Factor VIII expression and regulation in health and disease

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Chapter 4

Elevated plasma factor VIII levels in anhepatic pigs

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ABSTRACT

Factor VIII is a plasma glycoprotein that plays an essential role in the hemostatic system. Deficiency of this protein causes a severe bleeding diathesis, known as hemophilia A. To date, most evidence supports that factor VIII is synthesized in the liver. Transplantation and organ perfusion studies suggest that the liver is but one site of a more widely dispersed factor VIII synthetic system, but the available data are conflicting. To further understand the significance of extra-hepatic factor VIII synthesis we studied the effect of total hepatectomy in pigs, a model of acute, irreversible liver failure, during a 24 hours follow up period on the expression of plasma and tissue factor VIII. Tissue factor VIII expression was detected before and 24 hours after hepatectomy, both at the mRNA level and immunohistochemically. In addition, the expression of plasma and tissue von Willebrand factor (VWF), the natural stabilizing carrier protein of factor VIII was measured. The results presented here show that total hepatectomy elicits a gradual and sustained 2-fold elevation of circulating factor VIII. Factor VIII messenger RNA levels in various organs before and after hepatectomy did not increase. The factor VIII half-life increased from 7.7 till 10.3 hours following hepatectomy. Also VWF levels were increased in anhepatic pigs. At the protein level prominent changes in cellular factor VIII distribution were seen in spleen and kidney. These observations indicate that both spleen and kidney may contribute to the regulation of plasma factor VIII levels, most likely at a post-translational level.

INTRODUCTION

Factor VIII is a plasma glycoprotein that plays an essential role in the hemostatic system. Deficiency of this protein causes a severe bleeding diathesis, known as hemophilia A. In human and other species the primary site of factor VIII synthesis is the liver. This is demonstrated by the observations that liver transplantation cures hemophilia A (1-3). Interestingly, also clinically significant extra-hepatic sites of factor VIII synthesis may exist, as evidenced by studies which show that liver transplantation from hemophilia A dogs to normal dogs did not result in hemophilia A (4). The observation that significant factor VIII mRNA levels have been detected in several organs, including kidney, spleen, lung and brain, is consistent with these findings (5,6). However, despite many organ transplantation and perfusion studies, the primary site or sites for extra-hepatic biosynthesis of factor VIII protein have not been definitively identified, though several observations suggest that the spleen could serve a significant role in regulating plasma factor VIII levels (4,7). Also other tissues have been proposed as sites of factor VIII synthesis, including lung, kidney and lymphatic tissue, but the available data are conflicting (8).

To further understand the significance of extra-hepatic factor VIII synthesis we studied the effect of total hepatectomy in pigs, a model of acute, irreversible liver failure, during a 24 hours follow up period on the expression of plasma and tissue factor VIII. Tissue sections of kidney, spleen and lung were obtained before and 24 hours after total
hepatectomy and analyzed for factor VIII mRNA expression levels and protein distribution. In addition, the expression of plasma and tissue von Willebrand factor (VWF), the natural stabilizing carrier protein of factor VIII, was measured. We have also studied the effect of hepatectomy on the clearance of factor VIII from the circulation. The results presented here suggest that both spleen and kidney contribute to the regulation of plasma factor VIII levels.

MATERIALS AND METHODS

The anhepatic pig model

Pigs (35-50 kg, n=6) were anaesthetized and total hepatectomy was performed as described previously (9,10). The infrahepatic caval vein and the portal vein were connected to the subdiaphragmatic caval vein using a 3-way vascular prosthesis. Postoperatively, all animals were kept under full anesthesia and mechanical ventilation until death. No blood transfusion, platelets, fresh frozen plasma or other substances containing coagulation factors were given during the experiment. This model has previously been shown to be a reliable, safe and effective clinical setting of acute liver failure, allowing the evaluation of extra-hepatic factor VIII synthesis for at least 24 hours (9,10). This study was approved by the Animal Ethical Committee of the University of Amsterdam.

