UNIVERSITY OF TROMSØ UIT

Faculty of Health Sciences
Department of Clinical Medicine

New insights into the clearance of tissue factor pathway inhibitor (TFPI) and unfractionated heparin (UFH)

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New insights into the clearance of tissue factor pathway inhibitor (TFPI) and unfractionated heparin (UFH)

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LIST OF PAPERS

This thesis is based on the following papers:

I. Cristina Ionica Øie, Ellen Brodin, Rupa Shree Appa, Bård Smedsrød, John-Bjarne Hansen. Asialoglycoprotein Receptor (ASGP-R) is Liver Parenchymal Cells is Involved in Elimination of Recombinant Human TFPI – manuscript

II. Oie, C.I., Olsen, R., Smedsrød, S., Hansen, J.B. Liver sinusoidal endothelial cells are the principal site for elimination of unfractionated heparin from the circulation. Am J Physiol Gastrointest Liver Physiol, 2008. 294(2): p. G520-8

III. Cristina Ionica Øie, Ellen Brodin, Rupa Shree Appa, Ida Hilden, Bård Smedsrød, John-Bjarne Hansen. Effect of Unfractionated Heparin on TFPI Elimination – manuscript

IV. Cristina Ionica Øie, Rupa Shree Appa, Ida Hilden, Helle Heibroch Petersen, Albrecht Gruhler, Bård Smedsrød, John-Bjarne Hansen. Rat Liver Sinusoidal Endothelial Cells (LSECs) express functional Low Density Lipoprotein Receptor-Related Protein-1 (LRP-1) – submitted

ABBREVIATIONS

AGE = advanced glycation end products
APP = amyloid precursor protein
ASGP-R = asialoglycoprotein receptor
ASOR = asialo-orosomucoid
CHO = Chinese hamster ovary
EC = endothelial cell
EEA1 = Early Endosome Antigen 1
EGF = epidermal growth factor
EPI = extrinsic pathway inhibitor
GAGs = glycosaminoglycans
GPI = glycosylphoaphatidyinositol
HDL = high-density lipoprotein
HSPGs = heparan sulphate proteoclycans
IL = interleukin 1
KCs = Kupffer cells
LACI = lipoprotein-associated coagulation inhibitor
LDL = low density lipoprotein
LDL-R = low-density lipoprotein receptor
LMWH = low molecular weight heparin
LRP/LRP-1 = low-density lipoprotein receptor-related protein
LSECs = liver sinusoidal endothelial cells
MANN/COLL-R = mannose/alpha-collagen receptor
MPS = mononuclear phagocyte system
NPCs = non-parenchymal cells
oxLDL = oxidized low density lipoprotein
PARs = protease-activated receptors
PCs = parenchymal cells
RES = reticuloendothelial system
SR =scavenger receptor
TNF = tumor necrosis factor
TF = tissue factor
TFPI = tissue factor pathway inhibitor
UFH =unfractionated heparin
uPA = urokinase-type plasminogen activators
VLDL = very low density lipoprptein
vWF = von Willebrand Factor

CONTENTS

1. INTRODUCTION

1.1. Structural organization of the liver: anatomical localization and characteristics of the major liver cell types – parenchymal cells (PCs) and sinusoidal endothelial cells (LSECs)

The structural organization of the cellular and vascular elements of the liver is adapted to its special function as a key organ interposed between the digestive tract and the rest of the body. Representing a central metabolic processor of the body the liver functions both to

i) produce an array of bio-molecules including for instance several coagulation enzymes and albumin as well as a great number of low molecular weight metabolites, and

ii) take up blood borne nutrient components absorbed in the intestine and bring about their subsequent metabolism, storage and distribution to blood and bile. The uptake function also includes the removal of physiological and foreign waste from the blood.

The liver tissue is arranged in lobules; each has a central vein which carries blood to the hepatic veins. The lobules consist of thin cords of liver cells, separated by spaces called sinusoids. The sinusoids are arranged in a radial pattern around the central vein (Figure 1).

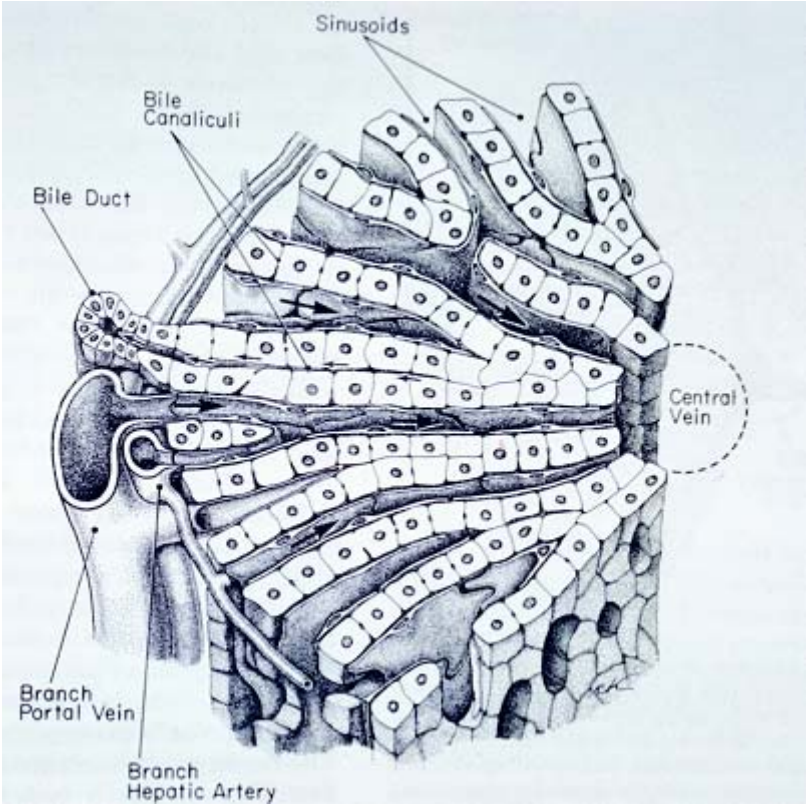

Figure 1. Schematic drawing of the liver sinusoids (reproduced with permission of Prof. Robert S McCuskey from A.W. Ham, "A Textbook of Histology", J.B. Lippincott Co., Philadelphia, 1965)

Sinusoidal cells consist of various cell types, usually classified in two major groups: the parenchymal cells (PCs) or hepatocytes and the non-parenchymal cells (NPCs). The NPCs consist of four cell types: liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), stellate cells and pit cells (also called liver associated lymphocytes or natural killer cells) (Figure 2).

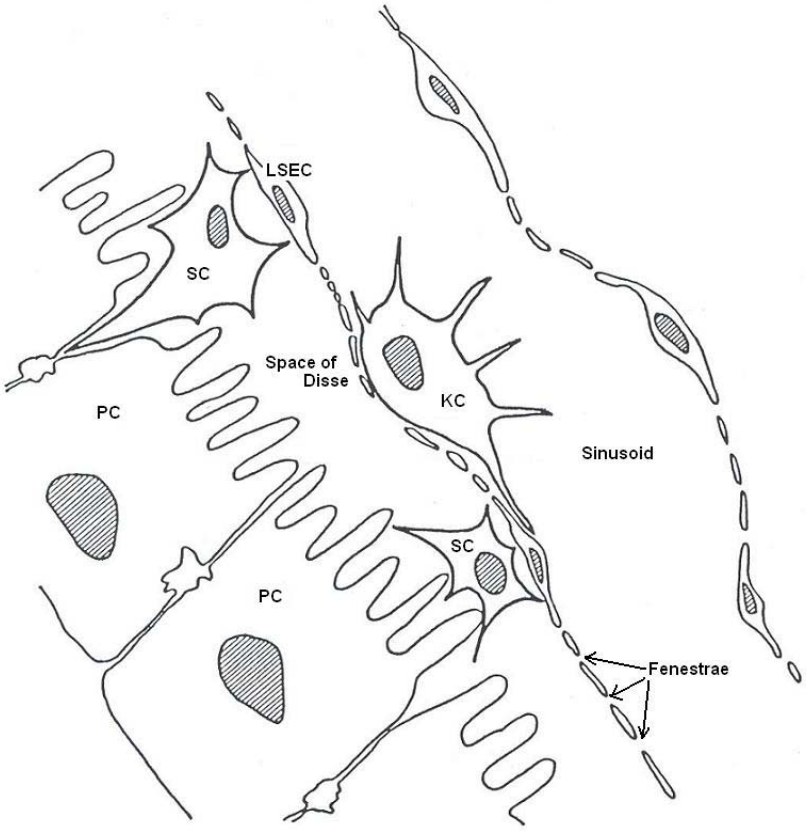

Figure 2. Schematic drawing of the liver sinusoidal cells (reproduced with permission of Dr. Grete Mørk Kindberg [1])

PCs are the largest cells in the liver, with a diameter of >20 μm and are polyhedral with eight or more surfaces; many of them are polyploid (up to octaploid) (Table 1). LSECs form a continuous lining of the liver sinusoids and represent a barrier between the PCs and the sinusoidal blood. In the early 1970s a number of electron microscopic studies by Eddie Wisse indicated that the LSECs were a unique cell type of the liver sinusoids [2, 3], that differ from other vascular endothelial cells in several aspects: (a) LSECs lack a basement membrane, (b) von Willebrand factor, or factor VIII related antigen (an often used immunohistochemical marker of vascular endothelial cells), is not expressed by LSECs. Rather, LSECs have been reported to synthesize procoagulant factor VIII [4], (c) the long cytoplasmic processes of LSECs contain abundant pores or fenestrae with a mean diameter of approximately 100 nm, which are arranged in so-called sieve plates and function as a barrier between particles in the

sinusoidal lumen and the PCs [5], (d) intracellularly, LSECs are rich in coated pits and vesicles and other organelles associated with endocytosis.

Cell Type	Number (%)	Volume (%)	Cells/g liver (x 10^6)	Cell diameter (μm)
Parenchymal cells	65	92.5	115	10-34
Non-parenchymal cells	35	7.5	63	6-15
Sinusoidal endothelial cells	21	3.3	38	6-11
Kupffer cells	8.5	2.5	15	7-15
Stellate cells	5.5	1.7	10	10-13
Pit cells	<1		<0.3	6-8

Table 1. Cellular composition of rat liver (reproduced with permission of Prof. Bård Smedsrød [6])

1.1.1. Receptor-mediated endocytosis

Endocytosis is the process of vesicle formation from the plasma membrane, and in this process, plasma membrane-associated proteins are internalised into membrane-bound transport vesicles. The endocytic pathway begins at the plasma membrane and ends up in the lysosomes, and along this pathway cargo is either destined for recycling back to the plasma membrane or for degradation. As cargo passes through early and late endosomes, communication with the *trans*-Golgi network and the secretory pathway takes place. The secretory pathway functions to deliver secretory and transmembrane proteins and lipids from the ER, via the Golgi apparatus, either to intracellular compartments, to the plasma membrane or to the exterior of the cell. At all stages in this vesicle-mediated transport between the plasma membrane and various intracellular compartments, recognition, tethering and fusion of vesicles are essential for proper destination of the cargo.

Endocytosis supports various cellular functions including nutrient uptake, waste removal, growth-factor signaling, and membrane homeostasis [7]. Categorically, endocytosis can occur by multiple mechanisms, and can be divided into phagocytosis or "cell eating" of large particles (> 200 nm) typically restricted to specialized cells such as macrophages and neutrophils [8] and pinocytosis or "cell drinking", the uptake of fluid and solutes that occurs in all cells.

Pinocytosis occurs by at least four basic mechanisms: 1) clathrin-dependent endocytosis, 2) caveolin-dependent endocytosis, 3) macropinocytosis, and 4) caveolae-, dynamin-, and clathrin-independent endocytosis [7]. These pathways are thought to vary mechanistically with respect to how the vesicles are formed, which cargo molecules are internalized, how entry is regulated, and to which intracellular destination the cargo is delivered.

Clathrin-mediated endocytosis is the best characterized endocytosis pathway (Figure 3). It ensures uptake of nutrients and waste and provides a way of transferring and regulating signalling from the exterior to the interior of the cell through surface receptor internalization. Receptor-mediated uptake of macromolecules via clathrin-coated pits constitutes a selective concentration mechanism that largely increases the efficiency of ligand internalization, such that even minor components of the extracellular environment can be taken up in large amounts without internalization of correspondingly large amounts of extracellular fluid.

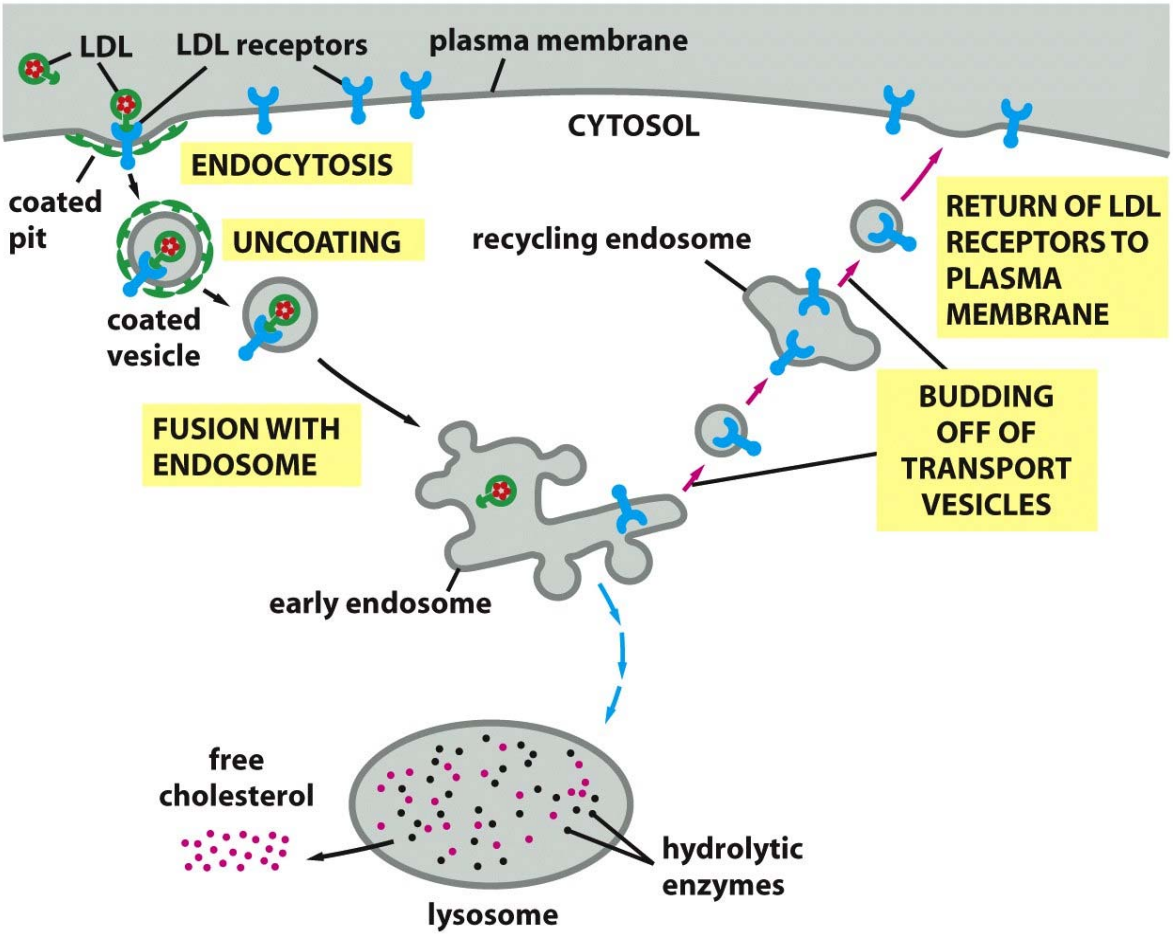

Figure 3. The receptor-mediated endocytosis of Low Density Lipoprotein (LDL) LDL receptor binds LDL at the plasma membrane and the complex assembly in a coated pit. The internalized pit is converted into a clathrin-coated vesicle that fuses with early endosomes (EEs) after the removal of the clathrin coat. The LDL dissociates from its receptor in the acidic environment of the EEs and ends up in lysosomes, where it is degraded. Reproduced with permission of Garland Science/Taylor and Francis LLC from Molecular Biology of the Cell 5th Ed. by Alberts et al.

Clathrin-mediated endocytosis is used for clearance of receptor-bound hormones and growth factors from the cell surface, control of the number of channels and transporters, internalization and degradation of extracellular material by some cells [9], recycling of synaptic vesicles in nerve terminals and is implicated in cell invasion by a variety of pathogens [10].

Clathrin is a vesicle coat protein involved in the assembly of membrane and cargo into transport vesicle at the plasma membrane [11]. Several proteins are involved in and essential for the formation of clathrin coated pits and vesicles and for the concentration of transmembrane receptors into these pits. The clathrin-mediated endocytosis starts with the binding of ligand to the luminal or extracellular domain of a transmembrane receptor, followed by concentration of receptor through interaction with the protein coat. Formation of an endocytic clathrin-coated vesicle is initiated by binding and oligomerisation of clathrin adaptor protein at the cytoplasmic surface of the plasma membrane. This leads to polymerization of clathrin into a lattice that pulls the plasma membrane inside [12].

Once the inward budding of the membrane is complete, interactions between the adaptor protein and the GTPase dynamin allow separation of the forming vesicle from the membrane [13]. After the pit is internalized and converted into a clathrin-coated vesicle, the clathrin coat is removed and the vesicle is able to fuse with target early endosomes, a process involving Rab5 and Early Endosome Antigen 1 (EEA1) [14]. Rab5 belongs to a superfamily of approximately 70 proteins that regulate many steps of membrane traffic [15] and EEA1 is a protein that binds phospholipid vesicles containing phosphatidylinositol 3-phosphate, which is necessary for endosomal trafficking [14]. Acidification of early endosomes leads to dissociation of most receptor-ligand complexes, and ligands destined for degradation travel with late endosomes to lysosomes, while receptor-enriched vesicles are recycled back to the

cell surface [16, 17]. The intracellular membrane trafficking system uses vesicles and tubulovesicular structures to deliver cargo proteins and lipids from one compartment to the next. Several proteins including the Rab guanosine triphosphate enzymes (GTPases) and the Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptors (SNAREs) are involved in recognition, docking and fusion of donor and acceptor compartments to ensure proper transport and delivery of cargo molecules [18].

1.1.2. Endocytic receptors on liver sinusoidal endothelial cells (LSECs) and parenchymal cells (PCs)

1.1.2.1. Liver sinusoidal endothelial cells (LSECs)

Eddie Wisse [3] was the first to present convincing evidence that the liver sinusoidal endothelial cell (LSEC) is a distinct cell type clearly different than the Kupffer cell and other sinusoidal liver cells. Moreover, Wisse's findings gave the first hint that these cells are active in endocytosis of blood plasma proteins [3]. At that time it was believed by most scholars that the cellular blood clearance system or the reticuloendothelial system (RES) was identical to the macrophages, or mononuclear phagocyte system (MPS). Wisse's suggestion that the LSECs endocytose plasma proteins was therefore not acted much upon. It was not until the beginning of the 1980's that scientists found the first evidence that the LSECs play an important role in the clearance of blood borne physiological macromolecular waste products [19, 20]. During the subsequent years several studies showed that the LSEC exhibits an unsurpassed blood clearance capacity [9]. In fact, an array of soluble waste colloids and macromolecules were shown to be removed by the LSECs and not by the KCs. Based on this solid body of evidence it was proposed by the end of the 1990's that the existing paradigm of "RES ≡ MPS" be shifted to "RES ≡ MPS + LSECs [9]. In spite of this, current text books in pathology and immunology still stick to the old incorrect paradigm of "RES ≡ MPS".

Moreover, most of the scientific literature published today still reveals that the old paradigm prevails. This is unfortunate, because it obscures interpretations of clearance studies. The novel paradigm holds that the LSECs are responsible for the clearance of blood borne colloids and soluble macromolecules (<200 nm), while KCs eliminate circulating particulate material (>200 nm).

Notably, animal species of all vertebrate classes contain a special population of scavenger endothelial cells [21]. In mammals and the 3 other land based vertebrate classes the LSECs carry the function of scavenger endothelial cells. However, in phylogenetically older vertebrates these cells are located in heart or kidney (bony fishes) or gills (cartilaginous and jawless fishes). In all vertebrates these cells play a pivotal homeostatic role by removing an array of physiological and foreign molecules from the blood [22]. To cope with the daily burden of grams of endocytosed material, LSECs are geared for rapid intracellular transport to degradation organelles [23], high capacity degradation of ligands by a large pool of active lysosomal enzymes, and release of degraded low molecular weight material to the surroundings [24].

