Factor VIII expression and regulation in health and disease
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Link to publication

Citation for published version (APA):

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Chapter 1

Introduction
INTRODUCTION

Factor VIII is a plasma protein, which functions as a cofactor in the intrinsic coagulation cascade. In the circulation factor VIII is present as inactive precursor, tightly associated with its carrier protein von Willebrand factor (VWF). After vascular damage, the coagulation system is triggered, eventually leading to fibrin formation. Activation of factor VIII is an intermediate step in this process. As a cofactor, activated factor VIII (factor VIIla) together with factor IXa will proteolytic convert factor X into factor Xa. Subsequently, this will lead to the formation of thrombin and thrombin-mediated conversion of fibrinogen into fibrin. Together with platelets fibrin forms an insoluble network, thereby arresting the bleeding at the site of vascular injury (1). Factor VIII deficiency causes a bleeding tendency, known as hemophilia A. Hemophilia A is an X-linked bleeding disorder affecting 1 in approximately 5000 males (2). The length of the factor VIII gene is 186 kb and is one of the largest genes known. The transcription comprises 26 exons, resulting in the synthesis of a 330 kDa protein. Factor VIII shows a distinct domain structure, arranged in a sequence, denoted as: A1-a1-A2-a2-B-a3-A3-C1-C2. Due to intracellular proteolysis factor VIII circulates in plasma as a heterodimeric protein consisting of metal ion-linked heavy -light chain species. The heterogeneous heavy chain (90-220 kDa) contains the A1-a1-A2-a2-B domain. The heterogeneity is caused by partial proteolytic cleavage of the B domain. The light chain (80 kDa) consists of domains a3-A3-C1-C2 (ref: 3-5).

Although the molecular structure of factor VIII and its functional properties are known for a few decades and insights into its cofactor function at the molecular level have been obtained (Table 1), the site of synthesis and cells responsible for factor VIII synthesis are less well understood. Similarly, little is known about the mechanisms responsible for the commonly observed fluctuations of the plasma concentration of factor VIII. This thesis will focus on these issues.

BIOSYNTHESIS OF FACTOR VIII

Our current knowledge on the biosynthesis and cellular distribution of factor VIII synthesis is limited and has been (and still is) a controversial issue for many years. Transplantation studies in both hemophilic animals and patients with hemophilia A clearly demonstrated that the liver is the primary site of factor VIII synthesis (6-9). After liver transplantation the factor VIII deficiency and associated hemorrhagic tendency is corrected. On the other hand, when normal dogs received a hemophilic liver, factor VIII levels never fell below 10-20% of the initial plasma factor VIII concentration, indicating an additional extra-hepatic source of factor VIII (10). The contribution of extra-hepatic tissues to the plasma factor VIII pool is clearly of significance. However, tissues and cells that are responsible for extra-hepatic factor VIII synthesis, have not been identified. Northern blot analysis revealed that factor VIII mRNA, besides in the liver, is present in a variety of tissues, such as kidney, spleen, lymph node and lung (11-14). Similarly, by immunohistochemical techniques factor VIII could be detected in spleen, lymph node, placenta and lung (15,16). Taken together,
these observations indicate that extra-hepatic factor VIII synthesis occurs, although the relative contribution of the various extra-hepatic tissues to the factor VIII plasma pool has not been established.