Plasma preparation and assays

Blood was collected (4.5 mL in 0.5 mL 3.1% sodium citrate) at 4 hourly intervals until 24 hours after hepatectomy. Samples were centrifuged at 2500 g for 15 min at 4°C and plasma was stored at -70°C until tested.

Factor V and factor VII procoagulant activity was measured using one-stage clotting assays. VWF antigen was measured by ELISA using a rabbit anti-human VWF polyclonal antibody (DAKO, Glostrup, Denmark) for both VWF capture and detection (11). Factor VIII activity was measured by a chromogenic assay according to the manufacturer’s instructions (Chromogenix, Milano, Italy) and a one stage clotting assay by reconstitution of human factor VIII deficient plasma. Pooled porcine plasma of 3 healthy pigs was used as standard. In two pigs human factor VIII was administered one week before and one hour after hepatectomy to determine the half-life of factor VIII under these conditions (see below). In these animals at every time point the measured plasma concentration of porcine factor VIII was corrected for injected human factor VIII, assuming that the specific activity of porcine factor VIII in the setting of a human test system is 4 times the activity of human factor VIII.
Plasma clearance of human factor VIII

Factor VIII clearance studies were performed (n=2) using human immunoaffinity purified factor VIII concentrate (Aafact®, Sanquin Plasma Products, Amsterdam, The Netherlands). Two pigs received one dose (100 U/kg) of factor VIII one week before heptectomy and 1 hour after heptectomy. Blood was collected (4.5 mL in 0.5 ml 3.1% sodium citrate) at 4 hourly intervals for 24 hours. Human factor VIII antigen was measured by ELISA according to a previously published method (12). The monoclonal antibodies used to detect human factor VIII do not crossreact with porcine factor VIII. Clearance data obtained from the average of experiments with two pigs were fitted to a bi-exponential equation which was used to calculate the mean residence time.

Tissue preparation and RNA extraction

Biopsies (5 x 5 mm) of spleen, kidney and lung were obtained 1 hour before total heptectomy and 24 hours after total heptectomy. All biopsies were divided in two parts and immediately frozen in liquid nitrogen. One part was used for immunohistochemical analysis and the other one for total RNA isolation. Total RNA was isolated from the tissues by acid guanidine thiocyanate-phenol-chloroform extraction as previously described (13), and then treated with RNase-free DNase (Promega Corp., Madison, WI, USA) to remove contaminating genomic DNA. Extracted RNA was quantified by measuring absorption at 260 nm.

Table 1. Sequences of PCR primers used for the determination of tissue factor VIII mRNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Position</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' factor VIII</td>
<td>5'-CGGAAGTGCACCTCCATTTTT-3'</td>
<td>988</td>
<td>187</td>
</tr>
<tr>
<td>3' factor VIII</td>
<td>5'-ACTCTGACGTGAGCCTCCAT-3'</td>
<td>1174</td>
<td></td>
</tr>
<tr>
<td>5' HPRT</td>
<td>5'-CTTTTGCCTGACCTGGATT-3'</td>
<td>85</td>
<td>233</td>
</tr>
<tr>
<td>3' HPRT</td>
<td>5'-GCTTGACAAAGGAAGCAAG-3'</td>
<td>317</td>
<td></td>
</tr>
</tbody>
</table>

Real-time quantitative PCR

The concentration of various mRNAs (i.e. factor VIII, hypoxanthine phosphoribosyl-transferase (HPRT)) was determined using a quantitative real-time RT-PCR method with specific primer combinations (14,15). Total tissue RNA (1 μg) was reverse transcribed. Real-time PCR of the cDNA was performed using a LightCycler rapid thermal cycler (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's instructions. Serial diluted solutions of the cDNA mixture were amplified using specific primers (Table 1). Amplified PCR product was measured using SYBR green. Serial dilutions of a cDNA
mixture, prepared by reverse transcribing a tissue RNA sample before hepatectomy, were used to prepare a standard curve. An undiluted sample of the standard was set as 100%. RNA levels were corrected for input differences and reverse transcription efficiencies by dividing all measured values by the corresponding mRNA levels of HPRT, a housekeeping gene. In each total RNA extract specific mRNA's were determined at least 2 times independently.

