C3, is well recognized today (2). The common pathway culminates in the formation of C5b-9, or the membrane attack complex, with the ultimate outcome being cell lysis (22).

Several of the soluble moieties released during complement activation, most notably C5a, promote the inflammatory activation of EC, neutrophils, and monocytes (23). Furthermore, the formation of C5b-9 in sublytic concentrations stimulates a number of cell types, including EC, platelets, and leukocytes, resulting in cytokine release and the upregulation of adhesion receptors (24). The immense cross talk between the complement system and the coagulation system has been suggested to both trigger and augment immunothrombosis (25).

Neutrophils

Roughly 60% of circulating leukocytes are neutrophils, making this the most abundant subset. Neutrophils have a short life span (5-50 hours) and are rapidly recruited to sites of inflammation, where their main function is to eradicate invading pathogens by phagocytosis, the release of bactericidal proteins or reactive oxygen species (ROS), or entrapment in neutrophil extracellular traps [NET; (26)].

Neutrophils recruited to activated EC participate in immunothrombosis by releasing ROS and proteases, which cause the degradation and shedding of anticoagulant mediators normally expressed on the EC surface (27). Neutrophils have also been reported to participate in TF dependent coagulation (28), and TF expression in neutrophils may be further augmented by C5a stimulation (29). Furthermore, NETs have been implicated in both platelet activation and the activation of FXII, further embroiling neutrophils in the process of immunothrombosis (1).

Monocytes

Monocytes are a heterogeneous population of cells constituting approximately 5% of circulating leukocytes. Monocytes are sub-characterized into a classic pro-inflammatory subtype and a nonclassical patrolling subtype (30). In contrast to the classic monocyte, the nonclassical subtype has been found to constantly interact with EC in a surveying capacity. Upon tissue damage or infection, the nonclassical monocyte quickly extravasates and begins the initial production of tumor necrosis factor (TNF) α and interleukin (IL)-1, contributing to the recruitment of classic monocytes and neutrophils (31). Intravascular monocytes may also contribute to the activation of hemostasis by expressing TF upon recruitment by activated platelets to sites of injury (4).

Following extravasation, monocytes gain phagocytic or antigen presenting capabilities depending on inflammatory stimuli and with a certain level of plasticity existing between phenotypes (32, 33). Furthermore, the extravasated monocytes, or macrophages, stimulate and modulate downstream inflammatory events in the parenchyma by secreting additional cytokines and chemokines, including (but not limited to) chemokine (C-C motif) ligand (CCL)-2, IL-6, and chemokine (C-X-C motif) ligand (CXCL)-8 (34).

Endothelial regulators of hemostasis

Healthy and undisturbed EC are covered by a substantial glycocalyx with a thickness ranging from approximately 0.5 to 4 um (35, 36). The glycocalyx is composed of GP and proteoglycans (PG), with polysaccharide side chains consisting of heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS). The glycocalyx is involved in multiple functional processes including permeability, the binding of growth factors and cytokines, and the binding of coagulation and complement regulators (37-39).

One such regulator of coagulation is antithrombin (AT), a serine protease inhibitor, or serpin, which interacts with and inactivates several components of the coagulation cascade, including thrombin, FVIIa, FIXa, FXa, FXIa, and FXIIa. The irreversible bond between AT and any of the activated serine proteases occurs at a 1:1 ratio. Moreover, AT activity is potentiated in a multifold fashion by a specific pentasaccaride motif present in heparin (see figure 2A), but less frequently expressed in HSPG presented by EC (40, 41). While the binding of AT by HS might differ from the one elicited by heparin (41, 42), it remains an important part of the anticoagulatory phenotype of the endothelium.

EC directly regulate activation of the TF-pathway through the expression of tissue factor pathway inhibitors (TFPI). EC express both the α and β isoforms of TFPI, formed by alternative splicing of the same pre-mRNA.

TFPIβ contains a C-terminal GPI anchor and remains membrane bound, while TFPIα is secreted but continues to be associated with the EC glycocalyx. Both isoforms inhibit TF-FVIIa and FXa; additionally, TFPIα has the ability to obstruct the entire prothrombinase complex (43).

Nearly all EC, aside from microvascular cells in the brain, constitutively express thrombomodulin (TM), a membrane bound GP that binds and neutralizes the coagulant function of thrombin. The binding of thrombin to TM potentiates the interaction between thrombin and protein C (PC), promoting the cleavage and subsequent transformation of PC into activated PC (APC). The interaction between thrombin and PC is further enhanced by the binding of PC to the endothelial protein C receptor (EPCR), which is an additional regulator expressed by EC. APC inhibits both FVa and FVIIIa, causing a reduction in thrombin formation (44).

The thrombin-TM complex also exerts anti-inflammatory properties by stimulating thrombin-activatable fibrinolysis inhibitor (TAFI); as the name suggests, TAFI inhibits fibrinolysis, as well as C3a and C5a, upon complement activation. The regulation of fibrinolysis is further facilitated by EC through the expression of tPA, uPA, and plasminogen activator inhibitor-1 (PAI-1). It is important to note that tPA, uPA, and PAI-1 have opposing functions during fibrinolysis, with the latter able to inhibit the former two (44, 45).

Furthermore, EC possess several means of inhibiting platelet adhesion and activation. The negatively charged glycocalyx acts as a repellant toward platelets and erythrocytes. Indeed, the glycocalyx is often referred to as *a red blood cell exclusion zone* formed along the endothelium (36). The glycocalyx has also been suggested to contribute to the shear dependent mechanotransduction that regulates nitric oxide (NO) production by the EC (46). Platelet activation is inhibited by the constitutive release of NO and prostacyclin (PGI_2), in addition to the expression of membrane bound CD39, a GP that catalyzes the hydrolysis of platelet agonist ADP to AMP (47).

Endothelial activation during acute inflammation

EC are activated by several inflammatory stimuli that trigger a more pro-inflammatory, procoagulant, and anti-fibrinolytic phenotype. To this end, two states of EC activation have been proposed; type I activation entailing a rapid and reversible response, and type II, which describes a long-lasting state that includes *de novo* protein synthesis (48). It should, however, be noted that there are several cases in which the signaling involved in type I and II activation are not mutually exclusive and that, furthermore, one stimulus may in fact alter the response of another (49, 50).

Type I activation

Type I EC activation is driven by G-protein coupled receptor (GPCR) signaling with agonists that include thrombin, histamine, and bradykinin (51). The downstream intracellular signaling triggers an increase in NO and PGI_2 production resulting in vasodilatation, an effect that is substantially more pronounced in arterial vessels (51). Signaling also produces cytoskeletal rearrangement, which increases the permeability of the endothelium (48). This effect is more noticeable in post-capillary venules, which are supported by fewer tight- and adherence junctions (52).

Furthermore, type I activation causes rapid exocytosis and content release of so-called Veibel-Palade bodies (VPB) present in the EC cytoplasm (53, 54). VPB are EC-specific intracellular vesicles, of which VWF and P-selectin constitute the majority of the contents (54). VWF is secreted in the form of ultralarge multimeres, which are rapidly cleaved by A disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) 13 present in the blood compartment. VWF binds platelets and collagen, while exerting a procoagulatory function by binding to FVIII and protecting it from inhibition by APC (55, 56). Unlike VWF, P-selectin contained in the VPB remain on the EC surface, facilitating interaction between the activated EC and platelets or leukocytes (57).

Type II activation

Additional EC activation markers are upregulated by *de novo* synthesis via the nuclear factor κB (NFκB) pathway upon prolonged stimulation by leukocyte-derived TNFα or IL-1 (48), or by hypoxia (58), bacterial lipopolysaccharide [LPS; (59)], or platelet-derived CD40L (60). The NFκB pathway has been implicated in the transcriptional upregulation of TF (61, 62), downregulation of TM by competitive inhibition (44), downregulation of NO production (63), downregulation of tPA and uPA, and the upregulation of PAI-1 (45), thus rendering the EC more procoagulant and anti-fibrinolytic upon activation. Upregulated expression of luminal adhesion proteins including E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular adhesion molecule (VCAM)-1 promotes leukocyte recruitment towards the activated EC (49, 64, 65) and the degradation of EC junction proteins (48, 66) facilitate the subsequent leukocyte extravasation. The expression of soluble chemokines such as CCL2 and CXCL8, which remain associated with the EC glycocalyx and stimulate leukocyte recruitment, is also increased during type II activation of EC (48, 53, 67).

Despite possessing the crucial function of maintaining a chemokine gradient during early inflammation, the glycocalyx is eventually shed of the EC surface. Conflicting data as to whether the shedding of the glycocalyx promotes or reduces the actual number of extravasating leukocytes significantly

limits our understanding of the event (68). Although EC-derived heparanase has been suggested to cause the cleavage of the HS side chains (69), the precise mechanism which causes shedding of the membrane-bound PG core proteins remains largely unclear (70). Increased plasma concentrations of glycocalyx constituents have, however, been reported in several clinical conditions associated with inflammation (70) suggesting that the shedding process is a genuine effects of EC activation.