Table 1. Characteristics of factor VIII and von Willebrand factor.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Factor VIII</th>
<th>Von Willebrand factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>X-chromosome</td>
<td>Chromosome 12</td>
</tr>
<tr>
<td>Size</td>
<td>186 kb</td>
<td>175 kb</td>
</tr>
<tr>
<td>Structure</td>
<td>26 exons</td>
<td>52 exons</td>
</tr>
<tr>
<td>mRNA</td>
<td>9 kb</td>
<td>9 kb</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structure</td>
<td>330 kD</td>
<td>250 kD→Dimer→Polymer</td>
</tr>
<tr>
<td>Synthesis</td>
<td>Liver, spleen,</td>
<td>Endothelial cells,</td>
</tr>
<tr>
<td></td>
<td>kidney, lung</td>
<td>megakaryocytes</td>
</tr>
<tr>
<td>Location</td>
<td>Blood plasma</td>
<td>Endothelial cells, extra-cellular matrix,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>blood platelets, blood plasma</td>
</tr>
<tr>
<td>Function</td>
<td>Cofactor of coagulation cascade</td>
<td>Chaperone of factor VIII, platelet adhesion and aggregation, biogenesis of Weibel-Palade bodies</td>
</tr>
</tbody>
</table>

The biosynthesis and the properties of factor VIII and VWF have been reported in comprehensive reviews (17-19).

The cellular distribution of factor VIII protein in the liver is still controversial. Some studies demonstrated factor VIII protein in sinusoidal endothelial cells (15,16,20,21), while other studies suggested that also hepatocytes are involved in factor VIII synthesis (22,23). Probably these discrepancies are due to different detection techniques. Factor VIII was detected in sinusoidal endothelial cells by immunoperoxidase staining (20)(Figure 1), while in hepatocytes factor VIII was detected by electron microscopy (23) and functional assay (22). Recently, factor VIII mRNA was primarily found in sinusoidal endothelial cells (14,24), supporting the view that this cell type contributes to factor VIII synthesis.

Spleen perfusion and transplantation studies supported the notion that this organ plays a role in the regulation of plasma factor VIII levels, either as a site of synthesis or as a storage reservoir (25,26). From these studies it can be speculated that the spleen may account for more than 15% of the factor VIII synthesis in the human body (25). In addition, in asplenic patients it was demonstrated that the factor VIII activity response was significantly lower after 1-deamino-8-D-arginine vasopressin (DDAVP) administration compared to normal subjects (27). Also the lung has been suggested as an extra-hepatic source of factor VIII synthesis (28). A small increase of plasma factor VIII levels was demonstrated by grafting normal lungs into hemophilic dogs. In contrast, virtually no factor
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VIII protein was detected in renal tissue, despite the presence of factor VIII mRNA, although in one study (15) a faint, scattered immunohistochemical staining of factor VIII was observed in the glomerulus of the kidney. Furthermore, a successful renal transplantation in a patient with hemophilia A had no effect on plasma factor VIII levels (29). Similarly, kidney transplantation was ineffective in restoring plasma factor VIII levels in hemophilic dogs (10). Overall, these observations are consistent with the view that besides the liver, also spleen and lung and perhaps kidney may play a role in the synthesis of factor VIII. However, the cell types that are responsible for factor VIII synthesis, notably cells of extra-hepatic tissue, have not been identified.

![Image of liver immunostaining](image_url)

**Figure 1.** Immunostaining of human liver with monoclonal antibody CLB-Cag A (a monoclonal antibody directed against factor VIII) and counter-stained with Mayer's haematoxylin. Strong cytoplasmic staining was observed in endothelial cells (ec) lining the sinusoids (s). Hepatocytes (hp) displayed no factor VIII staining (20).

**VON WILLEBRAND FACTOR AND THE INTERACTION WITH FACTOR VIII**

Von Willebrand factor (VWF) plays a dual role in hemostasis: it is required for proper adhesion and aggregation of platelets at sites of vascular injury, and serves as a carrier protein for factor VIII in plasma (19,30). VWF forms a tight, non-covalent complex with factor VIII, thereby regulating its function and biological half-life (18). VWF protects factor VIII from phospholipid-dependent proteolysis, by factor IXa, factor Xa and activated protein C. In addition, VWF inhibits factor VIII interaction with low-density lipoprotein receptor-related protein (LRP), thereby suppressing cellular uptake and degradation of factor VIII (31)(see below).