So far, three specific receptors for endocytsois have been identified and characterized in LSECs, i.e., the scavenger receptor (SR), the mannose/collagen receptor (MANN/COLL-R), and the Fcγ receptor.

The scavenger receptor (SR)

LSECs express a number of SRs, including SR-A (a.k.a. macrophage scavenger receptor) [25, 26], SR-B (SR-B1 and CD36) [27] and SR-H (Stabilin 1/FEEL-1 and Stabilin 2/FELL-2/HARE) [26, 28-31]. The other SRs, namely SR-C (not found in mammals), SR-D (CD68: macrophage specific), SR-E (LOX-1), SR-F (SREC) and SR-G (SR-PSOX) are not

normally expressed on LSECs, but LOX-1 expression can be induced on LSEC with *in vivo* exposure to malondialdehyde-acetaldehyde-bovine serum albumin [32].

Despite the expression of three SR sub classes in LSEC, the main work-horse SR in LSEC appears to be Stabilin 2 (possibly together with Stabilin 1), based on the following: i) SR-A knockout mice clear SR ligands equally well as wild type mice [33-35], and cultured LSECs from the same knockout mice endocytose and degrade SR ligands equally well as wild types [36, 37]; ii) an antibody to CD36 that inhibits CD36 mediated uptake of SR ligands in other cells has no effect on the uptake of SR ligands by LSEC [38].

Several categories of waste substances are eliminated by LSECs via the SRs: i) N-terminal propeptides of types I and III procollagen (PINP and PIIINP) [39], and ii) atherogenic molecules: oxidized LDL (oxLDL) [40] and advanced glycation end products (AGEs) [41]. Clearance of these substances represents an important physiological mechanism contributing to maintaining homeostasis, and preventing atherosclerosis.

The mannose/collagen receptor (MANN/COLLA-R)

The mannose receptor (MANN-R) is an endocytic protein expressed in macrophages [42], dendritic cells [43], LSEC [9], kidney mesanglial [44], tracheal smooth muscle [45], and retinal pigment epithelial cells [46]. The receptor is a 180 kDa monomeric transmembrane glycoprotein [47] and shows affinity for D-mannose, L-fucose and N-acetyl-D-glucosamine residues, and mediates endocytosis and phagocytosis of glycoproteins, glycolipids and particles that expose these monosaccharides in terminal positions of their carbohydrate side chains [42].

The MANN-R mediates receptor-mediated endocytosis of various soluble macromolecules, such as tissue plasminogen activator (tPA) [48], C-terminal propeptide of

type I procollagen [49], salivary amylase [50] and lysosomal enzymes [51-55]. Soluble mannose-terminated waste glycoproteins in blood circulation end up in liver, in the LSECs, rather than in KCs or other macrophages [9], probably due to the higher level of receptors on LSECs than on KCs [56].

Until recently it was believed that LSECs express a separate receptor, the collagen α-chain receptor (COLLA-R), that recognizes only denatured collagen [57]. However, research conducted to identify the COLLA-R expressed on rat and pig LSECs resulted in purification of a 180 kDa cell surface protein [58], which was identified as the MANN-R. Thus, the MANN-R and COLLA-R in LSECs represent differently located binding domains of the same receptor protein.

The Fcγ receptor

In humans, Fcγ receptors (FcγRs) have been classified into 3 major classes comprising multiple isoforms: FcγRI/CD64 (FcγRIa, FcγRib, and FcγRIc), FcγRII/CD32 (FcγRIIa, FcγRIIb, and FcγRIIc), and FcγRIII/CD16 (FcγRIIIa and FcγRIIIb). All these receptors except FcγRIIIb, which is a glycosyl phosphatidyl inositol anchored variety, are type 1 transmembrane proteins that, despite similarities in their extracellular domains, differ by ligand-binding specificity, cellular distribution, and regulatory functions [59]. These receptors recognize the Fc domain of immunoglobulin G (IgG), and are expressed on virtually all cells of the immune system [60-62].

The liver is the main organ for uptake of IgG-immune complexes (IgG-ICs) from the circulation [62, 63]. FcγR-mediated elimination of circulating IgG-ICs has generally been assumed to be mediated by KCs [64, 65]. However, while internalization of IgG-opsonised particles occurs via phagocytosis in the KCs [8], small immune complexes and soluble

aggregated IgG are internalized through receptor-mediated endocytosis in clathrin-coated pits by LSECs [62, 63, 66]. Studies using monoclonal antibodies to Fc-receptors and immunohistochemistry suggested that the FcγRII, and not FcγRIII is expressed in human LSECs [67]. By RT-PCR and Western blotting, it was demonstrated that purified rat LSECs express only one FcγR, the FcγRIIb2 [59]. Moreover, binding of ICs to the LSECs was completely blocked by the presence of the monoclonal antibody against the ligand binding site of FcγRIIb2 [59]. A recent study showed that an antibody (SE-1) which is specific to LSECs in rat does in fact recognize the antigen CD23b (FcγRIIb2) [68]. FcγRIIb2 may therefore be considered a biomarker for LSECs.

1.1.2.2. Liver parenchymal cells (PCs)

The PCs are equipped with the following major endocytosis receptors: the asialoglycoprotein receptor (ASPG-R), the low-density lipoprotein receptor (LDL-R), the low-density lipoprotein receptor-related protein (LRP) and the scavenger receptor class B type I (SR-BI).

The asialoglycoprotein receptor (ASPG-R)

The hepatic ASGP-R represents the “pioneer endocytosis receptor” as it was discovered and characterized already in 1974 [69]. This receptor is one of the best characterized model system for receptor-mediated endocytosis via the clathrin-coated pit pathway [69-73]. ASGP-R mediates the endocytosis and degradation of a wide variety of desialylated glycoproteins and neoglycoproteins that contain terminal galactose (Gal) or N-acetylgalactosamine (Gal-NAc) residues on their N-linked carbohydrate chains [69]. The most commonly used test ligand is asialoorosomucoid (ASOR), which contains tri-and tetra-antennary N-linked glycans. The ASGP-R is expressed along the entire surface of PC,

enriched in clathrin-coated pits, most abundantly over the sinusoidal plasma membrane domain, and one PC contains 1-5 x 10^5 binding sites [52, 74]. The affinity of the ASGP-R increases for mono-, di-, tri-, and tetra-antennary oligosaccharides [75]. The ligand receptor interaction is reversible on addition of competing Gal-exposing derivates, in the absence of Ca^{2+} and on acidification [76, 77]. Recycling of internalized ASGP-Rs back to the plasma membrane occurs with high efficiency [78, 79]. In isolated rat PCs, it takes about 5-7 min for internalized ASGP-Rs to reappear on the cell surface [80, 81].

Involvement of the ASGP-R in the clearance of coagulation proteins was also recently shown. Coagulation Factor VIII (FVIII) is recognized by ASGP-R as a ligand *in vitro*, and its binding is driven by the oligosaccharide structures of the FVIII B domain, which has 15 complex-type N-linked oligosaccharide branches [82]. Studies on mice deficient in ST3Gal-IV, an enzyme that mediates attachment of sialyl groups to terminal galactose residues, showed that the half-life of endogenous von Willebrand Factor (vWF) is reduced 2-fold in mice [83]. Enzymatic removal of sialyl groups reduces the half-life of vWF in rabbits from 240 min to 5 min [84]. The importance of the nature of terminal carbohydrate residues is most strikingly illustrated by RIIIS/J-mice, a strain characterized by vWF levels that are several fold lower compared with other strains. This level is caused by a rapid clearance of vWF through ASGP-R due to a surplus of terminal N-acetylgalactosamine residues [85].

The low-density lipoprotein receptor (LDL-R)

The low-density lipoprotein receptor (LDL-R) plays a critical role in the homeostatic control of blood cholesterol by mediating the removal of cholesterol-containing lipoprotein particles from circulation [86]. The most important physiological ligand for the receptor is

low density lipoprotein (LDL) [87, 88], which carries approximately 65% to 70% of plasma cholesterol in humans.

The LDL-R consists of five distinct domains with individual function: 1) ligand binding domain at the N-terminus containing complement-type repeats involved in LDL binding; 2) epidermal growth factor (EGF) precursor-homology repeats that contain YWTD motifs responsible for ligand dissociation; 3) an O-linked sugar domain acting as a spacer; 4) a membrane-spanning domain for anchorage; and 5) a cytoplasmic tail (NPxY motif) involved in internalization of LDL particles into coated pits [89, 90] (Figure 4).

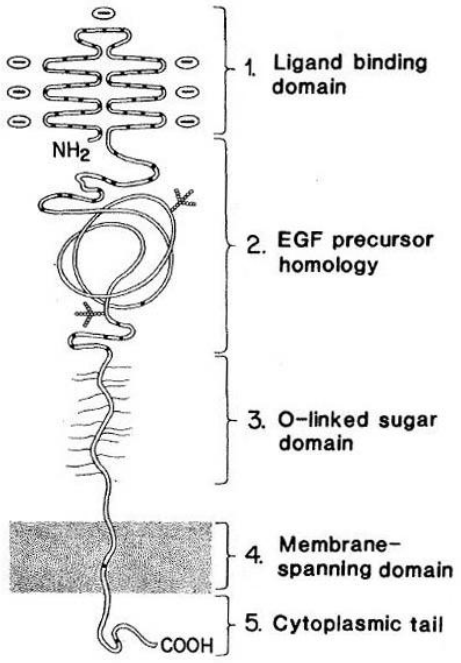

Figure 4. **Structure of the LDL receptor** From Brown, M.S. and J.L. Goldstein, *A receptor-mediated pathway for cholesterol homeostasis.* Science, 1986. 232(4746): p. 34-47., Reproduced with permission of AAAS and Dr. Brown.

There are eight members of the family besides LDL-R. These are: the low-density lipoprotein receptor-related protein (LRP), Megalin, very-low-density lipoprotein (VLDL) receptor, apoER2 and SorLa/LRP11, LRP1b, MEGF7, LRP5/6 (Figure 5). Each member of this receptor family undergoes receptor-mediated endocytosis; yet each member is expressed

in a number of different tissues and has a wide range of different ligands, not specific to the recognition of the LDL particle [91-93].

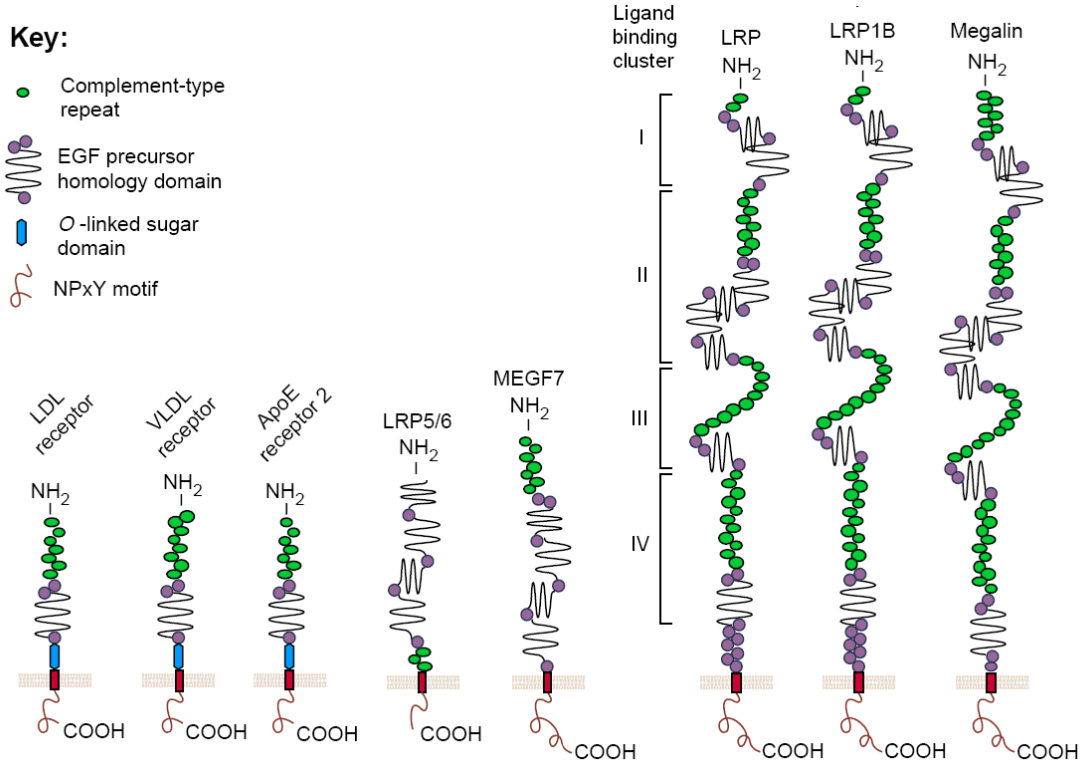

Figure 5. Structural organization of mammalian receptors of the low-density lipoprotein (LDL) receptor family (modified with permission of Nykjaer and Moestrup [91, 94])

Low-density lipoprotein receptor – related protein (LRP)

The low density lipoprotein receptor-related protein (LRP) is one of the largest member of the family (600 kDa). LRP is a multifunctional endocytic scavenger receptor expressed in a number of different cell types, mostly in hepatocytes, macrophages, trophoblasts, neurons, activated astrocytes and fibroblasts, pneumocytes and smooth muscle cells [95-97]. Mature LRP contains two subunits, a 515 kDa extracellular domain (α-chain), noncovalently attached to a 85 kDa intracellular and transmembrane domain (β-chain), which are produced by proteolytic cleavage from a single polypeptide precursor of 600 kDa in the trans-Golgi network [95, 98, 99].

Due to its high expression in the liver, LRP mediates the cellular uptake of lipoprotein particles containing apoE and lipoprotein lipase [100-102]. It cooperates with LDL receptor in the removal of cholesterol containing remnant lipoproteins from the circulation; thus acting as a chylomicron remnant receptor [100, 101, 103]. Despite lipoprotein recognition, it has a wide range of other ligands from urokinase-type plasminogen activators (uPA), amyloid precursor protein (APP), tissue factor pathway inhibitor (TFPI), protease/inhibitor complexes, toxins, viruses and activated α_2-macroglobulin [93, 99, 104-107]. All these ligands deviate both in structure and function and, characteristically, do not cross-compete for binding to LRP [108]. The major ligand-binding sites within LRP are contained in clusters II and IV (Figure 5) and most ligands bind equally well to both clusters, suggesting a functional duplication within LRP [109].

LRP gene depletion in mice has demonstrated a failure of LRP $^{-/-}$ embryos to develop after implantation. Thus, LRP seems to be important during foetal development, and mutations within the LRP gene can induce embryonic death within 10 days. [99]. In addition, LRP has been associated with the progression of Alzheimer's disease [110-112].

The Scavenger Receptor Class B type I (SR-BI)

SR-BI is a cell surface glycoprotein of 82 kDa [113] consisting of a horseshoe-like large extracellular loop, short N- and C-terminal cytoplasmic domains, adjacent N- and C-terminal transmembrane domains, and the bulk of the protein in a heavily N-glycosylated, disulfide-containing extracellular domain [114].

SR-BI is highly expressed in organs with critical roles in cholesterol metabolism (liver) and steroidogenesis (adrenal, ovary, testis) [114-116]. SR-BI has binding specificity for high-density lipoprotein (HDL) and mediates selective uptake of lipids from HDL without

endocytic uptake of the lipoprotein itself [115, 117]. Besides HDL, SR-BI binds a wide array of ligands, including anionic phospholipids [118], advanced glycation end products (AGE) [119], apoptotic cells [120], oxidized LDL (oxLDL), maleylated BSA, apoB-containing lipoproteins, including LDL and VLDL [121], apoE, an important ligand for VLDL removal from the circulation [122] and native and modified lipoproteins [115, 123-125].

1.2. Tissue factor pathway inhibitor (TFPI)

1.2.1. History

TFPI is an endogenous anticoagulant protein, a serine protease inhibitor, and the only known regulator of the TF-dependent pathway of blood coagulation. It is known to play an important role in the control of thrombogenesis at both cellular and plasmatic sites. Experiments performed early in the second half of the last century demonstrated the presence of an endogenous inhibitor of TF-induced coagulation activation [126-128]. Subsequently, Hjort (1957) reported that convertin, now known as TF-FVIIa catalytic activity, was inhibited by a component present in serum [129]. He described the calcium dependence and reversibility of the inhibition by calcium chelators, and suggested from indirect methods, that the inhibitor to convertin was calcium dependent, and named the inhibitor anticonvertin. The nature of this inhibition remained obscure until 25 years later, when Dahl et al. published a chromogenic substrate assay system, for the determination of the inhibitor [130]. They demonstrated that anti-convertin activity was present in two high molecular weight peaks, identified by gel filtration of plasma. Then Sanders et al. reported the remarkable finding that inhibition of TF-FVIIa requires the presence of FX [131], and it was quickly shown that FXa rather than FX was responsible for the inhibition of TF-FVIIa. The inhibitor was isolated in 1987 by two independent groups [132, 133], and was cloned and characterized in 1988 by Wun et al. [134].

The name of the inhibitor varied during the years [anithromboplastin, anticonvertin, the factor VIIa/tissue factor inhibitor, tissue factor inhibitor, extrinsic pathway inhibitor (EPI) and lipoprotein-associated coagulation inhibitor (LACI)], until a consensus meeting of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis in 1991 agreed on the name “Tissue Factor Pathway Inhibitor” (TFPI).

1.2.2. Molecular structure

The translated product of the human TFPI mRNA is a 304 amino acid protein. The removal of a classical 28 residue signal peptide yields the mature 276 amino acid TFPI [134]. TFPI contains 3 Kunitz-type domains arranged in tandem, each containing 3 disulphide bonds. There is a nonuniform charge distribution within the TFPI molecule. The amino-terminal contains several negatively charged acidic residues. The carboxy-terminal sequence includes 15 positively charged basic amino acids (Figure 6). The predicted molecular weight of the polypeptide backbone of TFPI is 32 kDa, and due to posttranslational modifications, the mature secreted protein size can increase to about 43 kDa [134]. TFPI is *N*-linked glycosylated at Asn117 and Asn167. *O*-linked carbohydrate attachment occurs at Ser174 and Thr175 [135].

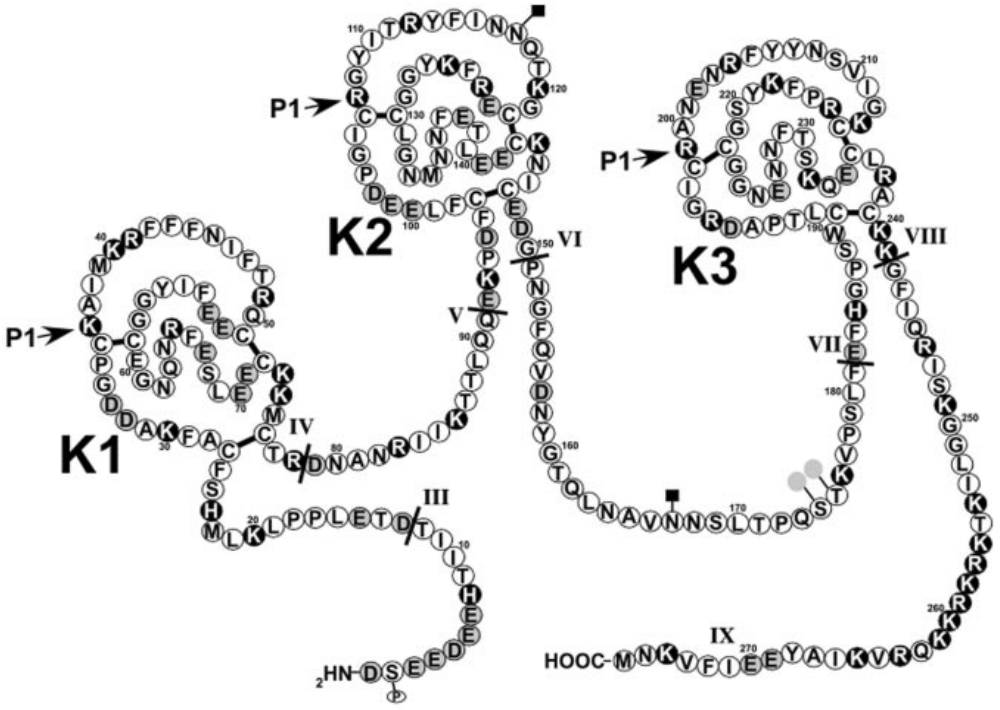

Figure 6. Primary amino acid sequence of mature TFPI Exon boundaries are numbered in roman numerals. *N*-linked glycosylation sites at Asn117 and Asn167 (black squares). *O*-linked glycosylation sites at Ser174 and Thr175 (gray circles). The Kunitz domains (K1, K2, K3) contain 3 disulphide bonds each. Black arrows indicate the P1 residues. P1 in K1 interacts with FVIIa in the TF-FVIIa complex. P1 in K2 interacts with FXa. The predicted P1 in K3 has no described inhibitory role. (Reproduced with permission of Dr. James Crawley [136])

The TFPI mRNA found in the endothelium and various cell types exists as a 4 kb and 1.4 kb species [137, 138]. The difference between the two mRNA sizes has been attributed to the presence of 2.6 kb additional, not translated sequences in the 4 kb band. The TFPI gene resides on the long arm of chromosome 2, at region 2q31-2q32.1, and contains nine exons, which are separated by eight introns [134]. Although the nucleotide sequence of the promoter region, containing several binding sites for transcription factors, has been determined, it remains to be established how endothelial cells specifically synthesize TFPI, and how the expression is regulated. Recently both the TGF-b-like response elements and GATA sequences have been reported [139-141]. The GATA-sequences act as cis-regulatory elements in the expression of several genes, and appear to be active in the expression of genes in the endothelium and haematopoietic cells [142, 143]. GATA-motifs in TFPI-gene may bind GATA-2 transcription factor, also expressed in the endothelium and thereby regulate TFPI gene expression [141, 144].