Kidney transplantation

Transplantation is the only curative approach to end stage renal disease, a disorder brought on by a number of underlying conditions such as diabetes, obesity, and hypertension (71). Kidney transplantation utilizing living donors are highly successful, with a 5-year graft survival rate of approximately 86% (72). The majority of transplanted kidneys are, however, currently derived from deceased donors (73) and these organs have a lower engraftment rate of approximately 77% 5 years post-transplantation (72). The discrepancy between living and deceased donors is commonly ascribed to the multiple physiological changes coinciding with brain death, as well as longer ischemia times during cold storage prior to implantation (74).

Following the initial catecholamine storm that is triggered by brain death, the systemic circulation of the donor is exposed to a period of hypotension and hypoperfusion (75). Hypoperfusion throughout the vasculature leads to extensive coagulation (76); approximately one-third of brain dead donors present signs of disseminating intravascular coagulation (77). Experimental transplantation models utilizing brain dead donors, as well as biopsies of human kidney grafts, have highlighted the vascular effects of brain death by reporting increased EC activation, which ultimately results in increased infiltration upon the restoration of blood flow in the recipient (78, 79).

Ischemia reperfusion injury and delayed graft function

Following removal from a deceased donor, kidneys are unavoidably exposed to ischemic storage for a period of approximately 20 hours prior to transplantation (80). Despite the substantial advances that have been made with regard to the formulation of preservation solutions (81); the hypoxic environment (74), the formation of danger-associated molecular patterns [DAMPs; (82)], and the temporary lack of shear stimulus on the EC (83) have all been implicated in contributing to the ischemic damage of the organ during storage. Consequently, disrupted blood flow is immediately detectable upon restoration of blood circulation to transplanted kidneys taken from deceased donors (84). The immediate perturbation of vascular function is defined as ischemia reperfusion injury (IRI), and activation of hemostasis

and the complement system is recognized as a substantial driver of subsequent events (85). Microthombi are rapidly formed (86), followed by measurable shedding of the EC glycocalyx within the first minutes of reperfusion (87). The increased platelet-, and neutrophil recruitment that occurs 20 minutes after connection of the renal vasculature to the recipient is subsequently associated with an increased risk of delayed graft function [DGF; (88)]. Approximately 25% of recipients of kidneys from deceased donors experience DGF, compared to only 5% of recipients with living donors (74, 89). DGF is generally defined as the need for dialysis during the first week after transplantation, or alternatively, by the absence of a decrease in serum creatinine levels for three consecutive days during the first week (90). DGF may have far-reaching repercussions, as it ultimately raises the risk of reduced long-term graft function (91).

Anticoagulation

Unfractionated heparin (UFH) and vitamin K antagonists, also known as Warfarin or Coumarin, were the first anticoagulants to be introduced into clinical settings. While the use of both drugs is rather limited today, their introduction paved the way for the development of several other modern anticoagulants (92).

UFH consists of a heterogeneous blend of highly sulfated polysaccharide chains (molecular weight 3-30 kDa) that are typically isolated from porcine intestinal mucosa (93). The anticoagulant properties of UFH, and of all heparins for that matter, stem from a specific pentasaccaride sequence (Figure 1A) that interacts with AT and subsequently potentiates the inhibitory effects of the latter. The conformational change in AT which is generated by heparin mainly targets the inhibition of FXa; however, UFH is also able to facilitate the interaction between AT and thrombin due to the longer chain lengths that stabilize the binding between the aforementioned (94).

The diminished clinical use of UFH is the result of several uncommon, albeit serous, side effects, including heparin induced thrombocytopenia and heparin induced osteoporosis. Furthermore, due to its propensity for unspecific interactions with plasma proteins and vascular cells, UFH demonstrates largely unpredictable pharmacokinetics upon administration (95). The unfavorable attributes of UFH prompted the development of low molecular weight heparins, which have since proven to possess more stable pharmacokinetics (95) and have exhibited fewer reported side effects (96). Modern anticoagulants are referred to as either direct thrombin inhibitors (97) or direct oral anticoagulants (98). Although anticoagulant therapy has improved immensely during the last few decades, there are still hurdles to overcome. Two major difficulties facing modern anticoagulation efforts involve a lack of reliable antidotes against several of the available treatments (99) and spe-

cifically treatment of patients with renal failure, as most drugs are cleared via the kidneys (100).

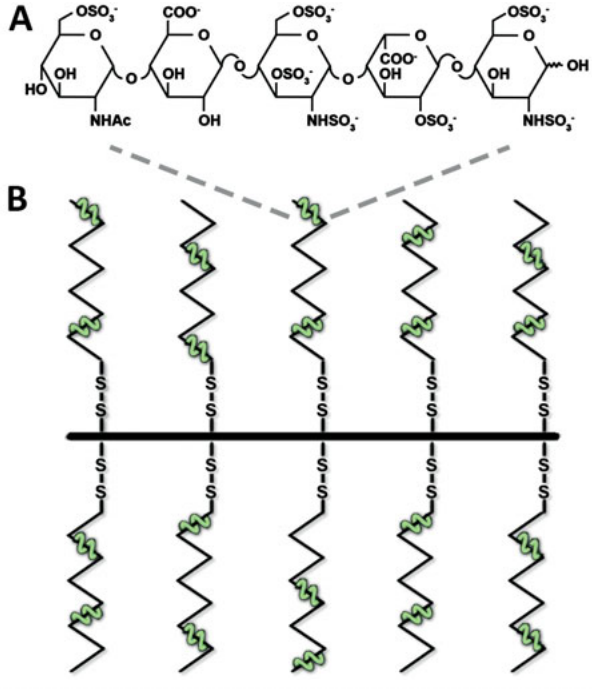

Figure 2. The pentasaccharide sequence responsible for the potentiating interaction between heparin and AT (A). A schematic illustration of the brush-like structure of CHC (B); the heparin side chains that are equipped with several pentasaccharide sequences each (stylized in green) are coupled to the PAV-backbone via disulfide bridges.

Corline heparin conjugate

Corline heparin conjugate (CHC) was developed roughly 20 years ago by Larsson et al (101) in an effort to improve the hemocompatibility of artificial surfaces. The brush-like conjugate (Figure 1B) consists of more than 20, typically 70, heparin molecules coupled to a polymeric amine carrier chain (PAV) by disulfide bridges (102). CHC has successfully been studied with the intent of reducing blood compartment activation during cardio pulmonary bypass (103, 104), it has been applied clinically to improve coronary stent lifetime (105) and preclinically to enhance pancreatic islet transplantation (106-109).

During most applications, surfaces are preconditioned with a linker molecule prior to the addition of CHC in order to facilitate binding of the conju-

gate. Artificial surfaces are pretreated with PAV, which supplies a surface of positively charged amine groups that may then bind to CHC (101, 102). Surfaces coated with CHC-PAV, also known as a Corline heparin surface (CHS), have routinely been used for experiments with whole blood over the last decade (110, 111). Islets of Langerhans, on the other hand, are coated with CHC via a multistep process involving the use of biotin and avidin prior to the application of CHC (106). CHC-coating has indeed improved the hemocompatibility of all of the aforementioned surfaces by reducing the activation of coagulation and complement system, in addition to reducing platelet and leukocyte recruitment.

Until recently, only the linker-facilitated coating techniques for CHC binding were available. The investigations in this thesis demonstrated binding of the conjugate in a one step process to activated or damaged EC thereby facilitating protection of the vasculature.

PRESENT INVESTIGATIONS

PAPER I

Background and aims

Studies of hemostasis and the early activation of the complement system have, for quite some time, been carried out utilizing human whole blood (110). Whole blood models are of great value when assessing interactions within the blood compartment; however, very few methods incorporate an interaction with the blood vessel endothelium. The aim of paper I was thus to develop an *in vitro* tool that would enable the study of interactions between human whole blood and primary human EC. The paper was published in the format of a video that illustrated the method, and representative results obtained by said method. The visualized experiment can be found at:

http://www.jove.com/video/52112

Results and discussion

The blood-EC chamber model was based on a previously published method for studying the interactions between artificial surfaces and whole blood (112). The fairly simplistic design of the model enabled the blood to be kept in motion by a gentle mechanism, which produced minimal background activation of the blood while in contact with a layer of cultured EC.

Early thrombotic and inflammatory events are in fact easily triggered in whole blood *ex vivo* [(8, 110) and personal experience]. Platelets are rapidly activated upon exposure to shear stress extremes (113), and the cascade-like functioning of the coagulation and complement systems increases the risk of accumulating activated components in the blood. As such, 0.75 IU/mL UFH was added to the blood to further reduce the risk of inadvertent activation. Additionally, during all whole blood experiments (in this and subsequent papers), an open system venipuncture procedure was used so as to avoid considerable activation of the blood prior to experimentation. The blood was thus acquired through a small gauge hypodermic needle coupled to heparin-coated silicon tubing and let out by gravity; as opposed to drawing the blood using vacuum extraction tubes.