The importance of the interaction between VWF and factor VIII is supported by the following observations. In patients with decreased plasma VWF levels, the plasma
concentration of factor VIII is decreased as well. Furthermore, infusion of factor VIII in patients with von Willebrand disease, type 3, a variant of von Willebrand disease characterized by a virtually complete VWF deficiency, the half-life of factor VIII is shorter than normally observed (32,33). In addition, VWF levels in severely affected hemophilia A patients, showed a positive correlation with the half-life of infused recombinant factor VIII (34). When VWF levels are increased by intranasal delivery of DDAVP, it was demonstrated that factor VIII half-life of infused recombinant factor VIII was prolonged in hemophilia A patients (35). Furthermore, it was demonstrated that individuals with blood group O and concomitant reduced VWF levels, the plasma factor VIII concentration in individuals with blood group O is also lower than in individuals with other blood groups. Similarly, factor VIII half-life of infused factor VIII in hemophilia A patients with blood group O is reduced (36). Together, these observations demonstrate a positive correlation between VWF- and factor VIII plasma levels.

**Figure 2. Dynamic equilibrium between bound and unbound factor VIII and VWF in blood.** Factor VIII and VWF form a tight, reversible complex. The input normally consists of endogenous synthesized factor VIII and VWF, but may also consist of therapeutically administrated factor VIII or VWF. This model gives accurate quantitative predictions of decreased and elevated steady-state concentrations of factor VIII in clinical conditions associated with decreased and elevated factor VIII levels (Adapted from (37)). (LRP, low-density lipoprotein receptor-related protein)

The kinetics of the interaction between factor VIII and VWF can be described by a mathematical model of law of mass action (37). This model assumes that in the circulation factor VIII and VWF in bound and unbound form, are in a dynamic equilibrium (Figure 2). This model confirms that in most physiological and pathophysiological conditions plasma factor VIII levels are governed by VWF. To date, no evidence has been presented that plasma VWF levels are influenced by factor VIII levels. For example, severe hemophilia A, due to complete absence of factor VIII protein, is not associated with VWF deficiency. Although VWF is a multivalent binding protein (17,19), only a fraction of potential factor VIII
binding sites that are available in VWF multimers, are occupied by factor VIII (38). The tight binding between factor VIII and VWF predicts that not only factor VIII biosynthesis is important in maintaining factor VIII plasma levels but also the synthesis of VWF.

**BIOSYNTHESIS OF VWF**

VWF is synthesized and stored in vascular endothelial cells and megakaryocytes (17,19). Before newly produced VWF leaves the endothelial cell, it undergoes a number of post-translational modifications, including polymerization and endoproteolytic cleavage of its propeptide. Together with its propeptide, VWF is either stored in intracellular secretory organelles, known as Weibel-Palade bodies, or secreted via the constitutive pathway. In both resting and activated endothelial cells VWF and propeptide are released in equimolar amounts (39). In healthy individuals plasma propeptide levels are about ten times lower, on a molar basis, compared to the plasma concentration of VWF (40,41). This is due to a four to five fold faster disappearance of propeptide from the circulation compared to mature VWF. So, the difference in steady state levels of each protein reflects the differences in their half-lives. This knowledge served as a basis to discriminate between acute and chronic vascular activation. It was demonstrated that elevated propeptide levels (together with elevated VWF concentrations) is a marker of acute, but transient endothelial cell activation. In patients with acute perturbation of the endothelium, like sepsis and thrombotic thrombocytopenic purpura (TTP), both VWF and propeptide are increased. In contrast, patients suffering from chronic diseases, like diabetes or venous thromboembolism only VWF is markedly elevated (41).

Not all vascular endothelial cells synthesize VWF to the same extent. VWF is mainly produced in lung tissue, as demonstrated by relatively high mRNA- and protein levels (42). Virtually no VWF synthesis is seen in the liver, a major site of factor VIII synthesis (vide supra). This raises the question: where do factor VIII and VWF form a complex, where do these proteins meet? To date there is no clear answer to this question. As VWF has been identified throughout the vascular endothelium whereas factor VIII synthesis is primarily confined to cells of non-vascular origin, it seems likely that complex formation occurs in the blood circulation.