To date, no patients with TFPI-deficiency have been reported, perhaps because homozygous TFPI deficiency is likely to be lethal. This is supported by observations of embryonic death, from unregulated consumptive coagulopathy in mice embryos, with TFPI gene disruption [145]. Additionally, a few polymorphism sites in the TFPI-gene have been reported in the normal population, and in patients with acute coronary syndrome, as well as with arterial and venous thrombosis [146-148], but do not seem to play a significant pathophysiological role in either arterial or venous thrombosis [149].

Plasma TFPI-levels vary across species [150]. The homology of rabbit and rat TFPI to human TFPI is about 60% [151, 152], whereas the homology of monkey TFPI to human TFPI is about 94% [153]. The differences between rabbit and rat TFPI, and those of humans or

monkeys, may be due to differences in the amino acid sequences, or post-transitional modification of TFPI.

TFPIα is a soluble form of TFPI. It is a 43 kDa glycoprotein, and consists of a highly negative acidic N-terminal region, followed by three tandem Kunitz-type inhibitory domains, and a highly positive C-terminal region. Studies of cultured endothelial cells and human placenta have demonstrated that TFPI associates with the cell surface through a glycosylphoaphatidyinositol (GPI)-anchor in a manner that is not dependent on glycosaminoglycans (GAGs) or altered by heparin. TFPIα is not directly bound to the GPI-anchor; instead it appears to bind to a GPI-anchored protein, a protein that appears to be necessary for the proper trafficking of TFPI to the cell surface [154-156]. Site-directed mutagenesis experiments indicate that domain 1 (K1) of TFPI binds to FVII and domain 2 (K2) binds to FXa, respectively [157]. The precise function of the third Kunitz-type domain is not fully understood. It appears that it has no inhibitory activity, but is probably involved in the association with lipoproteins [158], and is mandatory for the anticoagulant function of TFPI in TF-induced coagulation *in vitro* [159, 160]. The region between residues 181 and 242, including the third Kunitz domain, contains a heparin binding site, but whether this site is important for TFPI-function or -physiology is not known [161]. The heparin-induced enhancement of TFPIs FXa-inhibition has been shown to become gradually greater, as more of the C-terminal portion is intact [162]. Truncated forms of TFPI, lacking most of their C-terminal domains, exhibit reduced affinity for vascular wall proteoglycans [163]. Whether or not the N-terminal acidic region of TFPI plays a role in physiological inhibition of TF-induced coagulation remains to be determined. Posttranslational modifications in the TFPI-molecule include *O*-linked glycosylation at Ser174 and Thr175, as well as three potential sites for *N*-linked glycosylation: Asn117, Asn167 and Asn228 [157, 164, 165]. The extent of N-linked glycosylation in human plasma TFPI appears to be less than that of $TFPI^{HepG2}$ or

$TFPI^{CHO}$; N-linked glycosylation is linked only at Asn117 and Asn167 [135, 166]. Although these posttranslational modifications do not seem to function directly in the inhibitory function of TFPI, they may influence its cell binding properties and plasma clearance, possible through their interactions with the basic carboxy-terminal. Recombinant bacterial TFPI ($TFPI^{E.Coli}$), lacking post-translational modifications, may show functional differences to TFPI, that is endogenously synthesized or released from endothelium [167]. $TFPI^{E.Coli}$ does not interact with cell surface in the same manner as endogenously expressed TFPIα [168]. The cellular binding of $TFPI^{E.Coli}$ is of relatively low affinity, and appears to involve an interaction with surface glycosaminoglycans [163, 167].

TFPIβ is an alternatively spliced form of TFPI, in which the Kunitz-3 domain and the C-terminal region of TFPIα are replaced with an alternatively spliced C-terminal region, that signals direct attachment of a GPI-anchor [169, 170]. Based on protein mass, TFPIβ is considerably smaller than TFPIα, suggesting a difference in post-translational modifications, i.e. TFPIβ containing O-linked carbohydrate with considerably greater sialic acid content than TFPIα [169]. Although TFPIβ accounts for only 20% of total surface TFPI, it is responsible for most of the FXa-dependent anti TF-FVIIa-activity, suggesting a potential alternative role for cell-surface TFPIα [169]. The binding of TFPIα to its cellular receptor appears to interfere with its interaction with TF-FVIIa, perhaps because of steric effects or by limiting the movement of TFPIα at the membrane surface. Piro and Broze suggested another role of cell-surface TFPIα than just the inhibition of TF-FVIIa. They speculated that the binding of endogenously expressed TFPIα, but not $TFPI^{E.Coli}$, may affect protease-activated receptors (PARs) signalling [169]. When $TFPI^{E.Coli}$ was used to attempt inhibition of excessive TF expression on vascular endothelial cells, coagulation was more efficiently blocked than cell-

signaling. Therefore, although TF procoagulant function may be blocked by $TFPI^{E.Coli}$, TF signaling function may continue [171].

Truncated forms of TFPI also exist in the circulation. They lack most of the carboxy-terminal and often also most of the K3 [172], and so also exhibit reduced inhibitory effects, and a lower affinity for GAGs. Although it is not known how the truncated forms are generated physiologically, *in vitro* data have demonstrated that TFPI is cleaved into degraded forms by various proteases that TFPI might encounter physiologically. These include thrombin, plasmin, neutrophil elastase, and certain matrix metalloproteinases [173, 174].

1.2.3. Mode of action

TFPI exerts its function by neutralizing the catalytic activity of FXa, and by feedback inhibition of the FVIIa-TF-complex, in the presence of FXa [175, 176]. In the first step, TFPI inhibits FXa in a 1:1 stoichimetric complex by binding at or close to the active site serine of FXa. This binding is reversible and does not require calcium ions. Heparin moderately enhances the inhibition of FXa by TFPI through a template mechanism in which the simultaneous binding of FXa and TFPI to the same heparin molecule increases their interaction [161, 177]. In the second step, TFPI associated with FXa inhibits the TF-FVIIa-complex in a stoichiometric complex which is calcium ion dependent. This leads to the formation of the stable quaternary complex TFPI-FXa-TF-FVIIa (Figure 7) which does not possess catalytic activity on FX and FIX. Experiments in a model system surprisingly showed that protein S enhances the inhibition of TF/FVIIa-catalyzed FX-activation by TFPI [178]. This observation provides an important role for protein S in the down regulation of coagulation, and suggests that the increased risk of venous thrombosis associated with protein

S deficiency may, in part, be explained by an impaired down-regulation of the extrinsic coagulation pathway by TFPI at low protein S concentrations.

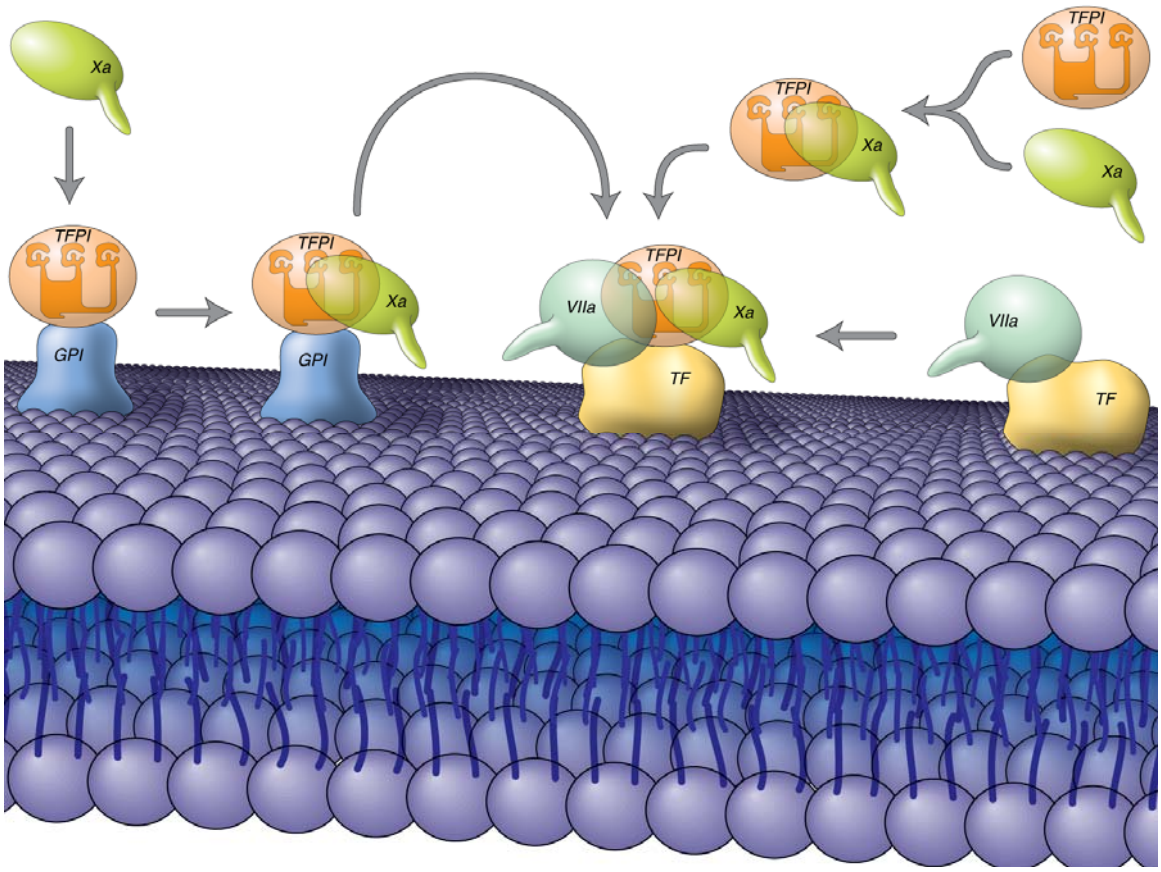

Figure 7. Mechanism for regulation of coagulation by TFPI

However, the requirement of FXa for the inhibition of TF-FVIIa by TFPI is not absolute, and very high concentrations of TFPI may inhibit the activation of FIXa by TF-FVIIa in the absence of FXa. This FXa-independent inhibition by TFPI is of questionable physiological relevance, but could be important when TFPI is used as a therapeutic agent, and when plasma levels of TFPI reach 10 to 50-fold that of normal plasma concentrations [179].

In addition to its direct function as an endothelial-bound anticoagulant, surface bound TFPI may also have an indirect anticoagulant impact, by regulating the clearance of FXa and FVIIa from the circulation via cellular internalisation and degradation [180].

1.2.4. Synthesis and intravascular distribution

TFPI is distributed in three pools *in vivo*; 80-85% is associated with vascular endothelial cells (EC); localized to the cell surface, the Golgi apparatus, and to the endocytic compartment [181]. In plasma, 10% circulates primarily in association with lipoproteins and a small amount in free form, and 3-5% is present within circulating/resting platelets [182, 183] (Figure 8).

TFPI secreted by endothelial cells circulates in plasma as a concentration of about 2.5 nmol/l [184, 185] but fluctuates temporally in humans because of their natural circadian rhythms [186, 187]. Plasma levels of total TFPI are increased 1.5-to 3-fold after administration of heparin [188].

The major site of TFPI production is in endothelial cells, which constitutively express the protein under normal physiological conditions [137], where it is thought to contribute, in part, towards maintaining lumen in an antithrombotic state. In immunohistochemical analysis of normal human tissues TFPI was found to be restricted to the micro vascular endothelium and megakaryocytes [189]. The expression of TFPI-gene by endothelium of different organs appears to vary, perhaps based on the physiologic demands of the tissue [190]. The human lung and heart appear to express the highest quantities of TFPI mRNA [190]. These findings are corroborated by studies in mouse [90]. Small amounts of TFPI are also expressed by monocytes [191], within platelets [192], macrophages [189], lung fibroblasts and vascular smooth muscle cells [137], laryngeal squamous epithelial cells [193], astrocytes [190], cardiomyocytes and mesanglial cells fibroblasts [194]. In addition, TFPI has been detected in macrophages and T cells in atherosclerotic lesions [195]. Importantly, cells that do not synthesize TFPI in the adult under physiological conditions are normal hepatocytes, erythrocytes, neutrophils and lymphocytes [137, 196]. In the human foetus, TFPI is expressed

in the endothelium, liver, as well as in epithelium of the lung, kidney and intestine [197]. Further, TFPI is abundantly expressed in placental syncytiotrophoblasts and cytotrophoblasts, where it helps maintain the blood in a fluid state [197].

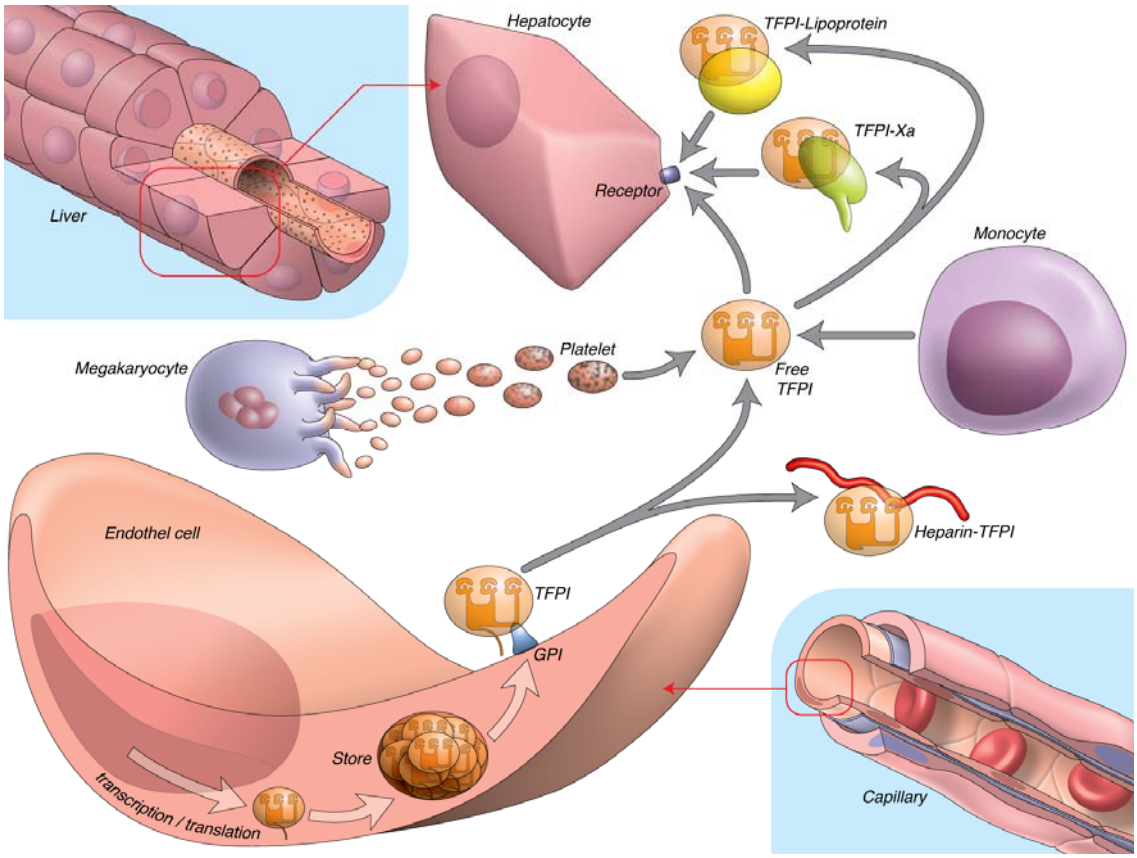

Figure 8. Distribution of TFPI *in vivo*

TFPI expression can be modulated in several cell types in response to various inflammatory stimuli. Shear stress applied to human endothelial cells is reported to increase the expression of TFPI twofold [198]. In addition, thrombin may release cellular TFPI from the vascular endothelium and contribute to an increase in endothelial surface TFPI [199]. Upregulators of TFPI expression *in vitro* that might be of physiological significance include endotoxin, interleukin (IL)-1, tumor necrosis factor (TNF)-α, platelet-derived growth factor, heparin, basic fibroblast growth factor, and elevated shear stress [144, 200-202]. However, endotoxin only slightly upregulates TFPI expression in normal human monocytes and in a

monocytoid cell line U937 [203, 204]. The exposure of different cell types to such agonists or conditions may represent a physiological mechanism by which local anticoagulant activity is upregulated in response to a given challenge.

1.2.5. Elimination and degradation

It was previously assumed that TFPI secreted from ECs became reattached to sulphated proteoglycans at the cell surface glycocalyx through electrostatic bounds between the positively charged C-terminus of TFPI and the negatively charged sulphate group in GAGs. Heparin was simply thought to displace TFPI from binding site on the EC surface and release it into the blood stream in the form of heparin-TFPI complexes. However, heparin treatment of cultured endothelial cells induces a significant release of TFPI in the medium without affecting its surface concentration [163, 201, 205]. This result suggests that TFPI is released from intracellular stores in response to heparin, rather than from the cell surface, or displaced from the membrane surface followed by a very rapid replacement of cell surface TFPI from intracellular stores [201].

Recently, more pieces of evidence have been gathered to point out a possibly more important role for the endogenous cell-associated TFPI than of the circulating form of the inhibitor in maintaining the anticoagulant properties of the endothelium. Studies in cultured endothelial cells have reported that endothelium-associated TFPI was bound to GPI membrane anchors and could be released by phospholipase treatment [170]. Furthermore, GPI-anchored TFPI is located in glycospingolipid-rich domains, in caveolae [154, 156] and mediates the formation and translocation of TF-FVIIa-FXa complexes in activated endothelial cells [154]. Thus, it is possible that the endothelium plays a key role in regulation of coagulation by TFPI.

A separate clearance system is responsible for the removal of TFPI from the circulation. The primary organs involved in TFPI clearance are the liver and kidneys [206]. Pharmacokinetic studies showed that recombinant $TFPI^{E.Coli}$ has a biphasic and rapid clearance from the circulation with a plasma half-life of approximately 2 minutes in rabbits [206] and less than 1 minute in rats [207]. These characteristics of $TFPI^{E.Coli}$ clearance are reminiscent of other ligands whose cellular uptake and degradation are mediated by the LRP. LRP is particularly abundant in liver, brain and placenta [208], and a closely related receptor, glycoprotein 330 (gp330) that binds many of the same ligands is located in the kidney [209].