Research began by confirming previously published work (111) regarding the benefits of a heparin-coated surface in whole blood experiments. The

chamber model was assembled with uncoated inner walls and blood. Results clearly indicated that despite the addition of a small amount of anticoagulant to the blood inside the chambers, immense clotting occurred within 30 minutes if the chambers were unprotected by a heparin surface. The formation of thrombin-antithrombin (TAT) complexes was used to quantify the activation of coagulation (114). Whereas the TAT concentration in plasma isolated immediately after procurement was typically < 5 μg/L (data not shown), the concentration of generated TAT in the plasma of the clotted chambers was well over 6000 μg/L, reflecting the immense activation of coagulation when the heparin surface was not present.

The effect of different volumes of blood added to the chambers was also assessed. Leaving space for the formation of an air bubble by neglecting to fill the chambers completely enabled the blood inside the chambers to be kept in motion when the chambers were subsequently rotated along a vertical axis. Previous studies have, however, proposed that the cascade systems in blood may be activated at an interface between blood and air (115-117). The chambers were thus filled with two different volumes of blood in order to study the effect of differently sized air bubbles on the activation of coagulation. Despite expectations that a larger air-blood interface would increase the activation of coagulation, the activation of coagulation was instead reduced by a larger air bubble. The reduction of TAT due to the presence of a larger air bubble and smaller blood volume may be explained by an increased mixing of the system with 1.5 mL of blood as compared to 1.75 mL. Indeed, the centermost volume of blood—which was not kept in motion by the air bubble—was increased when a larger volume of blood was added, resulting in a smaller air bubble. Reduced mixing presumably increased the likelihood of interaction between coagulation factors (118); thus, by increasing the volume of blood not being mixed, the activation of coagulation was also enhanced.

Interestingly, TAT formation remained similar between the two different blood volumes in the presence of human umbilical vein EC (HUVEC), which supported the notion of active endothelial regulation of coagulation. To further examine the interaction between EC and blood, HUVEC stimulated with TNFα were evaluated in contact with whole blood. In accordance with previous publications (34), a greater number of neutrophils adhered to TNFα stimulated HUVEC following blood contact as compared to unstimulated cells; results were quantified by immunofluorescent staining. Again, no difference was observed between varying blood volumes in the presence of EC, regardless of TNFα stimulation. Plasma samples collected following blood contact with the endothelial cells were again evaluated to examine the activation of coagulation; moreover, TNFα stimulated cells caused an approximate doubling of TAT in the plasma samples, in accordance with previous observations (44, 45, 61, 62).

PAPER II

Background and aims

Whereas one of the experiments in paper I demonstrated the importance of coating an artificial surface with heparin conjugate prior to blood contact in order to eliminate clotting, paper II investigated the feasibility of coating a damaged vascular wall with heparin conjugate. The aim of paper II was thus to evaluate whether the binding of CHC to activated EC and collagen would have a reducing effect on the activation of hemostasis and leukocyte recruitment.

Results and discussion

An investigation was conducted to determine whether CHC would bind to EC and to the underlying collagen layer upon which the cells were cultured. HUVEC treated with CHC were stained with avidin-Texas Red [which binds to the heparin chains of the conjugate (119)] and were shown to display a strong signal close to the cell junctions; additionally, results showed a clear binding to any exposed collagen between cells. A weaker, but still distinct signal was also recovered over the entire surface when the cells were imaged with super resolution structured illumination microscopy. No effect was seen on any of the investigated functional parameters; treatment did not seem to interfere with the migration, proliferation, or viability of the treated EC, suggesting that CHC formed an inert layer on the cultured EC.

Heparin is known to bind to EC, platelets, and a wide range of growth factors and chemokines (120). It should thus be noted that the results of the *in vitro* experiments presumably did not reflect the complex chemical environment present *in vivo*. However, the effects of CHC treatment on EC in the presence of vascular endothelial growth factor (VEGF)-A were also investigated, as the binding of the latter to its receptor has been shown to be modulated by heparin (121). No changes were observed in the phosphorylation of downstream intracellular signaling events when cells were simultaneously treated with CHC and VEGF-A (unpublished data).

In order to assess hemocompatibility, i.e., the anticoagulant and antiplatelet effects of CHC bound to collagen, a comparison of previously characterized CHC bound to a PAV-preconditioned surface was conducted. A previously published blood loop model (111) was used to place non-anticoagulated human whole blood in contact with the two different surfaces, which were applied to the inner wall of polyvinyl chloride (PVC) tubing. The ends of the PVC tubing were joined together subsequently forming a loop, and the blood inside the loops was then rotated for one hour in a heating cabinet at 37°C. No difference in hemocompatibility was ascertained between CHC-collagen and CHC-PAV, demonstrating the feasibility of re-

taining the functional protection of an exposed vascular basement membrane. Interestingly, UFH treated collagen had no effect on platelet adherence, platelet activation, or the activation of coagulation.

The discrepancy between CHC and UFH was hypothesized to be due to structural differences among the two heparins; whereas CHC would be able to simultaneously bind a surface and engage in interactions with AT, UFH would not retain availability of its active AT binding sites upon being bound to a surface. Quartz crystal microbalance with dissipation (QCM-D) was used to investigate the binding of AT to either CHC or UFH treated collagen. Following the addition of a layer of collagen to the sensor surface, CHC or UFH was adsorbed onto the collagen. While CHC was shown to readily bind to collagen, no measurable binding of UFH to the collagen layer was registered, in contrast to the findings of previous publications (122). However, it should be noted that the collagen layer formed during the QCM-D measurements was thick, and thus the possibility exists that the binding of UFH was concealed by the viscoelastic properties of collagen. Subsequent binding of AT to CHC, but not to UFH, confirmed the availability of active sites on CHC following the binding to collagen.

CHC treatment was then investigated in relation to two different models of EC disruption. The first model was made to resemble a vascular wound where the basement membrane was exposed to the blood compartment. A scratch was made in a monolayer of HUVEC cultured on collagen and was subsequently assayed in the blood-EC chamber model. Upon blood contact, the exposed collagen triggered the extensive recruitment of platelets consistent with observations made during the blood loop experiments. TSP-1, one of the many mediators secreted by activated platelets, was measured in the plasma isolated from blood, demonstrating a time dependent increase in platelet activation; all was in accordance with previous publications regarding the effects of platelet-collagen interactions (14).

Whether the accompanying increase in TAT formation should be attributed to TF secretion from the activated platelets (11, 12) or to the activation of FXII by collagen and platelet aggregates (4) remains to be resolved. Nonetheless, the pretreatment of cells and exposed collagen with CHC, but not with UFH, prior to blood contact reduced both platelet activation and the activation of coagulation as it completely inhibited the binding of platelets to the wound.

A contemporary publication illustrated the possibility of utilizing CHC during organ preservation prior to transplantation. When added to the preservation solution, porcine kidneys subjected to hypothermic machine perfusion showed the binding of CHC to the renal vasculature (123). Consequently, the CHC treatment of hypoxic HUVEC in the blood-EC chamber model was evaluated in order to investigate the effects on a model of IRI. HUVEC were placed in 1% O_2 at 4°C for 16 hours prior to blood contact in order to simulate the effects of organ storage prior to transplantation.

In accordance with previous findings (124-126), the hypoxic HUVEC elicited a procoagulatory phenotype, triggering increased TAT formation and platelet adhesion. However, the hypoxic HUVEC were unable to trigger measurable platelet activation (as assessed by the concentration of TSP-1). Whether the increased platelet adhesion resulted from increased P-selectin (57) or VWF (55) expression on the EC surface remains unclear. Increased platelet recruitment may also have been the result of fibrin deposits on hypoxic EC (127); fibrin is a weaker platelet agonist than collagen, which would explain the lack of increased platelet degranulation. Furthermore, the proinflammatory activation of hypoxic HUVEC was evidenced by the increased recruitment of $CD16^{+}$ neutrophils. CHC treatment prior to blood contact was, however, able to reverse the effects of hypoxia, with TAT formation, platelet adhesion, and leukocyte recruitment similar to that seen in normoxic HUVEC.

PAPER III

Background and aims

Paper III was a continuation of paper II, whereby the proposed treatment of vascular damage with CHC was investigated in experimental models of clinical kidney transplantation. The aims of paper III were to determine the properties of CHC-EC interactions, to investigate CHC-treatment of the renal endothelium during static cold storage, and to examine the effects on transplantation outcomes.

Results and discussion

The kinetics of fluorescein isothiocyanate (FITC) labeled CHC binding under cold storage conditions to cultured microvascular EC were measured using two different methods: a static saturation binding assay and a Ligand Tracer assay, which followed the binding in real time. The ensuing results from both assays were analyzed, assuming a model where CHC binds to only one site on the EC. While the limitations of this binding model are acknowledged, the model was chosen because no definite knowledge as to the real relationship of the binding was available, and the appearance of matching between the measured binding curves and fitted curves was satisfactory.

The saturation binding assay suggested an equilibrium dissociation coefficient (K_D) of approximately 40 µg/mL, suggesting that upon reaching a steady binding state at this concentration, half of the binding sites would be occupied on the EC. However, the saturation binding assay is limited with regard to resolution (128), as there is no way of determining whether the binding has in fact reached a steady state upon termination of the experiment—in this case, four hours. Hence, the real time binding was monitored with the Ligand Tracer assay using three different concentrations of CHC in an attempt to surmise how long it would take to reach a steady state. The binding reached a steady state after approximately two hours with 50 and 100 µg/mL CHC, and after approximately one hour with 500 µg/mL CHC.