Immunohistochemistry

Acetone-fixed frozen tissue sections were blocked for endogenous biotin by incubating the slides with an avidin/biotin blocking kit according to the manufacturer's instructions (Vector Laboratories, Inc, Burlingame, CA, USA). The slides were then incubated with normal goat serum and rinsed in PBS/0.05% Tween. Subsequently, the slides were incubated overnight at 4°C with polyclonal anti-factor VIII IgG (2.5 μg/mL). The antiserum was obtained by immunizing rabbits with albumin-free recombinant factor VIII (Kogenate®, Bayer, Leverkusen, Germany) according to standard procedures. The slides were then washed with PBS/0.05% Tween and sequentially treated with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 minutes. After washing the slides, they were incubated with streptavidin FITC (Vector Laboratories) labeled. Next, the sections were incubated with a monoclonal antibody against VWF (CLB-Rag 20) for one hour. This antibody was raised against human VWF (16) and crossreacts with the porcine protein. After washing the slides, the monoclonal antibody was detected by incubating the slides with horse anti-mouse labelled Texas Red (Vector Laboratories) for 30 minutes. Finally the slides were washed and mounted with Vectashield mounting medium (Vector Laboratories) and examined by confocal laser scanning microscopy.

Statistical analysis

Statistical significance was calculated using Student t test for all analysis. Unpaired or paired tests were performed as indicated in the figure legends.

RESULTS

Anhepatic model

No complications were encountered during the surgical procedure. Total operation time was 145 ± 11 min. Following total hepatectomy, all 6 pigs showed rapid stabilization of vital parameters and continued to have stable hemodynamical and ventilatory parameters for at least 16 hours. Platelet numbers were measured in one animal. Similar to a previous study (9), the platelet number gradually declined after hepatectomy from 211 to 156x10^9/L during the evaluation period.
Plasma levels of coagulation factors in pigs after total hepatectomy

As monitored with the chromogenic assay, plasma factor VIII concentration first decreased from 0.65 to 0.45 U/mL during surgery and complete disconnection of the liver from the circulation and subsequently gradually increased to 0.92 U/mL after 24 hours ($P = 0.01$, paired $t$-test, fig.1). A similar pattern was observed when the plasma factor VIII concentration was determined with the one-stage clotting assay (data not shown). VWF levels before and 1 hour after total hepatectomy did not differ (respectively, 0.85 U/mL and 0.84 U/mL). Thereafter, similar to factor VIII levels, VWF gradually increased about 2-fold in these pigs during the observation period ($P = 0.004$). In contrast to the gradual and progressive increase in factor VIII and VWF levels, factor V and factor VII decreased gradually after hepatectomy, demonstrating the liver dependency of these two coagulation factors. Factor V levels decreased with 50 percent after 10 hours, while factor VII decreased with 50 percent after 3 hours, consistent with a shorter circulatory life of factor VII compared to factor V.

![Graph](image)

Figure 1. Effect of total hepatectomy on plasma concentration of factor VIII (triangles), VWF (circles), factor V (squares) and factor VII (diamonds) in pigs. Pigs ($n = 6$) underwent total hepatectomy and at the time points indicated blood was collected and clotting factor were measured. At time zero the liver was completely disconnected from the circulation. Surgery started about 3 hours earlier. Data are depicted as mean ± SEM. From 2 animals samples were taken till 16 and 20 hours after hepatectomy. Plasma concentration of clotting factors is expressed as the percentage of normal porcine levels (arbitrarily 1U/mL). The average values given at time point minus 3 hours are baseline values.
Plasma factor VIII clearance after heptectomy

To determine the effect of heptectomy on the factor VIII half-life in the circulation we measured in two pigs clearance of human, plasma-derived factor VIII injected intravenously 7 days before and 1 hour after heptectomy. Factor VIII antigen levels were measured at various time points after infusion. The mean residence time was 7.7 hours before and 10.3 hours after heptectomy (fig. 2). In both pigs the clearance of factor VIII decreased after heptectomy. The half-life of human factor VIII following intravenous administration was within the expected range (17). No antibody formation against factor VIII could be detected after the second infusion with factor VIII concentrate, as measured by ELISA and inhibitor (Bethesda) assay (data not shown).