Both *in vivo* and *in vitro* studies have demonstrated that LRP in liver mediates the cellular degradation of $TFPI^{E.Coli}$. Furthermore, *in vitro* studies in HepG2 cells have shown that receptor-associated protein (RAP), an inhibitor of all the ligand interactions with LRP, inhibits degradation of $TFPI^{E.Coli}$ [167, 210]. The initial hepatic cell surface binding in this cell culture requires the carboxy-terminus of TFPI, and is inhibited by clinically achievable levels of heparin [207]. Thus, it was initially proposed that LRP-mediated clearance of $TFPI^{E.Coli}$ involves a two-step process, in which TFPI first binds to heparan sulphate proteoglycans (HSPGs) on the liver endothelial surface, before its transfer to LRP and subsequent internalization. However, later studies demonstrated that receptor-mediated endocytosis of $TFPI^{E.Coli}$ by LRP occurred independently of HSPGs [211]. More recently, it was shown that recombinant human full-length TFPI expressed in mouse C127 cells ($TFPI^{C127}$, glycosylated) had substantially prolonged survival in the circulation and an apparently different route of elimination independent of LRP [167]. Although it has never been demonstrated whether glycosylation of the molecule could explain the difference between the two TFPI species, it must be considered that data from clearance studies using bacterially expressed TFPI (non-glycosylated) may not reflect precisely the normal physiological clearance of endogenous TFPI.

1.3. Heparin

1.3.1. History

Heparin was first discovered in 1916 by Jay McLean. He was investigating procoagulant preparations when he isolated fat soluble phosphatides from heart and liver tissues that inhibited blood coagulation. This type of fat soluble anticoagulant present in the liver was termed heparin in 1918. In the beginning, heparin was isolated from dog liver but was scarce, causing toxic side reactions and extremely expensive. It was not until the early 1930s that Connaught Medical Research Laboratories (Toronto, Canada) developed a method for making available a purified, plentiful and inexpensive supply safe for human use. Instead of using dog liver as a source they changed to beef liver, and later to beef lungs and intestines [212].

The first human trials began in May 1935, which soon involved hundreds of complex surgical cases where Connaught's heparin played an essential role. By 1937 it was clear that Connaught's heparin was easily available, safe and effective blood anticoagulant, and by the early 1940s it was available both for experimental and clinical use. Its routine application to medical procedures did not occur however until after the Second World War.

1.3.2. Structure

Heparin is a naturally occurring polysaccharide produced in the granules of mast cells that are closely associated with the immune response. Thus, the ability of heparin to regulate the major activities of the complement cascade is an area of active interest [213].

The biosynthesis of heparin includes formation in ER/Golgi of a polypeptide core containing characteristic extended ser-gly sequences. Following substitution of these serine

residues with gal-gal-xyl sequences the polysaccharide chain is built by adding alternating D-glucuronic acid and N-acetyl-D-glucosamine units. The resulting highly sulphated polysaccharide chains are converted to heparin through a series of modification reactions [214-216].

Heparin is normally polydisperse, with a molecular weight within 3-30 kDa, and an average molecular weight of about 15 kDa. Heparin has the highest negative charge density of any known biological macromolecule. This is the result of its high content of negatively charged sulfo- and carboxyl-groups [216]. The main repeating structure of heparin is a disaccharide of alternating N- and 6-O-sulfated alfa-D-glucosamine and 2-O-sulfated alfa-L-iduronic acid (90%) [217], with a minor proportion of N-acetyl glucosamine and beta-D-glucuronic acid (10%). Determination of the structural motif in heparin with high affinity for antithrombin came from Petitou et al. 2003 [218] (Figure 9).

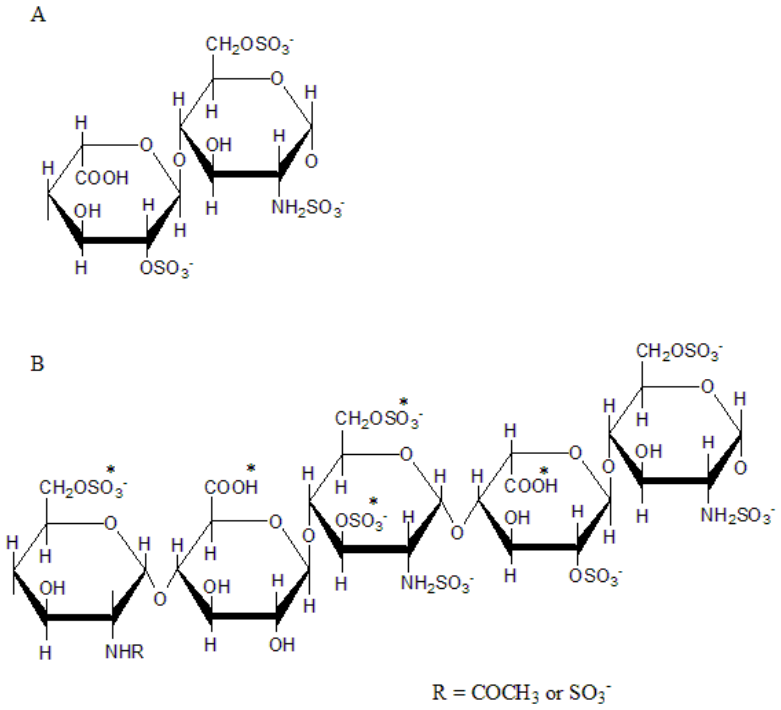

Figure 9. Structure of heparin
A) The main repeating unit of heparin. B) the pentasaccharide in heparin, which is the minimal structure with high affinity for antithrombin. (Reproduced with permission of the American Physiological Society [219])

1.3.3. Mode of action as an anticoagulant

Heparin exerts its anticoagulant effects by accelerating the interaction between the enzyme inhibitor antithrombin (AT) and various serine proteases, such as thrombin, FX and FIXa [220, 221]. Binding of heparin to AT causes a conformational change that results in its active site being exposed. The activated AT then inactivates thrombin and other proteases involved in blood clotting, among them factor Xa. Thus, heparin inhibits blood coagulation by binding to AT, thereby promoting inactivation of the protease factors of the coagulation cascade mechanism. In the absence of heparin, the reactions between AT and coagulation proteases are slow. With optimal amounts of heparin present, these reactions can be accelerated up to 2000-fold, thereby efficiently preventing the formation of fibrin in blood [222]. The reason for heparin's desirability as an anticoagulant is its safety, rapid onset of activity, and reversibility [223]. For controlling heparin therapy and in assay techniques, several other inorganic and organic substances have been reported to have antagonistic properties to heparin, such as protamine, clupein, polylysine, lysozyme, polybrene, toluidine blue, fuchsin, and tryptophan [223].

1.3.4. Pharmacology

Heparin and low molecular weight (LMW) heparins are the most commonly used clinical anticoagulants. The main clinical use of heparin is in acute coronary syndrome, e.g. myocardial infarction, vascular and cardiac surgery, atrial fibrillation, deep-vein thrombosis and pulmonary embolism.

Heparin is involved in a rising number of other physiological and pathological processes, such as tumor cell metastasis and immune cell migration and inflammation [224-228]. For instance, heparin is able of attenuating the neurotoxic and proinflammatory activity

of amyloid-beta protein, which is implicated in the pathogenesis of the Alzheimer disease because of its neurotoxicity and ability to trigger local inflammation [224]. This suggests that heparin could represent a new strategy to reduce the progressive neurodegeneration in the Alzheimer disease. Furthermore, peripheral treatment with the low molecular weight Enoxaparin has been shown to reduce plaques and beta-amyloid accumulation in a mouse model of the Alzheimer disease, which offers promise as a tool for slowing the progression of the disease [225].

1.3.5. Elimination

Because heparin is a widely prescribed drug, it is very important to understand its clearance mechanisms. Heparin clearance involves a combination of rapid saturable and much slower clearance kinetics [229-231]. The elimination of heparin from blood can follow three potential pathways. The first pathway is attachment to the vascular endothelium [232]. This binding was assumed to contribute significantly to the rapid phase of the UFH clearance. This assumption was supported by experimental studies on vascular endothelial cells *in vitro*, showing binding to saturable and specific binding sites at the endothelial surface. However, the capacity for internalization of heparin was reported to be very low in these cells [233-235]. The second pathway is by uptake and metabolism by cells of the RES. Studies in hepatectomized dogs [236] and patients with liver cirrhosis [237] showed increased circulatory half life of intravenously administered UFH in the circulation, indicating that the liver plays a crucial role in UFH removal. Both in humans [238] and in animal models [239, 240], the plasma half-life of heparin increased with dose. Whole body autoradiography of rats injected with tritiumlabeled heparin showed that the ligand was retained by the organs belonging to the RES, with the largest uptake in the liver [241]. However, little is known about the cells involved in the clearance of UFH from the circulation. The first evidence was

published in 1939 by Asplund et al. [242], when they observed heparin in the "sternzelln" of the liver sinusoids of rabbits, rats, and guinea pigs, but not in liver parenchymal cells (PCs) following injection of single and repeated doses of heparin. More than 40 years later, Hiebert observed that repeated administration of heparin into normal and atherosclerotic rabbits was succeeded by heavy accumulation of the polysaccharide in Kupffer cells (KCs) and in endothelial-like cells lining the liver sinusoid [243]. Most studies on distribution of heparin in liver performed *in vitro* using fractionated heparin report that the KCs and the PCs represent the main cellular site of uptake [244-247]. A specific binding and uptake via scavenger-like receptors in KCs and PCs has been suggested [246, 248]. The third pathway of heparin elimination involves the kidney, either via direct excretion into the urine, or by involvement of cellular clearance implicating a metabolic process. LMWH binds to the endothelial cells much less than UFH and is therefore more dependent on the renal, non-saturable elimination process than UFH [249]. It was shown that in nephrectomised rabbits, about 40% of UFH and 70% of the LMWH was eliminated by the kidney and that the elimination in case of the LMWH was due to urinary excretion [250]. In the same study RES was blocked by dextran sulphate, a ligand for the SR, which resulted in a prolonged half-life for UFH but not for LMWH.

1.4. Heparin and TFPI

Numerous proteins of physiological and pathophysiological importance interact with heparin [216]. These interactions lead to an interest in using heparin in roles outside its normal application as an anticoagulant/antithrombotic agent, and offer a large number of potential therapeutic applications for heparin.

In 1963, Nordøy [251] reported that polybrene completely neutralized the anticoagulant effect of heparin added to plasma *ex vivo*, whereas polybrene was unable to

abolish the anticoagulant effect in plasma collected from persons receiving heparin. This phenomenon was referred to as the post-heparin effect, and later attributed to TFPI [252]. TFPI and heparin are known to exert synergistic inhibitory effects on TF-induced coagulation *in vitro* [104, 253], and TFPI may account for as much as 30-50% of the prolongation in a diluted prothrombin time assay, caused by a bolus administration of heparin [252].

Although both UFH and LMWH are known to upregulate the synthesis and release of TFPI in endothelial cells *in vitro* [201, 205], prolonged administration of UFH, but not LMWH, is known to cause partial depletion of both plasma-free TFPI and heparin-releasable-TFPI *in vivo* [254, 255]. The reason for this discrepancy is unknown. Only trace amounts of endogenous TFPI is excreted into the urine in a native, non-degraded form under physiological conditions in humans [256], suggesting passive leakage rather than active excretion in the kidneys. Prolonged UFH treatment has been shown to decrease excretion of TFPI into the urine in humans, most probably by abrogating renal leakage due to formation of large molecular weight UFH-TFPI complexes in the circulation [256]. Furthermore, i*n vivo* studies have shown that LMWH prolonged the β half-life clearance of $TFPI^{BHK}$ in rabbits [257], and both LMWH and UFH inhibited binding of $TFPI^{E.Coli}$ to rat hepatoma MH_1C_1 cells *in vitro* [207]. $TFPI^{E.Coli}$ was shown to bind to the endothelial cell surface proteoglycans or glycosaminoglycans [207], but inhibition of this interaction with protamine promoted increased $TFPI^{E.Coli}$ degradation by LRP-positive cells (rat hepatoma MH_1C_1 cells and mouse embryonic fibroblasts heterozygous PEA10 cells), most probably by increasing the availability of TFPI for receptor-mediated endocytosis [211]. Thus, it remains a puzzle to understand the physiology and pharmacology beyond the apparent paradox of depleted intravascular stores, increased endothelial synthesis and inhibited elimination of TFPI by UFH.

2. AIMS OF THE STUDY

2.1. To carry out a more detailed pharmacokinetics study during the first phase of clearance, to identify the organ(s) involved in removal of recombinant human TFPI purified from BHK cells ($TFPI^{BHK}$) from the circulation, and to determine the hepatocellular site of uptake, as well as to identify the main cell (s) and their receptor(s) involved in the clearance.

2.2. To study the role of liver sinusoidal endothelial cells (LSECs) in the clearance of unfractionated heparin (UFH)

2.3. To study the mechanism of TFPI depletion during UFH treatment by investigating the effect of UFH *in vivo* and *in vitro* on the clearance of a recombinant full length glycosylated TFPI purified from baby hamster kidney cells ($TFPI^{BHK}$) as compared to recombinant full length non-glycosylated TFPI purified from *E.Coli* ($TFPI^{E.Coli}$)

2.4. To investigate if low density lipoprotein receptor-related protein-1 (LRP-1) is expressed on LSECs

3. SUMMARY OF THE RESULTS

Paper I: Asialoglycoprotein Receptor (ASGP-R) in Liver Parenchymal Cells is Involved in Elimination of Recombinant Human TFPI

We here report on a study carried out to determine the early clearance kinetics, and organ, cell(s) and receptor(s) responsible for the clearance of full length TFPI purified from BHK cells ($TFPI^{BHK}$). Following intravenous administration, $^{125}I\text{-}TFPI^{BHK}$ was cleared with a biphasic elimination curve, and with a significantly slower $t_{1/2}\alpha$ compared to recombinant human TFPI from *E.Coli* ($TFPI^{E.Coli}$) (1.95±0.10 versus 1.42±0.07 min, respectively, $p<0.001$). Studies on organ and cell distribution revealed that liver parenchymal cells (PCs) were responsible for 96% of the uptake of $TFPI^{BHK}$ and 81% of $TFPI^{E.Coli}$, whereas the non-parenchymal cells (NPCs) were responsible for uptake of 4% and 19%, respectively. Pre-administration of excessive amounts of unlabeled $TFPI^{BHK}$ prolonged blood clearance of $^{125}I\text{-}TFPI^{BHK}$. Unlabelled $TFPI^{BHK}$ inhibited endocytosis of $^{125}I\text{-}TFPI^{BHK}$ in PCs *in vitro*, whereas blocking of LDL-receptor related protein-1 (LRP-1) by receptor-associated protein (RAP) affected neither blood clearance nor endocytosis of $^{125}I\text{-}TFPI^{BHK}$ in PCs. In addition, $TFPI^{BHK}$ was also found in the kidneys and this could be reduced in the presence of RAP. Asialoorosomucoid (ASOR), a potent inhibitor of the asialoglycoprotein receptor (ASGP-R), prolonged the circulatory survival of ^{125}I-m-TFPI by 1.5-fold ($p<0.001$). *In vitro*, ASOR and other ASGP-R antagonists significantly inhibited endocytosis of $^{125}I\text{-}$ $TFPI^{BHK}$ in PCs. Moreover, unlabelled $TFPI^{BHK}$ markedly decreased endocytosis of ^{125}I-asialofetuin. In conclusion, our findings suggest that ASGP-R mediated endocytosis in the liver is involved in the clearance of $TFPI^{BHK}$.

Paper II: Liver Sinusoidal Endothelial Cells are the Principal Site for Elimination of Unfractionated Heparin from the Circulation

The mechanism of elimination of blood borne heparin was studied. To this end unfractionated heparin (UFH) was tagged with FITC, which served as both a visual marker and a site of labeling with ^{125}I-iodine. UFH labeled in this manner did not alter the anticoagulant activity or binding specificity of the glycosaminoglycan. Labeled heparin administered intravenously to rats (0.1 IU/kg) had a circulatory $t_{1/2}$ of 1.7 min, which was increased to 16 min upon coinjection with unlabeled UFH (100 IU/kg). At 15 min after injection, 71% of recovered radioactivity was found in liver. Liver cell separation revealed the following relative uptake capacity, expressed per cell: liver sinusoidal endothelial cell (LSEC)-parenchymal cell-Kupffer cell = 15:3.6:1. Fluorescence microscopy on liver sections showed accumulation of FITC-UFH only in cells lining the liver sinusoids. No fluorescence was detected in parenchymal cells or endothelial cells lining the central vein. Fluorescence microscopy of cultured LSECs following binding of FITC-UFH at 4°C and chasing at 37°C, showed accumulation of the probe in vesicles located at the periphery of the cells after 10 min, with transfer to large, evenly stained vesicles in the perinuclear region after 2 h. Immunogold electron microscopy of LSECs to probe the presence of FITC following injection of FITC-UFH along with BSA-gold to mark lysosomes demonstrated colocalization of the probe with the gold particles in the lysosomal compartment. Receptor-ligand competition experiments in primary cultures of LSECs indicated the presence of a specific heparin receptor, functionally distinct from the hyaluronan/scavenger receptor (Stabilin 2). The results suggest a major role for LSECs in heparin elimination.

Paper III: Unfractionated Heparin Promotes Elimination of Recombinant TFPI in a Rat Model

Background: Tissue factor pathway inhibitor (TFPI) plays an important role for the anticoagulant effect of heparin. Depletion of intravascular TFPI by treatment with unfractionated heparin (UFH), and not by low molecular weight heparin (LMWH), has been suggested to explain the superiority of LMWH in treatment of both arterial and venous thrombosis. The present study was undertaken to investigate the impact of UFH on clearance kinetics, and organs and cells responsible for the clearance of recombinant human full length TFPI purified from baby hamster kidney cells ($TFPI^{BHK}$) and from *E.Coli* ($TFPI^{E.Coli}$).

Methods: Male Sprague-Dawley rats were used as research animals. $TFPI^{BHK}$ and $TFPI^{E.Coli}$ were labelled with ^{125}I, and used to study clearance *in vivo.*

Results: Surface Plasmon Resonance (SPR) analysis revealed that both types of TFPI bound to UFH *in vitro*, but $TFPI^{E.Coli}$ exhibited a faster association rate and a much slow dissociation rate. Intravenous administration of 100 IU/kg UFH immediately prior to TFPI decreased the circulatory survival ($t_{1/2}\alpha$) of $TFPI^{BHK}$ from 1.99 ± 0.10 min to 1.17 ± 0.13 min ($p<0.001$) without affecting the fast clearance of $TFPI^{E.Coli}$. Presence of UFH significantly increased the circulatory survival during the slow $t_{1/2}\beta$ phase of $TFPI^{E.Coli}$ from 27.44 ± 1.91 min to 36.88 ± 1.87 min ($p<0.05$) without affecting the $t_{1/2}\beta$ of $TFPI^{BHK}$. Hepatocellular distribution of radiolabeled ligands showed that both forms of TFPI were mainly taken up by PCs in the absence of UFH (≥ 90%). UFH administration switched the hepatocellular distribution of $TFPI^{E.Coli}$ from PCs towards LSECs, without affecting the distribution of $TFPI^{BHK}$.

Conclusions: Our findings revealed a specific increase in the elimination of $TFPI^{BHK}$ during UFH treatment. This observation may represent the underlying mechanism for depletion of endogenous TFPI in humans during UFH treatment.

Paper IV: Rat Liver Sinusoidal Endothelial Cells (LSECs) express functional Low Density Lipoprotein Receptor-Related Protein-1 (LRP-1)

The low density lipoprotein receptor-related protein-1 (LRP-1), a member of the LDL receptor family, is a large, multifunctional endocytic receptor highly expressed in liver parenchymal cells (PCs), neurons, activated astrocytes and fibroblasts. Receptor associated protein (RAP), the antagonist for all known ligands to members of the LDL receptor family, was used in this study as a tool to investigate whether liver sinusoidal endothelial cells (LSECs) express LRP-1, and, if so, to what extent they contribute to the clearance of exogenously administered RAP. We found that ^{125}I-RAP was rapidly cleared from the circulation with the liver as the principal site of elimination. Liver cell separation following administration of radiolabeled-RAP showed accumulation mainly in parenchymal cells (PCs) (91%) and the rest in liver sinusoidal endothelial cells (LSECs) (8%). Studies *in vitro* showed that RAP endocytosis is specific and is followed by degradation. Excess amounts of unlabeled ligands to known endocytosis receptors in LSECs had no inhibitory effect on uptake of RAP, suggesting the involvement of a yet unknown receptor in these cells. Immunofluorescence assay using a monoclonal antibody to LRP-1 showed positive staining in LSECs. Ligand blot analyses using total cell proteins and ^{125}I-RAP followed by mass spectrometry further confirmed and identified LRP-1 in LSECs. ***Conclusion:*** An important implication of the expression of LRP-1 in LSECs is that these cells may contribute together with the parenchymal cells to the rapid removal of blood borne ligands for LRP-1, including proatherogenic ligands, thus preventing the development of atherosclerosis.