The dissociation phase was also monitored in real time with the Ligand Tracer assay. Dissociation of bound CHC from the EC occurred very slowly, as only about 3% of the bound CHC detached per hour; this suggested that the binding of CHC to EC occurred at very high avidity. Furthermore, the apparent K_D was calculated from the association and dissociation rate constants obtained from a curve fitting of the measured binding. The real time binding experiments suggested a K_D of approximately 1 µg/mL; i.e., much lower than that obtained from the saturation binding assay. The difference between K_D values may reflect the difference in the experimental conditions

of the two assays, or may suggest that true binding is more complex than what was assumed in the binding model.

Whereas the saturation binding assay was static, i.e., the CHC-containing solution was added to the cells and incubated without any motion, the real time binding measurements were obtained under conditions of flow, as the solution was continuously passed over the cells. It was only possible to speculate as to whether conditions of shear may have affected any binding sites on the EC, or whether the conformation of CHC itself may have been altered under shear. However, in generalized terms it would appear that under static conditions, fewer binding events seemed to occur.

A more tangible explanation of the differing K_D values would involve the utilization of an incorrect binding model. Previous work on heparin-EC interactions suggests the lack of a specific receptor for heparin and indicates that the binding was wholly dependent on electrostatic interactions (129). As such, binding would not rely on one singular site, but on several. A more accurate model would thus have accounted for multiple binding sites exhibiting varying affinities on the EC. However, without more information regarding the nature of the binding, such a model could not have been reliably implemented.

Content with the knowledge that the binding of CHC to EC demonstrated high avidity, radioactively labeled CHC was used in order to quantify the amount that would bind to the renal vasculature of kidneys treated under cold storage conditions. The choice of porcine kidneys was based on similarities to the human kidney (130) and donors were rendered brain dead prior to examination in order to mirror the most commonly utilized clinical donors (73). The labeling of CHC with indium-111 (^{111}In) was facilitated by the use of a previously characterized chelation linker that had demonstrated high stability *in vivo* (131, 132). During decay, ^{111}In emits gamma radiation by electron capture and has a half-life of 2.8 days, making it suitable for use in radioactive tracers (133).

It was first verified that the kidney vasculature had been wholly perfused via the renal artery, both when adding the labeled CHC diluted in University of Wisconsin (UW) preservation solution and when removing any unbound CHC with normal saline by measuring the flow-through from the renal vein in a gamma-counter. The retained radioactivity was then measured in the kidney tissue, which was separated according to cortex and medulla. While it was definitively concluded that the CHC had in fact bound within the kidney, it was not possible to conclusively determine whether binding had occurred to a similar extent throughout the entire renal vasculature.

Additional kidneys were subsequently treated in a similar fashion and imaged with single-photon emission computed tomography (SPECT)/computed tomography (CT) in order to evaluate the location of the bound CHC. The resolution of SPECT/CT did not allow for specific detection in the vasculature, but it did reveal clues about distribution with regard to major kidney

anatomy. The majority of the bound CHC was present in the cortex and outer medulla. Interestingly, the hot spots, which implied a higher concentration of CHC, present in the kidney images prior to the saline wash seemed to correspond reasonably well to areas which registered a higher binding after washing. Autoradiography was performed on biopsy sections of the treated kidneys and it was found that most CHC seemed to be bound to the cortex and outer medulla. These results were unlike those seen from an examination of mouse kidneys treated with FITC-labeled CHC under cold storage conditions, where the strongest signal originated from the medulla.

The most logical reason for the discrepancy in binding within murine and porcine kidneys involves a difference in the conditions that the organs were subjected to prior to treatment. The murine kidneys were explanted from previously healthy donors and the CHC-treatment was immediately applied to the kidneys upon procurement. The porcine kidneys, on the other hand, were subjected to circulatory shock triggered by the event of brain death in the donor (75), followed by static overnight cold storage prior to CHC-treatment. Prior research has suggested that medullary and cortical circulation are differently regulated within the kidney, and that (some) regulators of vascular tone may have a more pronounced effect on the medullary vessels (134, 135). As such, a more marked vasoconstriction of the vessels in the medulla could account for the fact that CHC was unable to reach the innermost part of the porcine kidney; this is further evidenced by the slightly lower signal registered in the medulla by the SPECT/CT during treatment. Furthermore, the CHC solution was perfused through the kidney solely via gravity, making it unlikely that the solution would have been able to force its way into the medulla in the case of significant vasoconstriction.

The functional effect of CHC-treated murine kidneys was investigated using a syngeneic transplantation model. Donor kidneys were explanted, stored for 5 hours in UW solution supplemented with CHC, and transplanted into nephrectomized littermates. In contrast to the many transplantation models focusing on long-term rejection (136), the recipients in this experimental setup were nephrectomized to enable assessment of the early renal function with serological parameters. The recipients were sacrificed 24 hours after transplantation and the histology of the kidneys was assessed.

The histology of control kidneys treated with UW alone showed signs of thrombotic events in the medulla; this was in contrast to the CHC treated kidneys, where thrombosis was not as evident. Immunofluorescent staining of platelets supported the histological findings, with slightly fewer platelets found in the CHC-treated kidneys. However, whether the platelets differed with respect to activation is yet to be investigated. The presence of infiltrating leukocytes was also investigated by staining for neutrophils and monocytes; preliminary results suggest a slight reduction of both cell populations in the CHC-treated kidneys.

Finally, renal function was examined by measuring the concentration of creatinine in the serum of the animals. Recipients of CHC-treated kidneys had a significantly lower serum creatinine concentration, suggesting that CHC-treatment may in fact have a protective effect on renal vasculature with regard to the capacity for creatinine filtration.

Currently, no data exists on the long-term effects of CHC treatment in transplanted kidneys. Preliminary *in vitro* data of cultured EC suggest that the cells consume and discard CHC over a 72-hour period (data not shown). As such, any protective effect might only be present during the very initial phases of transplantation. Multiple reports on human kidney transplantation do suggest that a very early perturbing effect may have severe consequences later on (91); but whether the early protective effect of CHC would translate into improved graft survival remains to be determined.

PAPER IV

Background and aims

Whereas papers II and III investigated the application of CHC on damaged EC prior to reintroducing contact with blood, paper IV was a study of the effects of CHC delivery to blood. The aim of paper IV was to study the anticoagulant and antiplatelet activity of CHC and to evaluate the potential targeting feature of enhanced binding to vascular damage.

Results and discussion

Heparins are characterized according to their capacity for potentiating AT dependent inhibition of FXa. The measurement of this capacity is specified in international units and the biological activity of a heparin is thus reported in the unit of IU/mg (137). In papers I-III the amount of CHC used was specified in µg/mL; however, to properly evaluate the anticoagulant capacity for paper IV, the FXa inhibiting capacity of CHC was quantified. Using a standard curve of UFH with known biological activity, it was ascertained that the activity for CHC was 106 IU/mg.

Activated partial thromboplastin time (APTT) is routinely used to monitor the anticoagulant status in the plasma of patients receiving UFH (95). This assay measures the time required for the formation of fibrin to occur in the plasma following the addition of an activator of the intrinsic pathway. The assay was used to compare CHC and UFH at similar concentrations, and UFH treated plasma had consistently longer fibrin formation times when compared to the corresponding CHC concentration. The difference in APTT was attributed to CHC's limited capacity for the facilitation of thrombin inhibition, as the investigated concentrations of the two heparins were similar with regard to FXa inhibition.

The effect on thrombus-formation was investigated in the collagen-coated blood loop assay, which was also used in paper II. In contrast to the experiments of paper II, where the collagen-coated loops were pre-treated with UFH or CHC prior to the addition of blood, the experiments of paper IV called for the addition of the heparins to the blood shortly before filling the loops. The effect of UFH and CHC on thrombus formation was compared at three different concentrations, and despite the noted dissimilarity of the APTT, both macroscopic clot formation and platelet consumption was similar across the examined groups.

The activation of thrombin in the loops was measured by an assessment of the concentration of prothombin fragment 1+2 (F1+2), which is released when prothrombin is cleaved into its active thrombin configuration (138). Blood supplemented with CHC prior to loop addition had consistently higher concentrations of F1+2, corroborating the APTT results. Furthermore, CHC

treated blood contained more activated platelets as compared to UFH, which may be an effect of the increased thrombin presence.

In order to rationalize the similarities in thrombus formation despite the reduced capacity for thrombin inhibition of CHC, the collagen-coated inner tubing wall was investigated following contact with blood. The number of platelet clusters adhering to the collagen was found to be lower when the blood was supplemented with CHC. Clear evidence of CHC-covered collagen was also found, suggesting that the uptake of CHC was extremely rapid and was able to confer protection against adhering platelets.

Interestingly, CHC was found bound to activated platelets, but not to resting platelets. The interaction between heparin and platelets has been characterized previously, and although some data suggest that the binding occurs via GPIIa-IIIb, most research indicates that there is not one specific binding site on platelets for heparin. When heparin binds to platelets, the latter is activated, as evidenced both by *in vitro* and *in vivo* studies (139). The increased binding of CHC to activated platelets may thus be explained by the fact that the platelets were simply activated by CHC. Preliminary data (not shown) on isolated platelets suggests that this is not the case; however, this question must be addressed further.