Figure 2. Plasma removal of human, plasma-derived factor VIII in pigs 7 days before and 1 hour after total heptectomy (n = 2). Levels of human factor VIII were determined by ELISA using monoclonal antibodies that do not cross-react with porcine factor VIII. Data of normal- (open circles) and anhepatic pigs (closed circles) represent mean ± range. The first sample collected after heptectomy was at 3 min after injection of factor VIII. The curves were analyzed using double-exponential fits.

Steady-state mRNA levels of factor VIII in kidney, lung and spleen biopsies.

To investigate whether any effect of heptectomy on factor VIII level was caused by a stimulation of its synthesis we measured factor VIII mRNA in lung, spleen and kidney in two pigs before and after total heptectomy. In the kidney there was no increase detected in factor VIII mRNA 24 hours after heptectomy (Table 2). Rather a decrease was observed (P = 0.02). Similarly, factor VIII mRNA of lung biopsies taken before and after heptectomy did not increase. Again, a decrease was detected, though not significant. In spleen factor VIII mRNA level could only be measured in one pig and was slightly lower compared to the factor VIII mRNA level before heptectomy.
Table 2. Effect of hepatectomy on factor VIII mRNA in kidney, lung and spleen

<table>
<thead>
<tr>
<th>Pig</th>
<th>#1</th>
<th>#2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>33</td>
<td>28</td>
<td>0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>48</td>
<td>21</td>
<td>n.s.</td>
</tr>
<tr>
<td>Spleen</td>
<td>58</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results are the mean. Data are given as % of changes in mRNA levels compared to the same tissue examined before hepatectomy, which is set at 100% (n = 2 animals). RNA analysis derived of the spleen from the second pig did not yield consistent results and was therefore not included. n.s.: not significant.

**Immunohistochemical evaluation of factor VIII and VWF expression before and 24 hours after hepatectomy.**

Immunohistochemical analysis of factor VIII antigen in kidney sections taken one hour before hepatectomy showed clear staining of the glomeruli (fig.3A,B). Factor VIII staining was also observed in stromal cells. No factor VIII was detected in tubular cells. The glomeruli also displayed intense staining for VWF, most likely endothelial cells. The staining co-localized with factor VIII, though not with the same intensity in all parts of the glomerulus. In addition, large vessels stained for VWF. Factor VIII antigen was not detected in these vessels. After total hepatectomy the cellular distribution of factor VIII in the kidney changed. Factor VIII staining was seen in clustered stromal cells (fig.3D). Interestingly, after hepatectomy factor VIII was also present in round vesicle-like structures in tubular cells (fig.3C). The cellular distribution of VWF was not affected by hepatectomy at the time-point examined. Pre-incubation of tissue specimen with plasma-derived human factor VIII abolished fluorescent staining, confirming the specificity of the antibody.

Immunostaining of lung tissue showed a few factor VIII-positive cells, seemingly randomly distributed throughout the tissue (fig.4B). The identity of these cells was not established. No changes were seen in cellular distribution of factor VIII after hepatectomy. This tissue displayed intense staining for VWF protein in endothelial cells of large vessels (fig.4). Differences in staining pattern before and after hepatectomy were not evident.