4. GENERAL DISCUSSION

4. 1. Methodological Considerations

4.1.1. Animal Models and Drug Clearance

Cell and tissue cultures, often suggested as an "alternative" to using animals, have been used in biomedical research for many years. But results obtained using such isolated systems may not reflect physiological processes in the intact body. To better understand the elimination mechanisms of different substances and drugs (Heparin and TFPI in the present study), the experiments must be conducted *in vivo*, under normal physiological conditions.

Several approaches are available to monitor the fate and distribution of exogenous ligands in the intact organism. The vast majority of the *in vivo* clearance studies are conducted using radiolabeled ligands, in which tissue distribution is usually reported as radioactivity accumulated in a particular organ at various times after administration [48, 57, 258-260]. In addition, the radioactivity in blood samples collected for a certain length period is used for calculating the decay in blood clearance. Iodogen was used in our studies for labelling of proteins with ^{125}I. Iodogen is an oxidizing agent that gives higher iodine incorporation than the other commonly used oxidizing reagents, chloramine-T and lactoperoxidase [261]. It can be used for a wide range of proteins and peptides, can permit iodine incorporation with minimal oxidation damage, and can produce tracers stable for up to 3 months [262].

The liver has been shown to be the primary organ involved in clearance and metabolism of a large number of physiological and non-physiological circulating macromolecules [22, 248, 263, 264]. However, in order to obtain information about which cell type(s) are involved in the clearance, quantification of radioactivity can be measured in different liver cells separated after injection of the protein labeled with ^{125}I-tyramine

cellobiose (TC) [39, 49] (paper IV) or ^{125}I-FITC (paper II). The technique of labeling macromolecules with TC was introduced in 1983 by Pittman and co-workers [265] and used in several studies since then [49, 266-269]. The advantages of using this technique are i) that the proteins derivatized with this adduct are recognized as unmodified proteins by biological systems, ii) that the TC ligand is labeled to a high specific radioactivity, and iii) that the ^{125}I-TC adduct is stably trapped intralysosomally after uptake and catabolism of the protein, allowing quantitation of the protein at the site of uptake hours or even days after the uptake [265]. Protein labeling with FITC or other fluorochromes have similar advantages when care is taken to modify the protein minimally by keeping the molecular ratio FITC:protein at a minimum. Usually, the isothiocyanate portion of FITC binds to primary amino groups of lysine residues of the ligand to be labeled. This interaction neutralizes the positive charge of the amino group of the ligand. In addition, an extra negative charge is incorporated by the carboxy group of FITC. Thus, with a high FITC:protein ratio it is likely that the net negative charge of the FITC-labeled ligand increases to the extent that it is recognized by the scavenger receptor (SR), a receptor known for its ability to bind negatively charged molecules [270, 271]. Since FITC is also trapped intralysosomally after internalization of FITC-conjugated ligands, it can be traced by fluorescence microscopy, and it may also be detected by gold-labeled FITC-antibodies at the electron microscopic level [23] (paper I and II).

4.1.2. Primary cells vs cell lines

To obtain more detailed information about binding and uptake mechanism and characterize the endocytic pathway involved in the uptake of the protein of interest, *in vivo* studies must as a rule be followed up with studies *in vitro* with isolated cells in culture. Cell lines are often used in such studies, since they are often readily available and easily maintained. Also, a cell line may exhibit many of the characteristics of the original tissue from which the cell line has been derived. As an example, the HepG2 cell line has been found

to exhibit many characteristics of normal hepatocytes, e.g. expressing the LRP-1 [272], ASGP-R [273], and synthesis of serum proteins [274]. However, hepatoma-derived cell lines may differ from primary cell cultures with regard to receptor expression, endocytic capacity and protein synthesis [137, 275-277]. Regarding LSECs, some laboratories culture these cells under conditions that allow cell proliferation and subsequent splitting and passaging before the cells are used in experiments several days or even weeks after isolation [278, 279]. However, many phenotypic features of LSECs change gradually when they are placed in a culture dish, and most importantly, many of the signature functions of LSECs, e.g. scavenger function and fenestration, are also rapidly lost during *in vitro* culture [280, 281]. For these reasons, true primary liver cell cultures (PCs and LSECs) were used in this study each time an *in vitro* experiment was required.

4.2. TFPI and heparin: Elimination and mechanisms of clearance

"How much?", "How often?" and "How long?" are important therapeutic questions when investigating a drug. To administer a drug optimally, knowledge is needed for the mechanism of absorption, distribution, and elimination. The present thesis aims to increase our understanding of how the liver contributes to the elimination of two important anticoagulants, namely unfractionated heparin (UFH) and tissue factor pathway inhibitor (TFPI).

Because of the central role it plays in thrombus generation, propagation, and stabilization, effective inhibition of thrombin is crucial in the prevention and treatment of thrombotic disorders.

Recombinant tissue factor pathway inhibitor (rTFPI) has been proposed to be a beneficial therapeutic agent as a natural anticoagulant to attenuate pathological clotting

activation. rTFPI purified from *E.Coli* ($TFPI^{E.Coli}$) or from different mammalian cell lines has been shown in animal studies to be effective against TF-induced coagulopathy [282], to reduce mortality from bacterial septic shock [283, 284], and to prevent arterial thrombosis [179]. In humans, initial results from phase I and II studies indicated that rTFPI is well tolerated, with no clinical significant bleeding side effect [285]. Unfortunately, these earlier encouraging results could not be repeated in a phase III trial (the OPTIMIST). There was no survival benefit with the administration of $TFPI^{E.Coli}$ in humans with severe sepsis [286]. However, patients who did not receive concomitant heparin appeared to benefit from the treatment with $TFPI^{E.Coli}$. As we have shown in paper III, $TFPI^{E.Coli}$ has a strong affinity binding to UFH, while $TFPI^{BHK}$ does not. Thus, it would be interesting to investigate the effect of a TFPI of mammalian origin in patients with severe sepsis.

Previous studies suggest that depending on the source, rTFPI may have different routes of elimination. The low density lipoprotein receptor-related protein (LRP-1), a multifunctional endocytic receptor, has been shown to be the main receptor responsible for the plasma clearance [210] and degradation of $TFPI^{E.Coli}$ in hepatoma-derived cell lines [106]. In addition, it has been shown that while the majority of $TFPI^{E.Coli}$ binds to cell surface HSPGs, LRP-1 can function independently from HSPGs in the binding and degradation of $TFPI^{E.Coli}$ [211]. On the other hand, LRP-1 is not involved in the clearance of rTFPI purified from mouse C127 fibroblasts ($TFPI^{C127}$) [167]. Notably, $TFPI^{C127}$ has a prolonged survival in the circulation compared to $TFPI^{E.Coli}$ and it does not compete with $TFPI^{E.Coli}$ for binding or degradation in HepG2 cells [167]. While $TFPI^{E.Coli}$ was mainly found in the liver after intravenous injection [207] (paper I), rTFPI purified from C127 fibroblasts and human SK hepatoma cells was found in liver and kidneys [206].

In our study we used recombinant human full-length TFPI purified from baby hamster kidney (BHK) cell line ($TFPI^{BHK}$), with the aims to i) carry out a more detailed pharmacokinetics study during the first phase of clearance, ii) to identify the organ(s) involved in removal of $TFPI^{BHK}$ from the circulation, iii) to determine the hepatocellular site of uptake, and iv) to identify the main cell (s) and their receptor(s) involved in the clearance (paper I). We found that $TFPI^{BHK}$ was cleared slower from the circulation than $TFPI^{E.Coli}$, and while $TFPI^{E.Coli}$ was cleared mainly by the liver, $TFPI^{BHK}$ was taken up in both liver and kidneys. As mentioned above, rTFPI from C127 fibroblasts and human SK hepatoma cells also accumulated in the kidneys [206]. However, the method used in that study, namely whole-body autoradiography, does not give a quantitative measurement of the ligand distribution in different organs. Besides, an error of the assessment of the rTFPI in the kidneys may be induced by evaluating the distribution in the organ without removing the blood. From our study, we could see that even 1h after injection, there is still a lot of $TFPI^{BHK}$ in the circulation (paper I). Based on our results and previous studies, we hypothesized that the possible mechanism for the detection of $TFPI^{BHK}$ in the kidneys is by renal reabsorption in the proximal tubule. Using the Opossum kidney (OK) cell line, we found that RAP significantly inhibited both binding and internalization of ^{125}I-$TFPI^{BHK}$, effect which was not seen on liver PCs. The results suggest that $TFPI^{BHK}$ may bind to megalin or/and cubilin in kidneys, two members of the LDL receptor family [287, 288], but it does not bind to LRP-1 in liver. With the goal of finding the receptor involved in its clearance, we found that $TFPI^{BHK}$ was inhibited both *in vivo* and *in vitro* by ligands to the ASGP-R, suggesting that this receptor is, at least partially involved in the removal of $TFPI^{BHK}$ from the circulation. Recombinant human $TFPI^{BHK}$ differs from bacterial $TFPI^{E.Coli}$ in molecular weight (42 kDa and 35 kDa, respectively) due to post-translational modifications in mammalian cells involving *N*-linked glycosylation at three potential sites; Asn 117, Asn 167, Asn 228 [157, 164, 165]. The

mammalian cells used for synthesis of $TFPI^{BHK}$ in our study, the BHK cells, are known to have a heterogenous glycosylation pattern. However, under certain culture conditions, changes in the oligosaccharide chains occur, and the product secreted by these cells is partially desialylated [289]. The carbohydrate moiety of plasma glycoproteins is known to play a key role in their plasma clearance [290]. Desialylation and subsequent exposure of the penultimate galactose is known to increase the rate of removal of many plasma glycoproteins from the circulation due to recognition by the ASGP-R. The finding that the liver ASGP-R is involved in the clearance of $TFPI^{BHK}$ adds to the important scavenger function of ASPG-R in designing clinical treatments to provide therapeutic levels of glycoproteins in circulation.

Traditional anticoagulants, such as heparin, are indirect inhibitors of thrombin. The main clinical use of heparin is in acute coronary syndrome, e.g. myocardial infarction, vascular and cardiac surgery, atrial fibrillation, deep-vein thrombosis and pulmonary embolism. Several studies indicated that the liver plays a key role in heparin elimination [248]. However, little is known about the liver cells and the mechanism involved in the clearance of unfractionated heparin (UFH). Most studies on distribution of heparin, performed *in vitro* using fractionated heparin, reveal that the PCs and KCs are the main cells for uptake [244-247]. However, our *in vivo* experiments showed that LSECs are the principal site for binding and uptake of UFH from the circulation (paper II). An unknown scavenger-like receptor was suggested to be involved in the binding and uptake of heparin [248]. As UFH has the highest negative charge density of any known biological macromolecule [216], it is a likely candidate ligand for the SRs. This group of receptors has only one feature in common, namely the ability to bind negatively charged macromolecules [22]. Our finding that neither hyaluronan, nor AGE-BSA, that are both high affinity ligands for Stabilin 2, or anti-Stabilin 2 antibody could inhibit the uptake of UFH, suggested that Stabilin 2 in LSECs is not involved in the uptake of UFH. Contrary to our finding, Harris and Weigel suggested shortly after the

publication of our paper II that Stabilin 2 is the systemic clearance receptor for heparin [291]. This conclusion from these authors was surprising in light of their previous publication that heparin does not bind Stabilin 2 [292]. Moreover, mapping the binding sites of 8 different ligands within Stabilin 2, the same authors showed that the receptor specifically and simultaneously bind to both heparin and HA, in a non-competitive manner [293]. This property of one and the same receptor to bind different ligands non-competitively is also observed in LRP-1 and MANN/COLLA-R [58, 108]. Of note, we recently purified a major heparin binding protein using heparin-sepharose affinity chromatography of rat LSECs (unpublished data). Although we have not yet characterized this protein, its apparent MW was around 70 kDa, which is clearly different than the MW of the Stabilin 2 of 270 kDa [26]. This evidence support our conclusion that heparin uptake in LSECs is via a receptor that is clearly different than Stabilin 2.

4.2.1. Clearance of TFPI during heparin treatment

Heparin is known to bind a large number of plasma proteins [294]. Formation of heparin-protein complexes during heparin treatment may have important clinical implications by affecting the elimination of the proteins. Administration of heparin is known to cause partial depletion of both plasma free TFPI and heparin-releasable TFPI *in vivo*, an effect not seen by low molecular weight heparin (LMWH) [254, 255]. As mentioned above, the primary organs involved in TFPI clearance are the liver and kidneys. Urinary loss of TFPI has been suggested to explain the selective depletion of intravascular TFPI during continuous UFH treatment. However, a recent study showed that only trace amounts of native TFPI were detected in the urine under basal conditions and during heparin treatment, with a more pronounced reduction during UFH treatment [256]. It was speculated that filtration of TFPI-heparin complexes probably depends on the molecular weight of the complexes; thereby explaining attenuated renal clearance of TFPI during UFH treatment. In paper III we aimed to

elucidate the mechanism of TFPI depletion during UFH clearance by investigating the effect of UFH *in vivo* and *in vitro* on the clearance of recombinant human full length glycosylated TFPI ($TFPI^{BHK}$) as compared to recombinant full length non-glycosylated TFPI ($TFPI^{E.Coli}$). We found that $TFPI^{BHK}$ binds weaker to heparin compared to $TFPI^{E.Coli}$, and that intravenous administration of UFH immediately prior to TFPI significantly decreased the circulatory survival of $TFPI^{BHK}$ during the alpha-phase of elimination, while the circulatory survival of $TFPI^{E.Coli}$ during the beta-phase of elimination was significantly increased. Administration of UFH did not affect the organ distribution of $TFPI^{BHK}$. Hepatocellular distribution of $TFPI^{BHK}$ was not affected by the presence of UFH, while the uptake of $TFPI^{E.Coli}$ was switched from PCs towards NPCs. Bregengaard et al. reported that the recovery of $TFPI^{BHK}$ after 2 min was increased to 46% in rabbits receiving low molecular weight heparin and $TFPI^{BHK}$ [257]. The discrepancy between the two studies could explain the selective depletion of $TFPI^{BHK}$ with UFH and not with LMWH. Interestingly, UFH administration only affected the distribution of $TFPI^{E.Coli}$ within the liver cells, and not of $TFPI^{BHK}$, by switching the distribution from the PCs towards the non-parenchymal cells (NPCs). As shown in paper II, LSECs, the largest population among the non-parenchymal cells, is the principal site of UFH elimination. This result suggests that in the presence of heparin, the scavenger receptor on LSECs may have higher affinity for binding of $TFPI^{E.Coli}$-UFH complexes than the HSPGs and/or LRP-1 receptor on PCs and/or LSECs for binding of $TFPI^{E.Coli}$ alone.

The mechanism by which heparin shorten the circulatory survival of $TFPI^{BHK}$ is not fully understood. Interestingly, the anatomical distribution and the hepatocellular distribution of $TFPI^{BHK}$ were not significantly affected by the presence of UFH. In accordance with previous findings in humans [256], only traces of $TFPI^{BHK}$ were detected in the urine of rats with or without pre-administration of UFH. Moreover, only slightly elevated levels of $TFPI^{BHK}$ were detected in the blood following UFH administration. These findings in addition

to the rapid clearance of TFPI in the presence of UFH clearly demonstrate that the mechanism for depletion of TFPI is not the urinary loss. Previously, we and others have shown that TFPI of mammalian origin, i.e. $TFPI^{BHK}$ and $TFPI^{C127}$ bind very weakly, or even fail to bind to HSPGs, and that their uptake is not mediated by the LRP-1 (paper I, [167, 295]). These findings along with the SPR analysis showing weaker $TFPI^{BHK}$-UFH interactions compared to $TFPI^{E.Coli}$-UFH interactions suggest that the faster clearance of $TFPI^{BHK}$ in the presence of UFH is caused by an enhanced binding affinity of the $TFPI^{BHK}$-UFH complexes to the yet unknown receptor(s) for $TFPI^{BHK}$ on PCs. Our findings may explain why prolonged treatment with UFH causes depletion of intravascular TFPI in humans.

4.3. LRP-1 expression in LSECs

Hepatocellular distribution of TFPI in paper I showed that while the majority of $TFPI^{E.Coli}$ was found in PCs, 19% was also found in NPCs. NPCs consist mainly of KCs and LSECs, with KCs eliminating particles (> 200 nm) from the circulation via phagocytosis, and LSECs removing colloids and soluble macromolecules (< 200 nm) via non-phagocytic receptor-mediated endocytosis [22]. Knowing that liver PCs LRP-1 is involved in the clearance and degradation of $TFPI^{E.Coli}$ [106, 210] we investigated whether LSECs also express LRP-1 (paper IV). Studies on hepatocellular distribution after intravenously injection of ^{125}I-RAP showed that, while the PCs were responsible for 90% of the RAP clearance, 8% was also found in LSECs. However, it may be argued that the apparent 8% uptake in LSECs reflects uptake in contaminating PCs. To eliminate the possibility of PC contamination in the LSEC fraction, *in vitro* cultures of LSECs were prepared, where the cells were extensively washed free of any contaminating PCs. This can be very easily visualized by light microscopy, as the two types of cells have very distinct morphology and size. A significant uptake of ^{125}I-RAP was observed in the super pure LSECs *in vitro*. As RAP is known to interact also with other members of the LDL receptor family, and not only with LRP-1, we performed an

immunofluorescence assay using LRP-1 antibody. Positive staining was observed in LSECs, which were identified by the presence of the LSEC specific marker, Stabilin 2. Furthermore, ligand blot analyses using LSECs total cell protein and ^{125}I-RAP followed by mass spectrometry showed the appearance of a single band, which was identified as LRP-1. The finding that LRP-1 is expressed in liver not only in PCs but also in LSECs, is novel, and gives insight into the role of these cells in the catabolism of a variety of molecules.

5. MAIN CONCLUSIONS

- The clearance of intravenously administered recombinant human TFPI purified from BHK cells ($TFPI^{BHK}$) has a significantly slower α-phase compared to $TFPI^{E.Coli}$. While $TFPI^{E.Coli}$ was cleared mainly by the liver, $TFPI^{BHK}$ was found both in liver and kidneys. In the liver, $TFPI^{BHK}$ was found mainly in PCs, while $TFPI^{E.Coli}$ was found in PCs, as well as in NPCs. The uptake of $TFPI^{BHK}$ in liver is not mediated by the LRP-1, as with $TFPI^{E.Coli}$, but by the ASGP-R. This finding has the important implication that any rTFPI in mammalian cell lines intended for use as a therapeutic agent in antithrombotic treatment must be specifically checked for its clearance by the ASGP-R.

- LSECs are the principal site for binding and uptake of unfractionated heparin (UFH). The receptor for endocytosis of UFH is distinct from the hyaluronan/scavenger receptor (Stabilin 2). Formation of heparin-protein complexes during heparin treatment may have important clinical implications by affecting the elimination of the proteins.

- In contrast to $TFPI^{E.Coli}$, $TFPI^{BHK}$ was found to bind weaker to UFH. During UFH treatment, $TFPI^{BHK}$ elimination was significantly increased without affecting the target organ and specific cells responsible for binding and endocytosis

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- We demonstrate for the first time that the expression of functional LRP-1 in liver is not restricted only to PCs, it is also found in LSECs. This suggests that the atheroprotective activity ascribed to LRP-1 in liver is operative in both PCs and LSECs. In addition, LSECs may work together with the PCs in cleaning the blood from numerous ligands with affinity to LRP-1.

6. FUTURE PERSPECTIVES

To improve anticoagulant treatment strategies in the future it is necessary to increase our knowledge about the mechanism of clearance of key molecular players. The present thesis focused on two major anticoagulants TFPI and heparin.

$TFPI^{BHK}$ was shown to be mainly cleared *in vivo* by the liver PCs. *In vitro* experiments carried out to study the details of the uptake mechanism were obtained using cells cultured in the absence of plasma components. Thus, the *in vitro* system used here is highly simplified compared to the physiological *in vivo* situation. To make more physiologically relevant experimental conditions *in vitro* it would be necessary to include factors such as FXa and TF, lipoproteins and platelets to study if they would influence the uptake process of $TFPI^{BHK}$.

Our findings that $TFPI^{BHK}$, which is glycosylated and thereby resembling endogenous TFPI, was eliminated faster in the presence of UFH may explain why prolonged treatment with UFH causes depletion of intravascular TFPI in humans. However, further studies are needed to understand the impact of UFH on cell binding and degradation of $TFPI^{BHK}$.