The rapid binding to collagen in the blood-endothelial chamber model was confirmed using the model of the vascular wound developed in paper II. There was a clear reduction in CHC as compared to UFH with regard to the amount of adhering platelets and leukocytes to the exposed collagen; moreover, a clear binding of CHC to cultured EC was found. Cultured EC are known to undergo phenotypic changes (140), including a clear reduction in the size of the apical glycocalyx (141). This prompted an investigation as to whether CHC would be able to target EC that had shed the luminal glycocalyx *in vivo*.

Intravenous (i.v.) injection of LPS has previously been reported to cause the shedding of the glycocalyx from the pulmonary endothelium (69). Thus, mice were injected with a high concentration of LPS i.v. followed by FITC labeled CHC in order to determine whether CHC-binding would target the affected lung vasculature. Control mice were injected with saline prior to receiving CHC, and animals were sacrificed two hours after the first injection. LPS treatment did indeed result in a significant accumulation of CHC in the lung vasculature. The lungs of LPS-treated animals that had not received CHC showed no signs of thrombosis, as surmised by immunofluorescent staining for platelets (data not shown). No difference in neutrophil recruitment could be surmised either (data not shown), suggesting that CHC was mostly bound by the EC. Taken as a whole, the results indicated that CHC may bind to EC that have shed the glycocalyx, but not to quiescent EC. Whether this confers a localized protecting effect against thrombosis is yet to be determined.

Conclusions

PAPER I

The blood-EC chamber model enables the study of sensitive interactions between whole blood and EC.

PAPER II

CHC binds EC and collagen and may reduce the thrombogenicity of activated endothelial cells and exposed basement membrane proteins.

PAPER III

CHC binds EC with high avidity and to the vasculature of porcine kidneys under cold storage conditions. CHC treatment improves the early function of transplanted murine kidneys.

PAPER IV

CHC does not facilitate thrombin inhibition to the same extent as UFH, and may target the binding of EC damage *in vivo*.

Future considerations

The work that we have performed in this thesis illustrates the importance of proper endothelial function with regard to the regulation of hemostasis and inflammation. We were able to develop a novel assay that accommodates the complexity of whole blood combined with cultured EC. We used this assay to model EC dysfunction and to investigate a potential treatment against the effects of a damaged blood vessel endothelium.

We have demonstrated the potential benefits of the blood-EC chamber model in this thesis whereby we have found it to be a very useful tool for investigating early events during thrombosis and activation of innate immunity. However, the method still lacks several parameters required for the simulation of a fully functional blood vessel. The most obvious deficits involves the nonlinear flow of blood over the EC which, aside from being nonequivalent to physiological blood flow, also prohibits studies of specific shear dependent interactions between the EC and blood components. A system that enables uniform flow over the EC surface would require the use of a more abrasive method for mobilizing the blood. Most pumps available today trigger hemolysis and activation of the blood compartment (142), making such a solution difficult to implement. We are, however, currently in search of a system that fulfills our demands of minimizing any external strenuous forces acting on the blood in order to reduce the unavoidable background activation caused by any *ex vivo* system.

Another evident limitation to the current blood-EC model is the timespan available for experimentation. We have to date stretched the incubation time to four hours (data not shown in this thesis), after which the pH and the energy stores present in the blood reach levels which would be considered pathological *in vivo*. The major benefit of a longer experimentation time could, however, enable studies of events taking place downstream of the very early activation that we have focused on in this thesis. It would, indeed, be very interesting to investigate whether any particular early event may be coupled to a specific downstream signature of either resolution of inflammation, wound healing, or activation of the adaptive immune system. Further development of the model to incorporate physiologically relevant conditions is thus an attractive objective, as it may facilitate the future evaluation of additional treatments with regard to both efficacy and toxicity.

The major focus of our work presented here has been to develop and evaluate a novel treatment using a heparin conjugate that enhances the he-

mocompatibility of the vascular endothelium. We applied the treatment to IRI during kidney transplantation and we investigated the possibility of delivery during cold storage in order to have a localized effect on the renal vasculature. Whereas we were able to determine that the heparin conjugate binds to cultured EC with high avidity and to the vasculature of explanted kidneys without difficulty, the treatment of kidneys removed from braindead donors elicited low binding to the renal medulla. We have yet to determine whether this has any implication on the efficacy of the treatment, and we are currently investigating alternative means of delivering the heparin conjugate [(123) and unpublished data]. We have begun to examine the application of treatment during hypothermic machine perfusion, a preservation strategy that is becoming more frequently utilized in clinical settings. However, we have not yet investigated the dispersion of the conjugate with regard to kidney morphology when applied by machine perfusion; to this end, we will pursue future efforts in this area.

The murine kidneys that were treated with the heparin conjugate prior to transplantation presented better early renal function as compared to controls. The preliminary histological investigations also suggested a reduced presence of platelets and leukocytes in the treated kidneys. We have yet to determine whether treatment has any effect on long-term graft function. A continuation of the current transplantation model using nephrectomized recipients should, however, reveal whether the early improvement has any impact on long-term survival.

Despite the wide range of side effects of UFH, the substantial clinical experience with regard to its use may ultimately be beneficial in translating treatment with the heparin conjugate to a clinical setting. In the final study of this thesis, we thus investigated the anticoagulant effect of the heparin conjugate in comparison to UFH. We found the heparin conjugate to be less effective than UFH in facilitating the inhibition of thrombin. We also determined that the conjugate quickly binds to exposed collagen, activated platelets, and damaged EC. We will continue to investigate the binding of the conjugate to platelets further with regard to its effects on activation in future studies.

The seemingly increased propensity for binding to damaged EC combined with the tendency to neglect quiescent EC that we found could, however, greatly benefit the anticoagulant treatment of acute conditions. Serious acute thrombotic complications such as disseminated intravascular coagulation pose major challenges in a clinical setting (143). Systemic thrombotic disorders are often difficult to treat with anticoagulants, as the massive consumption of coagulation factors increase the inherent bleeding risk that comes with treatment. An anticoagulant that would favor binding to thrombotic lesions could thus be very beneficial. We have, however, yet to investigate the heparin conjugate with regard to *in vivo* efficacy, biodistribution, and

clearance mechanisms before we draw any definite conclusions regarding the suitability of systemic treatment.

Finally, the translational outlook for treatment with the heparin conjugate is to date overall primarily positive. The manufacturer of the conjugate has recently carried out initial toxicity studies and so far no unpredictable adverse effects have been found upon systemic delivery (personal communication). Extensive preclinical toxicity studies are currently in progress, furthermore; the first clinical trial involving CHC-coated islets of Langerhans is in its final preparatory stage with plans to start enrollment this year (144). Preparations for clinical safety studies of the treatment in kidney grafts are presently underway with plans for initiation upon the finalization of the ongoing toxicity studies.

Populärvetenskaplig sammanfattning

Insidan av kroppens blodkärl är täckta av ett tunt endotelcellslager som tillsammans med blodet, har en viktig roll som transportörer av näring och syre till kroppens alla organ. Utöver delaktighet i transportörsrollen ser friska endotelceller till att blodet inte levrar sig. På grund av den omfattande kommunikationen mellan levringssystemet och kroppens tidiga inflammationssystem kan levring aktivera inflammation och vice versa. I situationer där endotelcellerna inte fungerar som de ska, ökar risken för både levring och inflammation.

Vid njurtransplantation genomgår endotelceller en process som skadar dem; i de fall då njuren kommer från en hjärndöd donator är endotelcellernas funktion redan kraftigt nedsatt vid organuttag och ytterligare försämring sker under njurens förvaring i väntan inför transplantationen. Resultatet av en försämrad endotelcellsfunktion är i värsta fall att njuren inte fungerar som den ska i mottagaren, med risk för att njuren inte överlever långsiktigt.

I denna avhandling har vi

- I utvecklat en ny metod för att studera endotelcellernas samspel med blodets levring och inflammatoriska respons,
- II använt den nya metoden för att utreda en behandling som skyddar blodet från den levring och inflammation som skadade endotelceller orsakar,
- III undersökt möjligheterna för att använda den nya behandlingen vid njurtransplantation,
- IV undersökt lokal behandling av endotelcellsskador i kroppen via systemisk administration.

I denna avhandling har vi således illustrerat vikten av de problem som skadade endotelceller kan ge upphov till. Vi har utvärderat en ny behandling som genom att skydda kärlbädden potentiellt kan förbättra funktionen hos transplanterade njurar, samt påbörjat utvärderingen av en ny behandling mot kärlskada.