**CATABOLISM OF FACTOR VIII**

Recently, it was demonstrated that LRP serves as a clearance receptor for factor VIII. LRP is a receptor with a broad ligand specificity (43,44). It mediates catabolism of a large number of proteins involved in blood coagulation and fibrinolysis, like IXa (45), Xa (46), plasminogen activators and their complexes with inhibitors (47,48). It is also involved in the transport of factor VIII from the cell-surface to the endosomal degradation pathway (31,49). LRP is expressed in a large number of tissues but most prominently by hepatocytes (50). Further, LRP is found on macrophages, smooth muscle cells, fibroblasts and monocytes.
In LRP-deficient mice factor VIII levels are about 2-fold higher than levels in control mice. In addition, in clearance experiments LRP-deficient mice display a 1.5-fold prolongation of factor VIII mean residence time (51). Similarly, administration of receptor-associated protein (RAP), a LRP ligand which efficiently inhibits binding and endocytosis of all known LRP ligands (52), triggers a sustained rise in factor VIII levels. Together, these observations strongly suggest that LRP contributes to the clearance of plasma factor VIII. The observation that injected radiolabelled factor VIII is predominantly found in the liver (53) is in agreement with the observation that LRP is mainly expressed on hepatocytes (50).

For many LRP ligands, LRP-mediated endocytosis is facilitated by cell-surface heparan sulfate proteoglycans (HSPGs). The contribution of HSPGs to factor VIII catabolism was demonstrated by studying factor VIII clearance in the presence of protamine sulfate, which prevents HSPGs from interaction with their ligands (54). In the latter study a prolongation of 1.6-fold was found for factor VIII half-life, indicating that HSPGs indeed contributes to factor VIII clearance (55). Current knowledge of the role of HSPGs and LRP in factor VIII catabolism suggests that HSPGs facilitates interaction of factor VIII/VWF complexes with LRP on the cell surface.

SCOPE OF THIS THESIS

The past decade has contributed considerably to our knowledge about the structure and function of factor VIII. However, as outlined above the cellular origin of factor VIII and its expression at the cellular level is still not well understood. Furthermore, it is not known if, and to what extent different cell types control the synthesis and assembly of factor VIII-VWF complexes. In this thesis we have studied the distribution of factor VIII and VWF gene expression in vivo on a quantitative basis (chapter 2). Particularly the liver, an important site of factor VIII synthesis, was studied in detail. Chapter 3 focuses on the phenomenon that in patients with severe liver disease factor VIII levels are markedly increased, while other hepatic coagulation factors are reduced. This is unexpected because in liver disease protein synthesis is, in general, severely affected. This prompted us to study factor VIII expression and expression of VWF and LRP, potential modulators of factor VIII plasma levels, in liver tissue of patients with hepatic disease.

Chapter 4 describes the role of extra-hepatic tissues in factor VIII synthesis in an anhepatic pig model. This model provides an unique opportunity to demonstrate extra-hepatic factor VIII synthesis in an unequivocal manner. Factor VIII gene expression and cellular distribution of factor VIII protein was studied in spleen, kidney and lung before and after hepatectomy.

Chapter 5 emphasizes the significance of the vascular endothelium as a reservoir of potentially active VWF. This study documents, as demonstrated by markedly increased VWF and propeptide levels, that in patients with *Plasmodium falciparum* malaria the vascular endothelium is substantially perturbed. This feature relates to disease activity. Expression of factor VIII in bone marrow cells is described in chapter 6. Factor VIII
deficient mice underwent a bone marrow transplantation receiving bone marrow from wildtype littermates. Subsequently, factor VIII expression in various tissues was studied, as well as the effect of transplantation on plasma factor VIII levels.

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