Further studies are warranted to elucidate the specific mechanism for binding and uptake of heparin in LSECs, as well as identification and characterization of the binding protein that seems to be specific for the heparin recognition and internalization in LSECs.

Although only one band was visible in the ligand blot in paper IV, RAP is known to associate with most of the LDL receptor family members [296]. Thus, it would be interesting to look more into the physiological importance of the LRP-1 expression in LSECs by investigating the clearance of a more specific ligand for this receptor, such as trypsin activated alpha 2-macroblobulin [297] or uPA-PAI-1 complexes [298]. Another interesting study could be to investigate whether LRP-1 compensates for the clearance of tissue plasminogen

activator (tPA) in the absence of the mannose receptor (MANN-R). tPA was shown to be taken up both in PCs and LSECs, by the LRP-1 and the MANN-R, respectively [299]. Clearance of SR-ligands was shown not to be affected by the lack of MANN-R in MANN-R KO mice [58]. However, with tPA being a ligand for both MANN-R and LRP-1, it would be interesting to investigate whether the LRP-1 clearance function in LSECs would be upregulated to compensate for the lack of MANN-R-mediated uptake in the MANN-R KO mice.

7. REFERENCES

1. **Kindberg, G.M.,** ***Intracellular transport of endocytosed compounds in liver cells : a study by means of subcellular fractionation*** **1990.**
2. **Wisse, E.,** ***An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids.*** **J Ultrastruct Res, 1970. 31(1): p. 125-50.**
3. **Wisse, E.,** ***An ultrastructural characterization of the endothelial cell in the rat liver sinusoid under normal and various experimental conditions, as a contribution to the distinction between endothelial and Kupffer cells.*** **J Ultrastruct Res, 1972. 38(5): p. 528-62.**
4. **Hellman, L., et al.,** ***Secretion of coagulant factor VIII activity and antigen by in vitro cultivated rat liver sinusoidal endothelial cells.*** **Br-J-Haematol, 1989. 73(3): p. 348-55.**
5. **Fraser, R., B.R. Dobbs, and G.W. Rogers,** ***Lipoproteins and the liver sieve: the role of the fenestrated sinusoidal endothelium in lipoprotein metabolism, atherosclerosis, and cirrhosis.*** **Hepatology, 1995. 21(3): p. 863-74.**
6. **Pertoft, H. and B. Smedsrod,** ***Separation and characterization of liver cells.*** **In: Pretlow TG, Pretlow TPNY, eds. Cell Separation Methods and Selected Applications. Volume 4. Academic Press, 1987: p. 1-24.**
7. **Conner, S.D. and S.L. Schmid,** ***Regulated portals of entry into the cell.*** **Nature, 2003. 422(6927): p. 37-44.**
8. **Aderem, A. and D.M. Underhill,** ***Mechanisms of phagocytosis in macrophages.*** **Annu Rev Immunol, 1999. 17: p. 593-623.**
9. **Smedsrod, B., et al.,** ***Scavenger functions of the liver endothelial cell.*** **Biochem J, 1990. 266(2): p. 313-27.**
10. **Pearse, B.M. and M.S. Robinson,** ***Clathrin, adaptors, and sorting.*** **Annu Rev Cell Biol, 1990. 6: p. 151-71.**
11. **Owen, D.J. and J.P. Luzio,** ***Structural insights into clathrin-mediated endocytosis.*** **Curr Opin Cell Biol, 2000. 12(4): p. 467-74.**
12. **Rapoport, I., et al.,** ***Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes.*** **Embo J, 1997. 16(9): p. 2240-50.**
13. **Hinshaw, J.E. and S.L. Schmid,** ***Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding.*** **Nature, 1995. 374(6518): p. 190-2.**
14. **Christoforidis, S., et al.,** ***The Rab5 effector EEA1 is a core component of endosome docking.*** **Nature, 1999. 397(6720): p. 621-5.**
15. **Stenmark, H. and V.M. Olkkonen,** ***The Rab GTPase family.*** **Genome Biol, 2001. 2(5): p. REVIEWS3007.**
16. **Harford, J., et al.,** ***Intracellular dissociation of receptor-bound asialoglycoproteins in cultured hepatocytes. A pH-mediated nonlysosomal event.*** **J Biol Chem, 1983. 258(5): p. 3191-7.**
17. **Wolkoff, A.W., et al.,** ***Intracellular segregation of asialoglycoproteins and their receptor: a prelysosomal event subsequent to dissociation of the ligand-receptor complex.*** **J Cell Biol, 1984. 98(2): p. 375-81.**
18. **Rothman, J.E.,** ***Mechanisms of intracellular protein transport.*** **Nature, 1994. 372(6501): p. 55-63.**
19. **Fraser, J.R., et al.,** ***Uptake of circulating hyaluronic acid by the rat liver. Cellular localization in situ.*** **Cell Tissue Res, 1985. 242(3): p. 505-10.**
20. **Eriksson, S., et al.,** ***Endothelial cells are a site of uptake and degradation of hyaluronic acid in the liver.*** **Exp-Cell-Res, 1983. 144(1): p. 223-8.**

21. **Seternes, T., K. Sorensen, and B. Smedsrod, *Scavenger endothelial cells of vertebrates: a nonperipheral leukocyte system for high-capacity elimination of waste macromolecules.* Proc Natl Acad Sci U S A, 2002. 99(11): p. 7594-7.**
22. **Smedsrod, B., *Clearance function of scavenger endothelial cells.* Comp Hepatol, 2004. 3 Suppl 1: p. S22.**
23. **Hellevik, T., et al., *Transport of residual endocytosed products into terminal lysosomes occurs slowly in rat liver endothelial cells.* Hepatology, 1998. 28(5): p. 1378-89.**
24. **Falkowska-Hansen, B., et al., *Endocytosis and degradation of serglycin in liver sinusoidal endothelial cells.* Mol Cell Biochem, 2006.**
25. **Hughes, D.A., I.P. Fraser, and S. Gordon, *Murine macrophage scavenger receptor: in vivo expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs.* Eur J Immunol, 1995. 25(2): p. 466-73.**
26. **McCourt, P.A., et al., *Characterization of a hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship to scavenger receptors.* Hepatology, 1999. 30(5): p. 1276-86.**
27. **Malerod, L., et al., *The expression of scavenger receptor class B, type I (SR-BI) and caveolin-1 in parenchymal and nonparenchymal liver cells.* Cell Tissue Res, 2002. 307(2): p. 173-80.**
28. **Adachi, H., et al., *Expression cloning of a novel scavenger receptor from human endothelial cells.* J Biol Chem, 1997. 272(50): p. 31217-20.**
29. **Hansen, B., et al., *Stabilin-1 and stabilin-2 are both directed into the early endocytic pathway in hepatic sinusoidal endothelium via interactions with clathrin/AP-2, independent of ligand binding.* Exp Cell Res, 2005. 303(1): p. 160-73.**
30. **Politz, O., et al., *Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues.* Biochem J, 2002. 362(Pt 1): p. 155-64.**
31. **Zhou, B., et al., *Identification of the hyaluronan receptor for endocytosis (HARE).* J Biol Chem, 2000. 275(48): p. 37733-41.**
32. **Duryee, M.J., et al., *Scavenger receptors on sinusoidal liver endothelial cells are involved in the uptake of aldehyde-modified proteins.* Mol Pharmacol, 2005. 68(5): p. 1423-30.**
33. **Ling, W., et al., *Oxidized or acetylated low density lipoproteins are rapidly cleared by the liver in mice with disruption of the scavenger receptor class A type I/II gene.* J Clin Invest, 1997. 100(2): p. 244-52.**
34. **Suzuki, H., et al., *A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection.* Nature, 1997. 386(6622): p. 292-6.**
35. **Van Berkel, T.J., et al., *Uptake and catabolism of modified LDL in scavenger-receptor class A type I/II knock-out mice.* Biochem J, 1998. 331 (Pt 1): p. 29-35.**
36. **Hansen, B., B. Arteta, and B. Smedsrod, *The physiological scavenger receptor function of hepatic sinusoidal endothelial and Kupffer cells is independent of scavenger receptor class A type I and II.* Mol Cell Biochem, 2002. 240(1-2): p. 1-8.**
37. **Matsumoto, K., et al., *Endocytic uptake of advanced glycation end products by mouse liver sinusoidal endothelial cells is mediated by a scavenger receptor distinct from the macrophage scavenger receptor class A.* Biochem J, 2000. 352 Pt 1: p. 233-40.**
38. **Nakajou, K., et al., *CD36 is not involved in scavenger receptor-mediated endocytic uptake of glycolaldehyde- and methylglyoxal-modified proteins by liver endothelial cells.* J Biochem, 2005. 137(5): p. 607-16.**

39. **Melkko, J., et al.,** ***Clearance of NH2-terminal propeptides of types I and III procollagen is a physiological function of the scavenger receptor in liver endothelial cells.*** **J Exp Med, 1994. 179(2): p. 405-12.**
40. **Van Berkel, T.J., Y.B. De Rijke, and J.K. Kruijt,** ***Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells.*** **J Biol Chem, 1991. 266(4): p. 2282-9.**
41. **Smedsrod, B., et al.,** ***Advanced glycation end products are eliminated by scavenger-receptor-mediated endocytosis in hepatic sinusoidal Kupffer and endothelial cells.*** **Biochem J, 1997. 322 (Pt 2): p. 567-73.**
42. **Pontow, S.E., V. Kery, and P.D. Stahl,** ***Mannose receptor.*** **Int Rev Cytol, 1992. 137B: p. 221-44.**
43. **Avrameas, A., et al.,** ***Expression of a mannose/fucose membrane lectin on human dendritic cells.*** **Eur J Immunol, 1996. 26(2): p. 394-400.**
44. **Liu, Z.H., et al.,** ***TNF-alpha and IL-1 alpha induce mannose receptors and apoptosis in glomerular mesangial but not endothelial cells.*** **Am J Physiol, 1996. 270(6 Pt 1): p. C1595-601.**
45. **Lew, D.B. and M.C. Rattazzi,** ***Mitogenic effect of lysosomal hydrolases on bovine tracheal myocytes in culture.*** **J Clin Invest, 1991. 88(6): p. 1969-75.**
46. **Shepherd, V.L., B.I. Tarnowski, and B.J. McLaughlin,** ***Isolation and characterization of a mannose receptor from human pigment epithelium.*** **Invest Ophthalmol Vis Sci, 1991. 32(6): p. 1779-84.**
47. **Ezekowitz, R.A., et al.,** ***Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells.*** **J Exp Med, 1990. 172(6): p. 1785-94.**
48. **Smedsrod, B. and M. Einarsson,** ***Clearance of tissue plasminogen activator by mannose and galactose receptors in the liver.*** **Thromb-Haemost, 1990. 63(1): p. 60-6.**
49. **Smedsrod, B., et al.,** ***Circulating C-terminal propeptide of type I procollagen is cleared mainly via the mannose receptor in liver endothelial cells.*** **Biochem J, 1990. 271(2): p. 345-50.**
50. **Niesen, T.E., et al.,** ***Metabolism of glycosylated human salivary amylase: in vivo plasma clearance by rat hepatic endothelial cells and in vitro receptor mediated pinocytosis by rat macrophages.*** **J Leukoc Biol, 1984. 36(3): p. 307-20.**
51. **Stahl, P.D., et al.,** ***Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages.*** **Proc Natl Acad Sci U S A, 1978. 75(3): p. 1399-403.**
52. **Hubbard, A.L., et al.,** ***An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of 125I-ligands among the liver cell types.*** **J Cell Biol, 1979. 83(1): p. 47-64.**
53. **Isaksson, A., et al.,** ***Uptake of beta-hexosaminidase by nonparenchymal liver cells and peritoneal macrophages.*** **Enzyme, 1983. 30(4): p. 230-8.**
54. **Smedsrod, B. and O.K. Tollersrud,** ***Sinusoidal liver endothelial cells recruit lysosomal enzymes from the circulation by mannose-receptor mediated endocytosis.*** **In Cells of the Hepatic Sinusoid. Edited by Wisse E, Knook DL, Wake K. Leiden, The Netherlands, 1995. 5: p. 180-183.**
55. **Elvevold, K., et al.,** ***Liver sinusoidal endothelial cells depend on mannose receptor-mediated recruitment of lysosomal enzymes for normal degradation capacity.*** **Hepatology, 2008. 48(6): p. 2007-15.**

56. Magnusson, S. and T. Berg, *Endocytosis of ricin by rat liver cells in vivo and in vitro is mainly mediated by mannose receptors on sinusoidal endothelial cells.* Biochem-J, 1993. 291(Pt 3): p. 749-55.
57. Smedsrod, B., S. Johansson, and H. Pertoft, *Studies in vivo and in vitro on the uptake and degradation of soluble collagen alpha 1(I) chains in rat liver endothelial and Kupffer cells.* Biochem J, 1985. 228(2): p. 415-24.
58. Malovic, I., et al., *The mannose receptor on murine liver sinusoidal endothelial cells is the main denatured collagen clearance receptor.* Hepatology, 2007. 45(6): p. 1454-61.
59. Mousavi, S.A., et al., *Receptor-mediated endocytosis of immune complexes in rat liver sinusoidal endothelial cells is mediated by FcgammaRIIb2.* Hepatology, 2007. 46(3): p. 871-84.
60. Hulett, M.D. and P.M. Hogarth, *Molecular basis of Fc receptor function.* Adv Immunol, 1994. 57: p. 1-127.
61. Ravetch, J.V. and J.P. Kinet, *Fc receptors.* Annu Rev Immunol, 1991. 9: p. 457-92.
62. Skogh, T., et al., *Hepatic uptake of circulating IgG immune complexes.* Immunology, 1985. 55(4): p. 585-94.
63. Johansson, A.G., et al., *Liver cell uptake and degradation of soluble immunoglobulin G immune complexes in vivo and in vitro in rats.* Hepatology, 1996. 24(1): p. 169-75.
64. Mannik, M., *Mechanisms of tissue deposition of immune complexes.* J Rheumatol Suppl, 1987. 14 Suppl 13: p. 35-42.
65. Skogh, T., *Tissue distribution of intravenously injected dinitrophenylated human serum albumin. Effects of specific IgG and IgA antibodies.* Scand J Immunol, 1982. 16(6): p. 465-75.
66. Lovdal, T., et al., *Fc receptor mediated endocytosis of small soluble immunoglobulin G immune complexes in Kupffer and endothelial cells from rat liver.* J Cell Sci, 2000. 113 (Pt 18): p. 3255-66.
67. Muro, H., et al., *Defect of Fc receptors and phenotypical changes in sinusoidal endothelial cells in human liver cirrhosis.* Am J Pathol, 1993. 143(1): p. 105-20.
68. March, S., et al., *Microenvironmental regulation of the sinusoidal endothelial cell phenotype in vitro.* Hepatology, 2009. 50(3): p. 920-8.
69. Ashwell, G. and A.G. Morell, *The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins.* Adv Enzymol Relat Areas Mol Biol, 1974. 41(0): p. 99-128.
70. Ciechanover, A., A.L. Schwartz, and H.F. Lodish, *Sorting and recycling of cell surface receptors and endocytosed ligands: the asialoglycoprotein and transferrin receptors.* J Cell Biochem, 1983. 23(1-4): p. 107-30.
71. Schwartz, A.L., *The hepatic asialoglycoprotein receptor.* CRC Crit Rev Biochem, 1984. 16(3): p. 207-33.
72. Breitfeld, P.P., et al., *Cell biology of the asialoglycoprotein receptor system: a model of receptor-mediated endocytosis.* Int Rev Cytol, 1985. 97: p. 47-95.
73. Stockert, R.J., *The asialoglycoprotein receptor: relationships between structure, function, and expression.* Physiol Rev, 1995. 75(3): p. 591-609.
74. Weigel, P.H. and J.A. Oka, *The large intracellular pool of asialoglycoprotein receptors functions during the endocytosis of asialoglycoproteins by isolated rat hepatocytes.* J Biol Chem, 1983. 258(8): p. 5095-102.
75. Rice, K.G. and Y.C. Lee, *Oligosaccharide valency and conformation in determining binding to the asialoglycoprotein receptor of rat hepatocytes.* Adv Enzymol Relat Areas Mol Biol, 1993. 66: p. 41-83.

76. **Ng, K.K., K. Drickamer, and W.I. Weis,** ***Structural analysis of monosaccharide recognition by rat liver mannose-binding protein.*** **J Biol Chem, 1996. 271(2): p. 663-74.**

77. **Hudgin, R.L., et al.,** ***The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins.*** **J Biol Chem, 1974. 249(17): p. 5536-43.**

78. **Baenziger, J.U. and D. Fiete,** ***Recycling of the hepatocyte asialoglycoprotein receptor does not require delivery of ligand to lysosomes.*** **J Biol Chem, 1982. 257(11): p. 6007-9.**

79. **Schwartz, A.L., S.E. Fridovich, and H.F. Lodish,** ***Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hepatoma cell line.*** **J Biol Chem, 1982. 257(8): p. 4230-7.**

80. **Weigel, P.H.,** ***Glycoconjugates composition, structure and function, in: H.J. Allen, E.C. Kisailus (Eds.), Mechanism and Control of Glycoconjugate Turnover, Marcel Dekker, New York.*** **1992: p. 421-497.**

81. **Weigel, P.H. and J.A. Oka,** ***Recycling of the hepatic asialoglycoprotein receptor in isolated rat hepatocytes. Receptor-ligand complexes in an intracellular slowly dissociating pool return to the cell surface prior to dissociation.*** **J Biol Chem, 1984. 259(2): p. 1150-4.**

82. **Bovenschen, N., et al.,** ***The B domain of coagulation factor VIII interacts with the asialoglycoprotein receptor.*** **J Thromb Haemost, 2005. 3(6): p. 1257-65.**

83. **Ellies, L.G., et al.,** ***Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands.*** **Proc Natl Acad Sci U S A, 2002. 99(15): p. 10042-7.**

84. **Sodetz, J.M., S.V. Pizzo, and P.A. McKee,** ***Relationship of sialic acid to function and in vivo survival of human factor VIII/von Willebrand factor protein.*** **J Biol Chem, 1977. 252(15): p. 5538-46.**

85. **Mohlke, K.L., et al.,** ***Mvwf, a dominant modifier of murine von Willebrand factor, results from altered lineage-specific expression of a glycosyltransferase.*** **Cell, 1999. 96(1): p. 111-20.**

86. **Brown, M.S. and J.L. Goldstein,** ***A receptor-mediated pathway for cholesterol homeostasis.*** **Science, 1986. 232(4746): p. 34-47.**

87. **Brown, M.S. and J.L. Goldstein,** ***Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity.*** **Proc Natl Acad Sci U S A, 1974. 71(3): p. 788-92.**

88. **Goldstein, J.L. and M.S. Brown,** ***Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia.*** **J Biol Chem, 1974. 249(16): p. 5153-62.**

89. **Ciznar, I., et al.,** ***Oral cholera vaccines containing B-subunit-killed whole cells and killed whole cells only. I. Cross-reacting antigens of members of family Vibrionaceae and the vaccines.*** **Vaccine, 1989. 7(2): p. 111-6.**

90. **Shimokawa, T., et al.,** ***Down-regulation of murine tissue factor pathway inhibitor mRNA by endotoxin and tumor necrosis factor-alpha in vitro and in vivo.*** **Thromb Res, 2000. 100(3): p. 211-21.**

91. **Nykjaer, A. and T.E. Willnow,** ***The low-density lipoprotein receptor gene family: a cellular Swiss army knife?*** **Trends Cell Biol, 2002. 12(6): p. 273-80.**

92. **Willnow, T.E., A. Nykjaer, and J. Herz,** ***Lipoprotein receptors: new roles for ancient proteins.*** **Nat Cell Biol, 1999. 1(6): p. E157-62.**

93. Gliemann, J., ***Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands.*** **Biol Chem, 1998. 379(8-9): p. 951-64.**

94. Herz, J. and D.K. Strickland, ***LRP: a multifunctional scavenger and signaling receptor.*** **J Clin Invest, 2001. 108(6): p. 779-84.**

95. Herz, J., et al., ***Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor.*** **Embo J, 1988. 7(13): p. 4119-27.**

96. Moestrup, S.K., J. Gliemann, and G. Pallesen, ***Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues.*** **Cell Tissue Res, 1992. 269(3): p. 375-82.**

97. Zheng, G., et al., ***Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/alpha 2MR, and the receptor-associated protein (RAP).*** **J Histochem Cytochem, 1994. 42(4): p. 531-42.**