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References

1. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. Nature Reviews Immunology. 2013;13(1):34-45.
2. Nilsson B, Teramura Y, Ekdahl KN. The role and regulation of complement activation as part of the thromboinflammation elicited in cell therapies. Molecular Immunology. 2014;61(2):185-90.
3. Land WG, Messmer K. The danger theory in view of the injury hypothesis: 20 years later. Frontiers in immunology. 2012;3:349.
4. Furie B, Furie BC. Mechanisms of thrombus formation. The New England journal of medicine. 2008;359(9):938-49.
5. Aird WC, ed. Endothelial Biomedicine: Cambridge University Press; 2007.
6. Maruyama Y. The human endothelial cell in tissue culture. Zeitschrift fur Zellforschung und mikroskopische Anatomie. 1963;60:69-79.
7. Nachman RL, Jaffe EA. Endothelial cell culture: beginnings of modern vascular biology. The Journal of clinical investigation. 2004;114(8):1037-40.
8. Ratner BD. The catastrophe revisited: blood compatibility in the 21st Century. Biomaterials. 2007;28(34):5144-7.
9. Smock KJ, Perkins SL. Thrombocytopenia: an update. International journal of laboratory hematology. 2014;36(3):269-78.
10. Semple JW, Italiano JE, Jr., Freedman J. Platelets and the immune continuum. Nature reviews Immunology. 2011;11(4):264-74.
11. Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. Platelets. 2001;12(5):261-73.
12. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. Blood reviews. 2009;23(4):177-89.
13. Projahn D, Koenen RR. Platelets: key players in vascular inflammation. J Leukoc Biol. 2012;92(6):1167-75.
14. Andrews RK, Berndt MC. Platelet physiology and thrombosis. Thrombosis research. 2004;114(5-6):447-53.
15. Nishimura S, Manabe I, Nagasaki M, Kakuta S, Iwakura Y, Takayama N, et al. In vivo imaging visualizes discoid platelet aggregations without endothelium disruption and implicates contribution of inflammatory cytokine and integrin signaling. Blood. 2012;119(8):e45-e56.
16. Jackson SP. The growing complexity of platelet aggregation. Blood. 2007;109(12):5087-95.
17. Cimmino G, Golino P. Platelet biology and receptor pathways. Journal of cardiovascular translational research. 2013;6(3):299-309.
18. Mackman N. The many faces of tissue factor. Journal of thrombosis and haemostasis : JTH. 2009;7 Suppl 1:136-9.
19. Dahlback B. Blood coagulation. Lancet. 2000;355(9215):1627-32.
20. Muller F, Renne T. Novel roles for factor XII-driven plasma contact activation system. Current opinion in hematology. 2008;15(5):516-21.

21. Law RHP, Abu-Ssaydeh D, Whisstock JC. New insights into the structure and function of the plasminogen/plasmin system. Current Opinion in Structural Biology. 2013;23(6):836-41.
22. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nature immunology. 2010;11(9):785-97.
23. Gasque P. Complement: a unique innate immune sensor for danger signals. Mol Immunol. 2004;41(11):1089-98.
24. Bohana-Kashtan O, Ziporen L, Donin N, Kraus S, Fishelson Z. Cell signals transduced by complement. Mol Immunol. 2004;41(6-7):583-97.
25. Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD. Complement and coagulation: strangers or partners in crime? Trends in immunology. 2007;28(4):184-92.
26. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nature reviews Immunology. 2013;13(3):159-75.
27. Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, Goosmann C, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. Nature medicine. 2010;16(8):887-96.
28. Darbousset R, Thomas GM, Mezouar S, Frere C, Bonier R, Mackman N, et al. Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. Blood. 2012;120(10):2133-43.
29. Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, et al. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. Journal of immunology. 2006;177(7):4794-802.
30. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003;19(1):71-82.
31. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science. 2007;317(5838):666-70.
32. Dal-Secco D, Wang J, Zeng Z, Kolaczkowska E, Wong CHY, Petri B, et al. A dynamic spectrum of monocytes arising from the in situ reprogramming of CCR2+ monocytes at a site of sterile injury. The Journal of Experimental Medicine. 2015;212(4):447-56.
33. Rua R, McGavern DB. Elucidation of monocyte/macrophage dynamics and function by intravital imaging. J Leukoc Biol. 2015;98(3):319-32.
34. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol. 2009;78(6):539-52.
35. Nieuwdorp M, Meuwese MC, Mooij HL, Ince C, Broekhuizen LN, Kastelein JJ, et al. Measuring endothelial glycocalyx dimensions in humans: a potential novel tool to monitor vascular vulnerability. Journal of applied physiology. 2008;104(3):845-52.
36. Reitsma S, Slaaf DW, Vink H, van Zandvoort MA, oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. Pflugers Archiv : European journal of physiology. 2007;454(3):345-59.
37. Pries AR, Secomb TW, Gaehtgens P. The endothelial surface layer. Pflugers Archiv : European journal of physiology. 2000;440(5):653-66.
38. Rosenberg RD, Rosenberg JS. Natural anticoagulant mechanisms. The Journal of clinical investigation. 1984;74(1):1-6.
39. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nature reviews Immunology. 2009;9(10):729-40.

40. Roemisch J, Gray E, Hoffmann JN, Wiedermann CJ. Antithrombin: a new look at the actions of a serine protease inhibitor. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis. 2002;13(8):657-70.
41. Shworak NW, Kobayashi T, de Agostini A, Smits NC. Anticoagulant heparan sulfate to not clot--or not? Progress in molecular biology and translational science. 2010;93:153-78.
42. Carlson TH, Kolman MR, Piepkorn M. Activation of antithrombin III isoforms by heparan sulphate glycosaminoglycans and other sulphated polysaccharides. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis. 1995;6(5):474-80.
43. Wood JP, Ellery PE, Maroney SA, Mast AE. Biology of tissue factor pathway inhibitor. Blood. 2014;123(19):2934-43.
44. Martin FA, Murphy RP, Cummins PM. Thrombomodulin and the vascular endothelium: insights into functional, regulatory, and therapeutic aspects. American journal of physiology Heart and circulatory physiology. 2013;304(12):H1585-97.
45. Medcalf RL. Fibrinolysis, inflammation, and regulation of the plasminogen activating system. Journal of thrombosis and haemostasis : JTH. 2007;5 Suppl 1:132-42.
46. Curry FE, Adamson RH. Endothelial glycocalyx: permeability barrier and mechanosensor. Annals of biomedical engineering. 2012;40(4):828-39.
47. Jones CI, Barrett NE, Moraes LA, Gibbins JM, Jackson DE. Endogenous inhibitory mechanisms and the regulation of platelet function. Methods in molecular biology. 2012;788:341-66.
48. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nature reviews Immunology. 2007;7(10):803-15.
49. Kaur J, Woodman RC, Ostrovsky L, Kubes P. Selective recruitment of neutrophils and lymphocytes by thrombin: a role for NF-kappaB. American journal of physiology Heart and circulatory physiology. 2001;281(2):H784-95.
50. Wang J, Al-Lamki RS, Zhang H, Kirkiles-Smith N, Gaeta ML, Thiru S, et al. Histamine antagonizes tumor necrosis factor (TNF) signaling by stimulating TNF receptor shedding from the cell surface and Golgi storage pool. J Biol Chem. 2003;278(24):21751-60.
51. Vanhoutte PM, Miller VM. Heterogeneity of endothelium-dependent responses in mammalian blood vessels. Journal of cardiovascular pharmacology. 1985;7 Suppl 3:S12-23.
52. Dejana E. Endothelial cell-cell junctions: happy together. Nature reviews Molecular cell biology. 2004;5(4):261-70.
53. Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(5):1002-7.
54. Metcalf DJ, Nightingale TD, Zenner HL, Lui-Roberts WW, Cutler DF. Formation and function of Weibel-Palade bodies. Journal of cell science. 2008;121(Pt 1):19-27.
55. Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood. 2002;100(12):4033-9.
56. Koppelman SJ, van Hoeij M, Vink T, Lankhof H, Schiphorst ME, Damas C, et al. Requirements of von Willebrand factor to protect factor VIII from inactivation by activated protein C. Blood. 1996;87(6):2292-300.

57. Massberg S, Enders G, Leiderer R, Eisenmenger S, Vestweber D, Krombach F, et al. Platelet-endothelial cell interactions during ischemia/reperfusion: the role of P-selectin. Blood. 1998;92(2):507-15.
58. Ichikawa H, Flores S, Kvietys PR, Wolf RE, Yoshikawa T, Granger DN, et al. Molecular mechanisms of anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. Circulation research. 1997;81(6):922-31.
59. Faure E, Equils O, Sieling PA, Thomas L, Zhang FX, Kirschning CJ, et al. Bacterial Lipopolysaccharide Activates NF-κB through Toll-like Receptor 4 (TLR-4) in Cultured Human Dermal Endothelial Cells: DIFFERENTIAL EXPRESSION OF TLR-4 AND TLR-2 IN ENDOTHELIAL CELLS. J Biol Chem. 2000;275(15):11058-63.
60. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature. 1998;391(6667):591-4.
61. Mackman N. Regulation of the tissue factor gene. Faseb J. 1995;9(10):883-9.
62. Kirchhofer D, Tschopp TB, Hadvary P, Baumgartner HR. Endothelial cells stimulated with tumor necrosis factor-alpha express varying amounts of tissue factor resulting in inhomogenous fibrin deposition in a native blood flow system. Effects of thrombin inhibitors. The Journal of clinical investigation. 1994;93(5):2073-83.
63. Mohamed F, Monge JC, Gordon A, Cernacek P, Blais D, Stewart DJ. Lack of Role for Nitric Oxide (NO) in the Selective Destabilization of Endothelial NO Synthase mRNA by Tumor Necrosis Factor–α. Arteriosclerosis, thrombosis, and vascular biology. 1995;15(1):52-7.
64. Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F, et al. Prolyl hydroxylase-1 negatively regulates IκB kinase-β, giving insight into hypoxia-induced NFκB activity. Proceedings of the National Academy of Sciences. 2006;103(48):18154-9.
65. Oitzinger W, Hofer-Warbinek R, Schmid JA, Koshelnick Y, Binder BR, de Martin R. Adenovirus-mediated expression of a mutant IkappaB kinase 2 inhibits the response of endothelial cells to inflammatory stimuli. Blood. 2001;97(6):1611-7.
66. McKenzie JAG, Ridley AJ. Roles of Rho/ROCK and MLCK in TNF-α-induced changes in endothelial morphology and permeability. Journal of Cellular Physiology. 2007;213(1):221-8.
67. Pober JS, Sessa WC. Inflammation and the blood microvascular system. Cold Spring Harbor perspectives in biology. 2015;7(1):a016345.
68. Marki A, Esko JD, Pries AR, Ley K. Role of the endothelial surface layer in neutrophil recruitment. J Leukoc Biol. 2015;98(4):503-15.
69. Schmidt EP, Yang Y, Janssen WJ, Gandjeva A, Perez MJ, Barthel L, et al. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. Nature medicine. 2012;18(8):1217-23.
70. Becker BF, Jacob M, Leipert S, Salmon AH, Chappell D. Degradation of the endothelial glycocalyx in clinical settings: searching for the sheddases. British journal of clinical pharmacology. 2015;80(3):389-402.
71. Hsu CY, Iribarren C, McCulloch CE, Darbinian J, Go AS. Risk factors for end-stage renal disease: 25-year follow-up. Archives of internal medicine. 2009;169(4):342-50.
72. Noordzij M, Kramer A, Abad Diez JM, Alonso de la Torre R, Arcos Fuster E, Bikbov BT, et al. Renal replacement therapy in Europe: a summary of the 2011 ERA-EDTA Registry Annual Report. Clinical kidney journal. 2014;7(2):227-38.

73. Horvat LD, Shariff SZ, Garg AX, Donor Nephrectomy Outcomes Research N. Global trends in the rates of living kidney donation. Kidney international. 2009;75(10):1088-98.
74. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet. 2004;364(9447):1814-27.
75. Chen EP, Bittner HB, Kendall SW, Van Trigt P. Hormonal and hemodynamic changes in a validated animal model of brain death. Critical care medicine. 1996;24(8):1352-9.
76. Lustenberger T, Talving P, Kobayashi L, Barmparas G, Inaba K, Lam L, et al. Early coagulopathy after isolated severe traumatic brain injury: relationship with hypoperfusion challenged. The Journal of trauma. 2010;69(6):1410-4.
77. Hefty TR, Cotterell LW, Fraser SC, Goodnight SH, Hatch TR. Disseminated intravascular coagulation in cadaveric organ donors. Incidence and effect on renal transplantation. Transplantation. 1993;55(2):442-3.
78. van der Hoeven JA, Molema G, Ter Horst GJ, Freund RL, Wiersema J, van Schilfgaarde R, et al. Relationship between duration of brain death and hemodynamic (in)stability on progressive dysfunction and increased immunologic activation of donor kidneys. Kidney international. 2003;64(5):1874-82.
79. Nijboer WN, Schuurs TA, van der Hoeven JA, Fekken S, Wiersema-Buist J, Leuvenink HG, et al. Effect of brain death on gene expression and tissue activation in human donor kidneys. Transplantation. 2004;78(7):978-86.
80. Seow YY, Alkari B, Dyer P, Riad H. Cold ischemia time, surgeon, time of day, and surgical complications. Transplantation. 2004;77(9):1386-9.
81. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie. 2011;38(2):125-42.
82. Zhao H, Perez JS, Lu K, George AJ, Ma D. Role of Toll-like receptor-4 in renal graft ischemia-reperfusion injury. American journal of physiology Renal physiology. 2014;306(8):F801-11.
83. Gallinat A, Fox M, Luer B, Efferz P, Paul A, Minor T. Role of pulsatility in hypothermic reconditioning of porcine kidney grafts by machine perfusion after cold storage. Transplantation. 2013;96(6):538-42.
84. Hattori R, Ono Y, Kato M, Komatsu T, Matsukawa Y, Yamamoto T. Direct visualization of cortical peritubular capillary of transplanted human kidney with reperfusion injury using a magnifying endoscopy. Transplantation. 2005;79(9):1190-4.
85. Damman J, Bloks VW, Daha MR, van der Most PJ, Sanjabi B, van der Vlies P, et al. Hypoxia and Complement-and-Coagulation Pathways in the Deceased Organ Donor as the Major Target for Intervention to Improve Renal Allograft Outcome. Transplantation. 2015;99(6):1293-300.
86. Schmitz V, Schaser KD, Olschewski P, Neuhaus P, Puhl G. In vivo visualization of early microcirculatory changes following ischemia/reperfusion injury in human kidney transplantation. European surgical research Europaische chirurgische Forschung Recherches chirurgicales europeennes. 2008;40(1):19-25.
87. Snoeijs MG, Vink H, Voesten N, Christiaans MH, Daemen JW, Peppelenbosch AG, et al. Acute ischemic injury to the renal microvasculature in human kidney transplantation. American journal of physiology Renal physiology. 2010;299(5):F1134-40.

88. Koo DD, Welsh KI, Roake JA, Morris PJ, Fuggle SV. Ischemia/reperfusion injury in human kidney transplantation: an immunohistochemical analysis of changes after reperfusion. The American journal of pathology. 1998;153(2):557-66.
89. Sharif A, Borrows R. Delayed graft function after kidney transplantation: the clinical perspective. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2013;62(1):150-8.
90. Yarlagadda SG, Coca SG, Garg AX, Doshi M, Poggio E, Marcus RJ, et al. Marked variation in the definition and diagnosis of delayed graft function: a systematic review. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2008;23(9):2995-3003.
91. Wu WK, Famure O, Li Y, Kim SJ. Delayed graft function and the risk of acute rejection in the modern era of kidney transplantation. Kidney international. 2015;88(4):851-8.
92. Hirsh J, O'Donnell M, Eikelboom JW. Beyond unfractionated heparin and warfarin: current and future advances. Circulation. 2007;116(5):552-60.
93. Chandarajoti K, Liu J, Pawlinski R. The design and synthesis of new synthetic low molecular weight heparins. Journal of thrombosis and haemostasis : JTH. 2016.
94. Hirsh J, Anand SS, Halperin JL, Fuster V. Mechanism of Action and Pharmacology of Unfractionated Heparin. Arteriosclerosis, thrombosis, and vascular biology. 2001;21(7):1094-6.
95. Hirsh J, Raschke R. Heparin and low-molecular-weight heparin: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest. 2004;126(3 Suppl):188S-203S.
96. Martel N, Lee J, Wells PS. Risk for heparin-induced thrombocytopenia with unfractionated and low-molecular-weight heparin thromboprophylaxis: a meta-analysis. Blood. 2005;106(8):2710-5.
97. Di Nisio M, Middeldorp S, Büller HR. Direct Thrombin Inhibitors. New England Journal of Medicine. 2005;353(10):1028-40.
98. Garcia D, Libby E, Crowther MA. The new oral anticoagulants. Blood. 2010;115(1):15-20.
99. Pernod G, Albaladejo P, Godier A, Samama CM, Susen S, Gruel Y, et al. Management of major bleeding complications and emergency surgery in patients on long-term treatment with direct oral anticoagulants, thrombin or factor-Xa inhibitors: Proposals of the Working Group on Perioperative Haemostasis (GIHP) – March 2013. Archives of Cardiovascular Diseases. 2013;106(6–7):382-93.
100. Hughes S, Szeki I, Nash MJ, Thachil J. Anticoagulation in chronic kidney disease patients—the practical aspects. Clinical kidney journal. 2014;7(5):442-9.
101. Larsson R. Heparin binding to improve biocompatibility. Encyclopedia of Biomaterials and Biomedical Engineering2004. p. 1368-76.
102. Thorslund S, Sanchez J, Larsson R, Nikolajeff F, Bergquist J. Functionality and stability of heparin immobilized onto poly(dimethylsiloxane). Colloids and surfaces B, Biointerfaces. 2005;45(2):76-81.
103. Johnell M, Elgue G, Larsson R, Larsson A, Thelin S, Siegbahn A. Coagulation, fibrinolysis, and cell activation in patients and shed mediastinal blood during coronary artery bypass grafting with a new heparin-coated surface. The Journal of thoracic and cardiovascular surgery.124(2):321-32.