98. Willnow, T.E., et al., ***RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors.*** **Embo J, 1996. 15(11): p. 2632-9.**

99. Ishibashi, S., R.E. Hammer, and J. Herz, ***Asialoglycoprotein receptor deficiency in mice lacking the minor receptor subunit.*** **J Biol Chem, 1994. 269(45): p. 27803-6.**

100. Anderson, R.G., et al., ***Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies.*** **J Cell Biol, 1982. 93(3): p. 523-31.**

101. Kowal, R.C., et al., ***Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins.*** **Proc Natl Acad Sci U S A, 1989. 86(15): p. 5810-4.**

102. Willnow, T.E., ***The low-density lipoprotein receptor gene family: multiple roles in lipid metabolism.*** **J Mol Med, 1999. 77(3): p. 306-15.**

103. Rohlmann, A., et al., ***Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants.*** **J Clin Invest, 1998. 101(3): p. 689-95.**

104. Valentin, S., et al., ***Synergism between full length TFPI and heparin: evidence for TFPI as an important factor for the antithrombotic activity of heparin.*** **Blood Coagul Fibrinolysis, 1992. 3(2): p. 221-2.**

105. Andrade-Gordon, P. and S. Strickland, ***Fractionation of heparin by chromatography on a tissue plasminogen activator-Sepharose column.*** **Proc Natl Acad Sci U S A, 1990. 87(5): p. 1865-9.**

106. Warshawsky, I., G.J. Broze, Jr., and A.L. Schwartz, ***The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor.*** **Proc Natl Acad Sci U S A, 1994. 91(14): p. 6664-8.**

107. Mast, A.E., et al., ***Glypican-3 is a binding protein on the HepG2 cell surface for tissue factor pathway inhibitor.*** **Biochem J, 1997. 327 (Pt 2): p. 577-83.**

108. Willnow, T.E., ***Receptor-associated protein (RAP): a specialized chaperone for endocytic receptors.*** **Biol Chem, 1998. 379(8-9): p. 1025-31.**

109. Schlepper-Schafer, J. and G.F. Springer, ***Carcinoma autoantigens T and Tn and their cleavage products interact with Gal/GalNAc-specific receptors on rat Kupffer cells and hepatocytes.*** **Biochim Biophys Acta, 1989. 1013(3): p. 266-72.**

110. van der Laan, L.J., et al., ***Macrophage scavenger receptor MARCO: in vitro and in vivo regulation and involvement in the anti-bacterial host defense.*** **Immunol Lett, 1997. 57(1-3): p. 203-8.**

111. Lambert, J.C., et al., ***Association at LRP gene locus with sporadic late-onset Alzheimer's disease.*** **Lancet, 1998. 351(9118): p. 1787-8.**
112. Wavrant-DeVrieze, F., et al., ***Association between the low density lipoprotein receptor-related protein (LRP) and Alzheimer's disease.*** **Neurosci Lett, 1997. 227(1): p. 68-70.**
113. Calvo, D. and M.A. Vega, ***Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family.*** **J Biol Chem, 1993. 268(25): p. 18929-35.**
114. Babitt, J., et al., ***Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae.*** **J Biol Chem, 1997. 272(20): p. 13242-9.**
115. Acton, S., et al., ***Identification of scavenger receptor SR-BI as a high density lipoprotein receptor.*** **Science, 1996. 271(5248): p. 518-20.**
116. Landschulz, K.T., et al., ***Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat.*** **J Clin Invest, 1996. 98(4): p. 984-95.**
117. Rigotti, A., et al., ***Scavenger receptor BI--a cell surface receptor for high density lipoprotein.*** **Curr Opin Lipidol, 1997. 8(3): p. 181-8.**
118. Rigotti, A., S.L. Acton, and M. Krieger, ***The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids.*** **J Biol Chem, 1995. 270(27): p. 16221-4.**
119. Ohgami, N., et al., ***Scavenger receptor class B type I-mediated reverse cholesterol transport is inhibited by advanced glycation end products.*** **J Biol Chem, 2001. 276(16): p. 13348-55.**
120. Murao, K., et al., ***Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes.*** **J Biol Chem, 1997. 272(28): p. 17551-7.**
121. Krieger, M., ***Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI.*** **Annu Rev Biochem, 1999. 68: p. 523-58.**
122. Thuahnai, S.T., et al., ***Scavenger receptor class B, type I-mediated uptake of various lipids into cells. Influence of the nature of the donor particle interaction with the receptor.*** **J Biol Chem, 2001. 276(47): p. 43801-8.**
123. Calvo, D., et al., ***CLA-1 is an 85-kD plasma membrane glycoprotein that acts as a high-affinity receptor for both native (HDL, LDL, and VLDL) and modified (OxLDL and AcLDL) lipoproteins.*** **Arterioscler Thromb Vasc Biol, 1997. 17(11): p. 2341-9.**
124. Stangl, H., M. Hyatt, and H.H. Hobbs, ***Transport of lipids from high and low density lipoproteins via scavenger receptor-BI.*** **J Biol Chem, 1999. 274(46): p. 32692-8.**
125. Gu, X., R. Lawrence, and M. Krieger, ***Dissociation of the high density lipoprotein and low density lipoprotein binding activities of murine scavenger receptor class B type I (mSR-BI) using retrovirus library-based activity dissection.*** **J Biol Chem, 2000. 275(13): p. 9120-30.**
126. Thomas, L., ***Studies on the intravascular thromboplastin effect of tissue suspensions in mice: II. A factor in normal rabbit serum which inhibits the thromboplastin effect of the sedimentable tissue component.*** **Bull Johns Hopkins Hosp, 1947. 81.**
127. Schneider, C.L., ***The active principle of placental toxin: thromboplastin; its inactivator in blood: antithromboplastin.*** **Am J Physiol, 1947. 149: p. 123-129.**

128. Biggs, R. and F.R. Mac, ***The reaction of haemophilic plasma to thromboplastin.*** **J Clin Pathol, 1951. 4(4): p. 445-59.**
129. Hjort, P.F., ***Intermediate reactions in the coagulation of blood with tissue thromboplastin; convertin, accelerin, prothrombinase.*** **Scand J Clin Lab Invest, 1957. 9(Suppl 27): p. 1-183.**
130. Dahl, P.E., et al., ***Inhibition of activated coagulation factor VII by normal human plasma.*** **Thromb Haemost, 1982. 48(3): p. 253-6.**
131. Sanders, N.L., et al., ***Inhibition of tissue factor/factor VIIa activity in plasma requires factor X and an additional plasma component.*** **Blood, 1985. 66(1): p. 204-12.**
132. Warn-Cramer, B.J., et al., ***Partial purification and characterization of extrinsic pathway inhibitor (the factor Xa-dependent plasma inhibitor of factor VIIa/tissue factor).*** **Thromb Res, 1987. 48(1): p. 11-22.**
133. Broze, G.J., Jr., et al., ***Isolation of the lipoprotein associated coagulation inhibitor produced by HepG2 (human hepatoma) cells using bovine factor Xa affinity chromatography.*** **Thromb Res, 1987. 48(2): p. 253-9.**
134. Wun, T.C., et al., ***Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains.*** **J Biol Chem, 1988. 263(13): p. 6001-4.**
135. Mori, Y., et al., ***Biochemical characterization of plasma-derived tissue factor pathway inhibitor: post-translational modification of free, full-length form with particular reference to the sugar chain.*** **J Thromb Haemost, 2009. 7(1): p. 111-20.**
136. Crawley, J.T. and D.A. Lane, ***The haemostatic role of tissue factor pathway inhibitor.*** **Arterioscler Thromb Vasc Biol, 2008. 28(2): p. 233-42.**
137. Bajaj, M.S., et al., ***Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: evidence that endothelium is the principal site of its synthesis.*** **Proc Natl Acad Sci U S A, 1990. 87(22): p. 8869-73.**
138. Girard, T.J., et al., ***Identification of the 1.4 kb and 4.0 kb messages for the lipoprotein associated coagulation inhibitor and expression of the encoded protein.*** **Thromb Res, 1989. 55(1): p. 37-50.**
139. Girard, T.J., et al., ***Structure of the human lipoprotein-associated coagulation inhibitor gene. Intro/exon gene organization and localization of the gene to chromosome 2.*** **J Biol Chem, 1991. 266(8): p. 5036-41.**
140. van der Logt, C.P., P.H. Reitsma, and R.M. Bertina, ***Intron-exon organization of the human gene coding for the lipoprotein-associated coagulation inhibitor: the factor Xa dependent inhibitor of the extrinsic pathway of coagulation.*** **Biochemistry, 1991. 30(6): p. 1571-7.**
141. Tyson, D.R., et al., ***Revised DNA sequence of exon 1 and 5' flanking region of the human tissue factor pathway inhibitor gene.*** **Thromb Res, 1993. 70(3): p. 269-73.**
142. Orkin, S.H., ***GATA-binding transcription factors in hematopoietic cells.*** **Blood, 1992. 80(3): p. 575-81.**
143. Lee, M.E., et al., ***Cloning of the GATA-binding protein that regulates endothelin-1 gene expression in endothelial cells.*** **J Biol Chem, 1991. 266(24): p. 16188-92.**
144. Ameri, A., et al., ***Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators.*** **Blood, 1992. 79(12): p. 3219-26.**
145. Broze, G.J., Jr., ***Tissue factor pathway inhibitor gene disruption.*** **Blood Coagul Fibrinolysis, 1998. 9 Suppl 1: p. S89-92.**
146. Moatti, D., et al., ***Polymorphisms of the tissue factor pathway inhibitor (TFPI) gene in patients with acute coronary syndromes and in healthy subjects : impact of the***

V264M substitution on plasma levels of TFPI. **Arterioscler Thromb Vasc Biol, 1999. 19(4): p. 862-9.**

147. **Arnaud, E., et al., *No link between the TFPI V264M mutation and venous thromboembolic disease.* Thromb Haemost, 1999. 82(1): p. 159-60.**
148. **Miyata, T., et al., *C-399T polymorphism in the promoter region of human tissue factor pathway inhibitor (TFPI) gene does not change the plasma TFPI antigen level and does not cause venous thrombosis.* Thromb Haemost, 1998. 80(2): p. 345-6.**
149. **Gonzalez-Conejero, R., et al., *The TFPI 536C-->T mutation is not associated with increased risk for venous or arterial thrombosis.* Thromb Haemost, 2000. 83(5): p. 787-8.**
150. **Zitoun, D., L. Bara, and M.M. Samama, *Compared activity of human, rat and rabbit plasma tissue factor pathway inhibitors.* Thromb Res, 1993. 72(3): p. 269-74.**
151. **Enjyoji, K., et al., *cDNA cloning and expression of rat tissue factor pathway inhibitor (TFPI).* J Biochem, 1992. 111(5): p. 681-7.**
152. **Wesselschmidt, R.L., T.J. Girard, and G.J. Broze, Jr., *cDNA sequence of rabbit lipoprotein-associated coagulation inhibitor.* Nucleic Acids Res, 1990. 18(21): p. 6440.**
153. **Kamei, S., et al., *Amino acid sequence and inhibitory activity of rhesus monkey tissue factor pathway inhibitor (TFPI): comparison with human TFPI.* J Biochem, 1994. 115(4): p. 708-14.**
154. **Sevinsky, J.R., L.V. Rao, and W. Ruf, *Ligand-induced protease receptor translocation into caveolae: a mechanism for regulating cell surface proteolysis of the tissue factor-dependent coagulation pathway.* J Cell Biol, 1996. 133(2): p. 293-304.**
155. **Ott, I., et al., *Reversible regulation of tissue factor-induced coagulation by glycosyl phosphatidylinositol-anchored tissue factor pathway inhibitor.* Arterioscler Thromb Vasc Biol, 2000. 20(3): p. 874-82.**
156. **Lupu, C., et al., *Tissue factor pathway inhibitor in endothelial cells colocalizes with glycolipid microdomains/caveolae. Regulatory mechanism(s) of the anticoagulant properties of the endothelium.* Arterioscler Thromb Vasc Biol, 1997. 17(11): p. 2964-74.**
157. **Girard, T.J., et al., *Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor.* Nature, 1989. 338(6215): p. 518-20.**
158. **Abumiya, T., et al., *An anti-tissue factor pathway inhibitor (TFPI) monoclonal antibody recognized the third Kunitz domain (K3) of free-form TFPI but not lipoprotein-associated forms in plasma.* J Biochem (Tokyo), 1995. 118(1): p. 178-82.**
159. **Nordfang, O., et al., *The C-terminus of tissue factor pathway inhibitor is essential to its anticoagulant activity.* Biochemistry, 1991. 30(43): p. 10371-6.**
160. **Wesselschmidt, R., et al., *Tissue factor pathway inhibitor: the carboxy-terminus is required for optimal inhibition of factor Xa.* Blood, 1992. 79(8): p. 2004-10.**
161. **Wesselschmidt, R., et al., *Structural requirements for tissue factor pathway inhibitor interactions with factor Xa and heparin.* Blood Coagul Fibrinolysis, 1993. 4(5): p. 661-9.**
162. **Lindhout, T., J. Franssen, and G. Willems, *Kinetics of the inhibition of tissue factor-factor VIIa by tissue factor pathway inhibitor.* Thromb Haemost, 1995. 74(3): p. 910-5.**
163. **Piro, O. and G.J. Broze, Jr., *Role for the Kunitz-3 domain of tissue factor pathway inhibitor-alpha in cell surface binding.* Circulation, 2004. 110(23): p. 3567-72.**

164. Broze, G.J., Jr., T.J. Girard, and W.F. Novotny, *Regulation of coagulation by a multivalent Kunitz-type inhibitor.* Biochemistry, 1990. 29(33): p. 7539-46.
165. Girard, T.J., et al., *Endogenous phosphorylation of the lipoprotein-associated coagulation inhibitor at serine-2.* Biochem J, 1990. 270(3): p. 621-5.
166. Novotny, W.F., et al., *Purification and characterization of the lipoprotein-associated coagulation inhibitor from human plasma.* J Biol Chem, 1989. 264(31): p. 18832-7.
167. Ho, G., et al., *Recombinant full-length tissue factor pathway inhibitor fails to bind to the cell surface: implications for catabolism in vitro and in vivo.* Blood, 2000. 95(6): p. 1973-8.
168. Smith, P.L., et al., *The asparagine-linked oligosaccharides on tissue factor pathway inhibitor terminate with SO4-4GalNAc beta 1, 4GlcNAc beta 1,2 Mana alpha.* J Biol Chem, 1992. 267(27): p. 19140-6.
169. Piro, O. and G.J. Broze, Jr., *Comparison of cell-surface TFPIalpha and beta.* J Thromb Haemost, 2005. 3(12): p. 2677-83.
170. Zhang, J., et al., *Glycosyl phosphatidylinositol anchorage of tissue factor pathway inhibitor.* Circulation, 2003. 108(5): p. 623-7.
171. Versteeg, H.H. and W. Ruf, *Emerging insights in tissue factor-dependent signaling events.* Semin Thromb Hemost, 2006. 32(1): p. 24-32.
172. Novotny, W.F., et al., *Purification and properties of heparin-releasable lipoprotein-associated coagulation inhibitor.* Blood, 1991. 78(2): p. 394-400.
173. Li, A. and T.C. Wun, *Proteolysis of tissue factor pathway inhibitor (TFPI) by plasmin: effect on TFPI activity.* Thromb Haemost, 1998. 80(3): p. 423-7.
174. Salemink, I., et al., *Factor Xa cleavage of tissue factor pathway inhibitor is associated with loss of anticoagulant activity.* Thromb Haemost, 1998. 80(2): p. 273-80.
175. Broze, G.J., Jr., et al., *The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action.* Blood, 1988. 71(2): p. 335-43.
176. Warn-Cramer, B.J., et al., *Modifications of extrinsic pathway inhibitor (EPI) and factor Xa that affect their ability to interact and to inhibit factor VIIa/tissue factor: evidence for a two-step model of inhibition.* Thromb Haemost, 1988. 60(3): p. 453-6.
177. Huang, Z.F., T.C. Wun, and G.J. Broze, Jr., *Kinetics of factor Xa inhibition by tissue factor pathway inhibitor.* J Biol Chem, 1993. 268(36): p. 26950-5.
178. Hackeng, T.M., et al., *Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor.* Proc Natl Acad Sci U S A, 2006. 103(9): p. 3106-11.
179. Haskel, E.J., et al., *Prevention of arterial reocclusion after thrombolysis with recombinant lipoprotein-associated coagulation inhibitor.* Circulation, 1991. 84(2): p. 821-7.
180. Ho, G., et al., *Receptor-mediated endocytosis of coagulation factor Xa requires cell surface-bound tissue factor pathway inhibitor.* J Biol Chem, 1996. 271(16): p. 9497-502.
181. Hansen, J.B., R. Olsen, and P. Webster, *Association of tissue factor pathway inhibitor with human umbilical vein endothelial cells.* Blood, 1997. 90(9): p. 3568-78.
182. Broze, G.J., Jr., et al., *Heterogeneity of plasma tissue factor pathway inhibitor.* Blood Coagul Fibrinolysis, 1994. 5(4): p. 551-9.

183. Lesnik, P., et al., ***Anticoagulant activity of tissue factor pathway inhibitor in human plasma is preferentially associated with dense subspecies of LDL and HDL and with Lp(a).*** **Arterioscler Thromb, 1993. 13(7): p. 1066-75.**

184. Novotny, W.F., et al., ***Plasma antigen levels of the lipoprotein-associated coagulation inhibitor in patient samples.*** **Blood, 1991. 78(2): p. 387-93.**

185. Dahm, A., et al., ***Opposite circadian rhythms in melatonin and tissue factor pathway inhibitor type 1: does daylight affect coagulation?*** **J Thromb Haemost, 2006. 4(8): p. 1840-2.**

186. Kanabrocki, E.L., et al., ***Day-night variations in blood levels of nitric oxide, T-TFPI, and E-selectin.*** **Clin Appl Thromb Hemost, 2001. 7(4): p. 339-45.**

187. Pinotti, M., et al., ***Daily and circadian rhythms of tissue factor pathway inhibitor and factor VII activity.*** **Arterioscler Thromb Vasc Biol, 2005. 25(3): p. 646-9.**

188. Gori, A.M., et al., ***Tissue factor reduction and tissue factor pathway inhibitor release after heparin administration.*** **Thromb Haemost, 1999. 81(4): p. 589-93.**

189. Werling, R.W., et al., ***Distribution of tissue factor pathway inhibitor in normal and malignant human tissues.*** **Thromb Haemost, 1993. 69(4): p. 366-9.**

190. Bajaj, M.S., et al., ***Transcriptional expression of tissue factor pathway inhibitor, thrombomodulin and von Willebrand factor in normal human tissues.*** **Thromb Haemost, 1999. 82(3): p. 1047-52.**

191. Ott, I., et al., ***Regulation of monocyte procoagulant activity in acute myocardial infarction: role of tissue factor and tissue factor pathway inhibitor-1.*** **Blood, 2001. 97(12): p. 3721-6.**

192. Novotny, W.F., et al., ***Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein associated coagulation inhibitor.*** **Blood, 1988. 72(6): p. 2020-5.**

193. Wojtukiewicz, M.Z., et al., ***Expression of tissue factor and tissue factor pathway inhibitor in situ in laryngeal carcinoma.*** **Thromb Haemost, 1999. 82(6): p. 1659-62.**

194. Yamabe, H., et al., ***Tissue factor pathway inhibitor production by human mesangial cells in culture.*** **Thromb Haemost, 1996. 76(2): p. 215-9.**

195. Crawley, J., et al., ***Expression, localization, and activity of tissue factor pathway inhibitor in normal and atherosclerotic human vessels.*** **Arterioscler Thromb Vasc Biol, 2000. 20(5): p. 1362-73.**

196. Osterud, B., M.S. Bajaj, and S.P. Bajaj, ***Sites of tissue factor pathway inhibitor (TFPI) and tissue factor expression under physiologic and pathologic conditions. On behalf of the Subcommittee on Tissue factor Pathway Inhibitor (TFPI) of the Scientific and Standardization Committee of the ISTH.*** **Thromb Haemost, 1995. 73(5): p. 873-5.**

197. Edstrom, C.S., D.A. Calhoun, and R.D. Christensen, ***Expression of tissue factor pathway inhibitor in human fetal and placental tissues.*** **Early Hum Dev, 2000. 59(2): p. 77-84.**