104. Johnell M, Elgue G, Thelin S, Larsson R, Siegbahn A. Cell Adhesion and Tissue Factor Upregulation in Oxygenators used during Coronary Artery Bypass Grafting are Modified by the Corline Heparin Surface. Scandinavian Cardiovascular Journal. 2002;36(6):351-7.
105. Christensen K, Larsson R, Emanuelsson H, Elgue G, Larsson A. Heparin coating of the stent graft — effects on platelets, coagulation and complement activation. Biomaterials. 2001;22(4):349-55.
106. Cabric S, Eich T, Sanchez J, Nilsson B, Korsgren O, Larsson R. A new method for incorporating functional heparin onto the surface of islets of Langerhans. Tissue engineering Part C, Methods. 2008;14(2):141-7.
107. Cabric S, Sanchez J, Johansson U, Larsson R, Nilsson B, Korsgren O, et al. Anchoring of Vascular Endothelial Growth Factor to Surface-Immobilized Heparin on Pancreatic Islets: Implications for Stimulating Islet Angiogenesis. Tissue Eng Pt A. 2010;16(3):961-70.
108. Cabric S, Sanchez J, Lundgren T, Foss A, Felldin M, Källen R, et al. Islet Surface Heparinization Prevents the Instant Blood-Mediated Inflammatory Reaction in Islet Transplantation. Diabetes. 2007;56(8):2008-15.
109. Leijon J, Carlsson F, Brannstrom J, Sanchez J, Larsson R, Nilsson B, et al. Attachment of Flexible Heparin Chains to Gelatin Scaffolds Improves Endothelial Cell Infiltration. Tissue Eng Pt A. 2013;19(11-12):1336-48.
110. Ekdahl KN, Hong J, Hamad OA, Larsson R, Nilsson B. Evaluation of the blood compatibility of materials, cells, and tissues: basic concepts, test models, and practical guidelines. Advances in experimental medicine and biology. 2013;735:257-70.
111. Andersson J, Sanchez J, Ekdahl KN, Elgue G, Nilsson B, Larsson R. Optimal heparin surface concentration and antithrombin binding capacity as evaluated with human non-anticoagulated blood in vitro. J Biomed Mater Res A. 2003;67A(2):458-66.
112. Hong J, Ekdahl KN, Reynolds H, Larsson R, Nilsson B. A new in vitro model to study interaction between whole blood and biomaterials. Studies of platelet and coagulation activation acid the effect of aspirin. Biomaterials. 1999;20(7):603-11.
113. Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, et al. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. Nature medicine. 2009;15(6):665-73.
114. Pelzer H, Schwarz A, Heimburger N. Determination of human thrombin-antithrombin III complex in plasma with an enzyme-linked immunosorbent assay. Thromb Haemostasis. 1988;59(1):101-6.
115. Gong J, Larsson R, Ekdahl K, Mollnes T, Nilsson U, Nilsson B. Tubing loops as a model for cardiopulmonary bypass circuits: Both the biomaterial and the blood-gas phase interfaces induce complement activation in anin vitro model. Journal of clinical immunology. 1996;16(4):222-9.
116. Kalman PG, Ward CA, McKeown NB, McCullough D, Romaschin AD. Improved biocompatibility of silicone rubber by removal of surface entrapped air nuclei. Journal of biomedical materials research. 1991;25(2):199-211.
117. Gaamangwe T, Peterson SD, Gorbet MB. Investigating the Effect of Blood Sample Volume in the Chandler Loop Model: Theoretical and Experimental Analysis. Cardiovascular Engineering and Technology. 2014;5(2):133-44.
118. Grabowski EF, Yam K, Gerace M. Evaluation of hemostasis in flowing blood. American journal of hematology. 2012;87 Suppl 1:S51-5.

119. Kett WC, Osmond RIW, Moe L, Skett SE, Kinnear BF, Coombe DR. Avidin is a heparin-binding protein. Affinity, specificity and structural analysis. Bba-Gen Subjects. 2003;1620(1-3):225-34.
120. Munoz EM, Linhardt RJ. Heparin-binding domains in vascular biology. Arteriosclerosis, thrombosis, and vascular biology. 2004;24(9):1549-57.
121. Soker S, Goldstaub D, Svahn CM, Vlodavsky I, Levi BZ, Neufeld G. Variations in the Size and Sulfation of Heparin Modulate the Effect of Heparin on the Binding of VEGF165 to Its Receptors. Biochemical and biophysical research communications. 1994;203(2):1339-47.
122. San Antonio JD, Lander AD, Karnovsky MJ, Slayter HS. Mapping the heparin-binding sites on type I collagen monomers and fibrils. J Cell Biol. 1994;125(5):1179-88.
123. Sedigh A, Larsson R, Brannstrom J, Magnusson P, Larsson E, Tufveson G, et al. Modifying the vessel walls in porcine kidneys during machine perfusion. The Journal of surgical research. 2014.
124. Dufourcq P, Seigneur M, Pruvost A, Dumain P, Belloc F, Amiral J, et al. Membrane thrombomodulin levels are decreased during hypoxia and restored by cAMP and IBMX. Thrombosis research. 1995;77(3):305-10.
125. Uchiyama T, Kurabayashi M, Ohyama Y, Utsugi T, Akuzawa N, Sato M, et al. Hypoxia Induces Transcription of the Plasminogen Activator Inhibitor-1 Gene Through Genistein-Sensitive Tyrosine Kinase Pathways in Vascular Endothelial Cells. Arteriosclerosis, thrombosis, and vascular biology. 2000;20(4):1155-61.
126. Ushigome H, Sano H, Okamoto M, Kadotani Y, Nakamura K, Akioka K, et al. The role of tissue factor in renal ischemic reperfusion injury of the rat. The Journal of surgical research. 2002;102(2):102-9.
127. Massberg S, Enders G, Matos FCD, Tomic LID, Leiderer R, Eisenmenger S, et al. Fibrinogen deposition at the postischemic vessel wall promotes platelet adhesion during ischemia-reperfusion in vivo. Blood. 1999;94(11):3829-38.
128. Hulme EC, Trevethick MA. Ligand binding assays at equilibrium: validation and interpretation. Br J Pharmacol. 2010;161(6):1219-37.
129. Barzu T, Van Rijn JL, Petitou M, Molho P, Tobelem G, Caen JP. Endothelial binding sites for heparin. Specificity and role in heparin neutralization. The Biochemical journal. 1986;238(3):847-54.
130. Sampaio FJB, Pereira-Sampaio MA, Favorito LA. The Pig Kidney as an Endourologic Model: Anatomic Contribution. Journal of Endourology. 1998;12(1):45-50.
131. Sugiura G, Kuhn H, Sauter M, Haberkorn U, Mier W. Radiolabeling strategies for tumor-targeting proteinaceous drugs. Molecules. 2014;19(2):2135-65.
132. Tolmachev V, Xu H, Wållberg H, Ahlgren S, Hjertman M, Sjöberg A, et al. Evaluation of a Maleimido Derivative of CHX-A″ DTPA for Site-Specific Labeling of Affibody Molecules. Bioconjugate Chemistry. 2008;19(8):1579-87.
133. Thakur ML. Gallium-67 and indium-111 radiopharmaceuticals. The International Journal of Applied Radiation and Isotopes. 1977;28(1):183-201.
134. Evans RG, Eppel GA, Anderson WP, Denton KM. Mechanisms underlying the differential control of blood flow in the renal medulla and cortex. Journal of hypertension. 2004;22(8):1439-51.
135. Sear JW. Kidney dysfunction in the postoperative period. British journal of anaesthesia. 2005;95(1):20-32.

136. Tse GH, Hughes J, Marson LP. Systematic review of mouse kidney transplantation. Transplant international : official journal of the European Society for Organ Transplantation. 2013;26(12):1149-60.
137. Alexander W. Heparin Revisions: A Call for Heightened Vigilance and Monitoring. Pharmacy and Therapeutics. 2009;34(11):634-8.
138. Hursting MJ, Butman BT, Steiner JP, Moore BM, Plank MC, Szewczyk KM, et al. Monoclonal antibodies specific for prothrombin fragment 1.2 and their use in a quantitative enzyme-linked immunosorbent assay. Clinical chemistry. 1993;39(4):583-91.
139. Horne M. Nonimmune heparin-platelet interactions: implications for the pathogenesis of heparin-induced thrombocytopenia. Fundamental and clinical cardiology. 2000;39:113-26.
140. Durr E, Yu J, Krasinska KM, Carver LA, Yates JR, Testa JE, et al. Direct proteomic mapping of the lung microvascular endothelial cell surface in vivo and in cell culture. Nature biotechnology. 2004;22(8):985-92.
141. Chappell D, Jacob M, Paul O, Rehm M, Welsch U, Stoeckelhuber M, et al. The glycocalyx of the human umbilical vein endothelial cell: an impressive structure ex vivo but not in culture. Circulation research. 2009;104(11):1313-7.
142. van Oeveren W, Tielliu IF, de Hart J. Comparison of modified chandler, roller pump, and ball valve circulation models for in vitro testing in high blood flow conditions: application in thrombogenicity testing of different materials for vascular applications. International journal of biomaterials. 2012;2012:673163.
143. Levi M, Ten Cate H. Disseminated intravascular coagulation. The New England journal of medicine. 1999;341(8):586-92.
144. ClinicalTrials.gov. Heparinized Islets in Clinical Islet Transplantation US 2016 [Available from: https://clinicaltrials.gov/ct2/show/NCT00678990

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