198. Grabowski, E.F., et al., ***Shear stress decreases endothelial cell tissue factor activity by augmenting secretion of tissue factor pathway inhibitor.*** **Arterioscler Thromb Vasc Biol, 2001. 21(1): p. 157-62.**

199. Lupu, C., et al., ***Thrombin induces the redistribution and acute release of tissue factor pathway inhibitor from specific granules within human endothelial cells in culture.*** **Arterioscler Thromb Vasc Biol, 1995. 15(11): p. 2055-62.**

200. Caplice, N.M., et al., ***Expression of tissue factor pathway inhibitor in vascular smooth muscle cells and its regulation by growth factors.*** **Circ Res, 1998. 83(12): p. 1264-70.**

201. Lupu, C., et al., ***Cellular effects of heparin on the production and release of tissue factor pathway inhibitor in human endothelial cells in culture.*** **Arterioscler Thromb Vasc Biol, 1999. 19(9): p. 2251-62.**
202. Westmuckett, A.D., et al., ***Fluid flow induces upregulation of synthesis and release of tissue factor pathway inhibitor in vitro.*** **Arterioscler Thromb Vasc Biol, 2000. 20(11): p. 2474-82.**
203. McGee, M.P., S. Foster, and X. Wang, ***Simultaneous expression of tissue factor and tissue factor pathway inhibitor by human monocytes. A potential mechanism for localized control of blood coagulation.*** **J Exp Med, 1994. 179(6): p. 1847-54.**
204. Rana, S.V., et al., ***Expression of tissue factor and factor VIIa/tissue factor inhibitor activity in endotoxin or phorbol ester stimulated U937 monocyte-like cells.*** **Blood, 1988. 71(1): p. 259-62.**
205. Hansen, J.B., et al., ***Heparin induces synthesis and secretion of tissue factor pathway inhibitor from endothelial cells in vitro.*** **Thromb Haemost, 2000. 83(6): p. 937-43.**
206. Palmier, M.O., et al., ***Clearance of recombinant tissue factor pathway inhibitor (TFPI) in rabbits.*** **Thromb Haemost, 1992. 68(1): p. 33-6.**
207. Warshawsky, I., et al., ***The carboxy terminus of tissue factor pathway inhibitor is required for interacting with hepatoma cells in vitro and in vivo.*** **J Clin Invest, 1995. 95(4): p. 1773-81.**
208. Krieger, M. and J. Herz, ***Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP).*** **Annu Rev Biochem, 1994. 63: p. 601-37.**
209. Willnow, T.E., et al., ***Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance.*** **J Biol Chem, 1992. 267(36): p. 26172-80.**
210. Narita, M., et al., ***Two receptor systems are involved in the plasma clearance of tissue factor pathway inhibitor in vivo.*** **J Biol Chem, 1995. 270(42): p. 24800-4.**
211. Warshawsky, I., et al., ***The low density lipoprotein receptor-related protein can function independently from heparan sulfate proteoglycans in tissue factor pathway inhibitor endocytosis.*** **J Biol Chem, 1996. 271(42): p. 25873-9.**
212. Marcum, J.A., ***The origin of the dispute over the discovery of heparin.*** **J Hist Med Allied Sci, 2000. 55(1): p. 37-66.**
213. Weiler, J.M., et al., ***Heparin and modified heparin inhibit complement activation in vivo.*** **J Immunol, 1992. 148(10): p. 3210-5.**
214. Lindahl, U., D.S. Feingold, and L. Roden, ***Biosynthesis of heparin.*** **Trends Biochem. Sci., 1986. 11(5): p. 221-25.**
215. Lindahl, U. and L. Kjellen, ***The biology of the Extracellular Matrix: Proteoglycans.*** **Academic Press, New York, 1987.**
216. Capila, I. and R.J. Linhardt, ***Heparin-protein interactions.*** **Angew Chem Int Ed Engl, 2002. 41(3): p. 391-412.**
217. Perlin, A.S., D.M. Mackie, and C.P. Dietrich, ***Evidence for a (1 leads to 4)-linked 4-O-(-L-idopyranosyluronic acid 2-sulfate)-(2-deoxy-2-sulfoamino-D-glucopyranosyl 6-sulfate) sequence in heparin. Long-range H-H coupling in 4-deoxy-hex-4-enopyranosides.*** **Carbohydr Res, 1971. 18(2): p. 185-94.**
218. Petitou, M., B. Casu, and U. Lindahl, ***1976-1983, a critical period in the history of heparin: the discovery of the antithrombin binding site.*** **Biochimie, 2003. 85(1-2): p. 83-9.**

219. Mulloy, B., *The specificity of interactions between proteins and sulfated polysaccharides.* An Acad Bras Cienc, 2005. 77(4): p. 651-64.
220. Abildgaard, U., *Highly purified antithrombin 3 with heparin cofactor activity prepared by disc electrophoresis.* Scand J Clin Lab Invest, 1968. 21(1): p. 89-91.
221. Holmer, E., K. Kurachi, and G. Soderstrom, *The molecular-weight dependence of the rate-enhancing effect of heparin on the inhibition of thrombin, factor Xa, factor IXa, factor XIa, factor XIIa and kallikrein by antithrombin.* Biochem J, 1981. 193(2): p. 395-400.
222. Bjork, I. and U. Lindahl, *Mechanism of the anticoagulant action of heparin.* Mol Cell Biochem, 1982. 48(3): p. 161-82.
223. Ehrlich, J. and S.S. Stivala, *Chemistry and pharmacology of heparin.* J Pharm Sci, 1973. 62(4): p. 517-44.
224. Bergamaschini, L., et al., *Heparin attenuates cytotoxic and inflammatory activity of Alzheimer amyloid-beta in vitro.* Neurobiol Aging, 2002. 23(4): p. 531-6.
225. Bergamaschini, L., et al., *Peripheral treatment with enoxaparin, a low molecular weight heparin, reduces plaques and beta-amyloid accumulation in a mouse model of Alzheimer's disease.* J Neurosci, 2004. 24(17): p. 4181-6.
226. Borsig, L., et al., *Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis.* Proc Natl Acad Sci U S A, 2001. 98(6): p. 3352-7.
227. Linhardt, R.J., *Heparin-induced cancer cell death.* Chem Biol, 2004. 11(4): p. 420-2.
228. Perretti, M. and C.P. Page, *Heparin and inflammation: a new use for an old GAG?* Gut, 2000. 47(1): p. 14-5.
229. de Swart, C.A., et al., *Kinetics of intravenously administered heparin in normal humans.* Blood, 1982. 60(6): p. 1251-8.
230. Olsson, P., H. Lagergren, and S. Ek, *The elimination from plasma of intravenous heparin. An experimental study on dogs and humans.* Acta Med Scand, 1963. 173: p. 619-30.
231. Bjornsson, T.D., K.M. Wolfram, and B.B. Kitchell, *Heparin kinetics determined by three assay methods.* Clin Pharmacol Ther, 1982. 31(1): p. 104-13.
232. Mahadoo, J., L. Heibert, and L.B. Jaques, *Vascular sequestration of heparin.* Thromb Res, 1978. 12(1): p. 79-90.
233. Barzu, T., et al., *Binding and endocytosis of heparin by human endothelial cells in culture.* Biochim Biophys Acta, 1985. 845(2): p. 196-203.
234. Barzu, T., et al., *Heparin degradation in the endothelial cells.* Thromb Res, 1987. 47(5): p. 601-9.
235. Vannucchi, S., et al., *Internalization and metabolism of endogenous heparin by cultured endothelial cells.* Biochem Biophys Res Commun, 1986. 140(1): p. 294-301.
236. Monkhouse, F.C., *Physiological factors concerned with the removal of injected heparin from the circulating blood.* Am J Physiol, 1954. 178(2): p. 223-8.
237. Teien, A.N., *Heparin elimination in patients with liver cirrhosis.* Thromb Haemost, 1977. 38(3): p. 701-6.
238. Dawes, J. and D.S. Papper, *Catabolism of low-dose heparin in man.* Thromb Res, 1979. 14(6): p. 845-60.
239. Bjornsson, T.D. and G. Levy, *Pharmacokinetics of heparin. II. Studies of time dependence in rats.* J Pharmacol Exp Ther, 1979. 210(2): p. 243-6.
240. Boneu, B., et al., *Evidence for a saturable mechanism of disappearance of standard heparin in rabbits.* Thromb Res, 1987. 46(6): p. 835-44.

241. Watanabe, J., et al., *Disposition of tritium-labelled heparin in rats.* J Pharmacobiodyn, 1982. 5(8): p. 627-37.
242. Asplund, J., U. Borell, and H. Holgreim, *Investigations on the storage og heparin in the animal organism as well as on the possibility of its reabsorption in the intestine and placenta.* Zeitschrift f. mirk.- anat. Forschung, 1939. 46: p. 16-67.
243. Hiebert, L., *The uptake of heparin by liver sinusoidal cells in normal and atherosclerotic rabbits.* Thromb Res, 1981. 21(4-5): p. 383-90.
244. Haba, M., et al., *Molecular weight dependency in the uptake of fractionated [3H]heparin in isolated rat Kupffer cells.* Biol Pharm Bull, 1996. 19(6): p. 864-8.
245. Nakamura, T., H. Yuasa, and J. Watanabe, *Uptake of fractionated heparin by two types of scavenger receptors in isolated rat Kupffer cells.* Biol Pharm Bull, 2000. 23(6): p. 743-7.
246. Watanabe, J., M. Haba, and H. Yuasa, *Uptake of fractionated 3H-heparin by isolated rat Kupffer cells.* Pharm Res, 1995. 12(7): p. 1092-5.
247. Watanabe, J., et al., *Uptake of low molecular weight fractionated [3H]heparin by rat hepatocytes in the primary culture.* Biol Pharm Bull, 1995. 18(3): p. 443-6.
248. Yuasa, H. and J. Watanabe, *Are novel scavenger-like receptors involved in the hepatic uptake of heparin?* Drug Metab Pharmacokinet, 2003. 18(5): p. 273-86.
249. Holmer, E., *Low molecular weight heparin.* In: Heparin. Editors: DA Lane and U Lindahl; Edward Arnold, Division of Hodder and Stoughton, London, Melbourne, Auckland, 1989.
250. Palm, M. and C. Mattsson, *Pharmacokinetics of heparin and low molecular weight heparin fragment (Fragmin) in rabbits with impaired renal or metabolic clearance.* Thromb Haemost, 1987. 58(3): p. 932-5.
251. Nordoy, A., *Difference in the heparin-neutralizing effect of protamine and polybrene as tested by thrombotest.* Scand J Clin Lab Invest, 1963. 15: p. 205-10.
252. Lindahl, A.K., U. Abildgaard, and R. Staalesen, *The anticoagulant effect in heparinized blood and plasma resulting from interactions with extrinsic pathway inhibitor.* Thromb Res, 1991. 64(2): p. 155-68.
253. Wun, T.C., *Lipoprotein-associated coagulation inhibitor (LACI) is a cofactor for heparin: synergistic anticoagulant action between LACI and sulfated polysaccharides.* Blood, 1992. 79(2): p. 430-8.
254. Hansen, J.B., et al., *Depletion of intravascular pools of tissue factor pathway inhibitor (TFPI) during repeated or continuous intravenous infusion of heparin in man.* Thromb Haemost, 1996. 76(5): p. 703-9.
255. Hansen, J.B., et al., *Differential effect of unfractionated heparin and low molecular weight heparin on intravascular tissue factor pathway inhibitor: evidence for a difference in antithrombotic action.* Br J Haematol, 1998. 101(4): p. 638-46.
256. Brodin, E., et al., *Intravascular release and urinary excretion of tissue factor pathway inhibitor during heparin treatment.* J Lab Clin Med, 2004. 144(5): p. 246-53; discussion 226-7.
257. Bregengaard, C., et al., *Pharmacokinetics of full length and two-domain tissue factor pathway inhibitor in combination with heparin in rabbits.* Thromb Haemost, 1993. 70(3): p. 454-7.
258. Beeby, T.L., et al., *Distribution of the recombinant coagulation factor 125I-rFVIIa in rats.* Thromb Haemost, 1993. 70(3): p. 465-8.
259. Fuchs, H.E., et al., *Regulation of factor IXa in vitro in human and mouse plasma and in vivo in the mouse. Role of the endothelium and the plasma proteinase inhibitors.* J Clin Invest, 1984. 73(6): p. 1696-703.

260. **Narita, M., et al.,** ***The low-density lipoprotein receptor-related protein (LRP) mediates clearance of coagulation factor Xa in vivo.*** **Blood, 1998. 91(2): p. 555-60.**
261. **Johnstone, A. and R. Thorpe,** ***Radiolabelling techniques.*** **Immunochemistry in Practice, Blackwell Scientific Publications, 1982: p. 102-112.**
262. **Salacinski, P.R., et al.,** ***Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen).*** **Anal Biochem, 1981. 117(1): p. 136-46.**
263. **Zhang, L., et al.,** ***The liver is a major organ for clearing simian immunodeficiency virus in rhesus monkeys.*** **J Virol, 2002. 76(10): p. 5271-3.**
264. **Martin-Armas, M., et al.,** ***Toll-like receptor 9 (TLR9) is present in murine liver sinusoidal endothelial cells (LSECs) and mediates the effect of CpG-oligonucleotides.*** **J Hepatol, 2006. 44(5): p. 939-46.**
265. **Pittman, R.C., et al.,** ***A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation in vivo.*** **Biochem J, 1983. 212(3): p. 791-800.**
266. **Smedsrod, B.,** ***Aminoterminal propeptide of type III procollagen is cleared from the circulation by receptor-mediated endocytosis in liver endothelial cells.*** **Coll-Relat-Res, 1988. 8(4): p. 375-88.**
267. **Out, R., et al.,** ***Scavenger receptor class B type I is solely responsible for the selective uptake of cholesteryl esters from HDL by the liver and the adrenals in mice.*** **J Lipid Res, 2004. 45(11): p. 2088-95.**
268. **Kaczmarczyk, A., et al.,** ***Plasma bikunin: half-life and tissue uptake.*** **Mol Cell Biochem, 2005. 271(1-2): p. 61-7.**
269. **Mulya, A., et al.,** ***Initial interaction of apoA-I with ABCA1 impacts in vivo metabolic fate of nascent HDL.*** **J Lipid Res, 2008. 49(11): p. 2390-401.**
270. **Goldstein, J.L., et al.,** ***Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition.*** **Proc Natl Acad Sci U S A, 1979. 76(1): p. 333-7.**
271. **Brown, M.S., et al.,** ***The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages.*** **J Supramol Struct, 1980. 13(1): p. 67-81.**
272. **Grimsley, P.G., et al.,** ***Low density lipoprotein receptor-related protein (LRP) expression varies among Hep G2 cell lines.*** **Thromb Res, 1997. 88(6): p. 485-98.**
273. **Schwartz, A.L., et al.,** ***Characterization of the asialoglycoprotein receptor in a continuous hepatoma line.*** **J Biol Chem, 1981. 256(17): p. 8878-81.**
274. **Knowles, B.B., C.C. Howe, and D.P. Aden,** ***Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen.*** **Science, 1980. 209(4455): p. 497-9.**
275. **Hjortoe, G., et al.,** ***Factor VIIa binding and internalization in hepatocytes.*** **J Thromb Haemost, 2005. 3(10): p. 2264-73.**
276. **Planas-Bohne, F., W. Jung, and M. Neu-Muller,** ***Uptake of 59Fe and 239Pu by rat liver cells and human hepatoma cells.*** **Int J Radiat Biol Relat Stud Phys Chem Med, 1985. 48(5): p. 797-805.**
277. **Bajaj, M.S., et al.,** ***Inhibitor of the factor VIIa-tissue factor complex is reduced in patients with disseminated intravascular coagulation but not in patients with severe hepatocellular disease.*** **J Clin Invest, 1987. 79(6): p. 1874-8.**
278. **Karrar, A., et al.,** ***Human liver sinusoidal endothelial cells induce apoptosis in activated T cells: a role in tolerance induction.*** **Gut, 2007. 56(2): p. 243-52.**

279. Rauen, U., et al., ***Injury to cultured liver endothelial cells during cold preservation: energy-dependent versus energy-deficiency injury.*** **Transpl Int, 1993. 6(4): p. 218-22.**
280. Krause, P., et al., ***Hepatocyte-supported serum-free culture of rat liver sinusoidal endothelial cells.*** **J Hepatol, 2000. 32(5): p. 718-26.**
281. Elvevold, K., et al., ***Long-term preservation of high endocytic activity in primary cultures of pig liver sinusoidal endothelial cells.*** **Eur J Cell Biol, 2005. 84(9): p. 749-64.**
282. Day, K.C., et al., ***Recombinant lipoprotein-associated coagulation inhibitor inhibits tissue thromboplastin-induced intravascular coagulation in the rabbit.*** **Blood, 1990. 76(8): p. 1538-45.**
283. Creasey, A.A., et al., ***Tissue factor pathway inhibitor reduces mortality from Escherichia coli septic shock.*** **J Clin Invest, 1993. 91(6): p. 2850-60.**
284. Carr, C., et al., ***Recombinant E. coli-derived tissue factor pathway inhibitor reduces coagulopathic and lethal effects in the baboon gram-negative model of septic shock.*** **Circ Shock, 1994. 44(3): p. 126-37.**
285. Braeckman, R., et al., ***Pharmacokinetics and pharmacodynamics (PK/PD) of recombinant tissue factor pathway inhibitor (rTFPI) in human volunteers.*** **Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada., 1997. September 28-October 1.**
286. Abraham, E., et al., ***Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial.*** **JAMA, 2003. 290(2): p. 238-47.**
287. Zhai, X.Y., et al., ***Cubilin- and megalin-mediated uptake of albumin in cultured proximal tubule cells of opossum kidney.*** **Kidney Int, 2000. 58(4): p. 1523-33.**
288. Hammad, S.M., et al., ***Megalin acts in concert with cubilin to mediate endocytosis of high density lipoproteins.*** **J Biol Chem, 2000. 275(16): p. 12003-8.**
289. Grabenhorst, E., et al., ***Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells.*** **Glycoconj J, 1999. 16(2): p. 81-97.**
290. Ashwell, G. and J. Harford, ***Carbohydrate-specific receptors of the liver.*** **Annu Rev Biochem, 1982. 51: p. 531-54.**
291. Harris, E.N., J.A. Weigel, and P.H. Weigel, ***The human hyaluronan receptor for endocytosis (HARE/Stabilin-2) is a systemic clearance receptor for heparin.*** **J Biol Chem, 2008. 283(25): p. 17341-50.**
292. Harris, E.N., J.A. Weigel, and P.H. Weigel, ***Endocytic function, glycosaminoglycan specificity, and antibody sensitivity of the recombinant human 190-kDa hyaluronan receptor for endocytosis (HARE).*** **J Biol Chem, 2004. 279(35): p. 36201-9.**
293. Harris, E.N. and P.H. Weigel, ***The ligand-binding profile of HARE: hyaluronan and chondroitin sulfates A, C, and D bind to overlapping sites distinct from the sites for heparin, acetylated low-density lipoprotein, dermatan sulfate, and CS-E.*** **Glycobiology, 2008. 18(8): p. 638-48.**
294. Cardin, A.D. and H.J. Weintraub, ***Molecular modeling of protein-glycosaminoglycan interactions.*** **Arteriosclerosis, 1989. 9(1): p. 21-32.**
295. Iversen, N., et al., ***Binding of tissue factor pathway inhibitor to cultured endothelial cells-influence of glycosaminoglycans.*** **Thromb Res, 1996. 84(4): p. 267-78.**
296. Bu, G. and M.P. Marzolo, ***Role of rap in the biogenesis of lipoprotein receptors.*** **Trends Cardiovasc Med, 2000. 10(4): p. 148-55.**
297. Boers, W., et al., ***Uptake of methylamine-activated alpha 2-macroglobulin by rat liver.*** **Ann N Y Acad Sci, 1994. 737: p. 428-30.**

298. **Nykjaer, A., et al.,** ***Purified alpha 2-macroglobulin receptor/LDL receptor-related protein binds urokinase.plasminogen activator inhibitor type-1 complex. Evidence that the alpha 2-macroglobulin receptor mediates cellular degradation of urokinase receptor-bound complexes.*** **J Biol Chem, 1992. 267(21): p. 14543-6.**

299. **Biessen, E.A., et al.,** ***Antagonists of the mannose receptor and the LDL receptor-related protein dramatically delay the clearance of tissue plasminogen activator.*** **Circulation, 1997. 95(1): p. 46-52.**

Paper I

Paper II

Paper III

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