In the spleen factor VIII protein was detected in reticular cells in the red pulp (fig.5). Although this could not be accurately quantified, the number of factor VIII positive reticular cells seemed to be increased after hepatectomy. VWF was detected in endothelial cells of large vessels and in platelets (fig.5A,B). Clear clusters of platelets containing VWF were seen in the red pulp (fig.5C,D). No changes were seen in cellular VWF distribution after total hepatectomy.
Figure 3. Immunohistochemical analysis of factor VIII and VWF protein distribution in porcine kidney sections before and 24 hours after hepatectomy. Factor VIII and VWF antigen was localized in endothelial cells of the glomeruli (small arrowhead) before (A) and 24 hours after total hepatectomy (B). In addition, VWF was detected in endothelial cells of larger vessels both before and after hepatectomy (large arrow, A, B). Only after total hepatectomy factor VIII was seen in vacuole-like structures present in tubular cells (C, small arrow). More factor VIII-positive stromal cells for factor VIII were seen after total hepatectomy (large arrowhead, D). Scale bars in panels is 20 μm.
Factor VIII levels in anhepatic pigs

Figure 4. Immunohistochemical analysis of factor VIII and VWF protein distribution in porcine lung sections before and after hepatectomy. Endothelial cells of large vessels displayed intense VWF staining (arrow), both before (A) and 24 hours after (B) total hepatectomy. Cellular staining of factor VIII was relatively weak. Scale bar is 20 μm.

Figure 5. Effect of hepatectomy on cellular distribution of factor VIII and VWF in spleen sections. Factor VIII staining was observed in reticular cells (small arrow) both before (A, C) and after (B, D) total hepatectomy. Hepatectomy resulted in an increase of factor VIII positive cells. The presence of VWF antigen was seen in endothelial cells of large vessels (large arrow) (A, B). Clusters of platelets (arrow head) displaying intense staining for VWF were observed in the red pulp (C, D). No difference in cellular VWF distribution was observed after total hepatectomy. Scale bar is 20 μm.
DISCUSSION

In the present study we have examined factor VIII synthesis in anhepatic pigs, an experimental clinical setting that permits unambiguous demonstration of the involvement of extra-hepatic tissue in controlling plasma factor VIII levels. We made the surprising observation that removal of the liver, the main site of factor VIII synthesis, does not decrease plasma factor VIII levels. Rather, after an initial decrease following surgery we observed a sustained and significant 2-fold increase of circulating factor VIII after total hepatectomy during a 24 hours follow-up period (fig.1). Apparently, the lack of hepatic factor VIII synthesis is not only adequately compensated by the factor VIII production by other tissues or reduced clearance; hepatectomy even displays a stimulatory effect on factor VIII levels. As expected, hepatic coagulation factors, including factors V and VII, gradually decreased under these conditions. To our knowledge, this finding represents the first unequivocal demonstration of the release of extra-hepatic factor VIII into the circulation.

As to the mechanisms underlying the changes in plasma factor VIII concentrations we considered the possibility that the increase of factor VIII was due to the effect of hepatectomy on the clearance of factor VIII. Experimental animal studies have shown that the liver plays an important role in the catabolism of factor VIII (18,19). It is conceivable, therefore, that elimination of hepatic clearance receptors contributes to elevated plasma factor VIII levels. Indeed, hepatectomy increased the half-life of injected human factor VIII (fig.2), although the difference between the half-life of injected factor VIII before and after hepatectomy was small. Thus, the delayed clearance of factor VIII may explain the rise in plasma factor VIII levels, at least in part. From the pharmacokinetics of factor VIII it appears that 20 to 24 hours after hepatectomy about 90% of infused factor VIII is cleared (fig.2) (probably by the kidney). It seems most likely that also a substantial part of endogenous factor VIII present at the time of hepatectomy will be cleared after 24 hours. As at this time point factor VIII levels are clearly elevated, this indicates that the increase in factor VIII is due to extra-hepatic de novo synthesis (20-24) and/or release from factor VIII storage pools. The existence of factor VIII storage pools was previously suggested by organ transplantation studies (4,25-27). Also increased levels of plasma VWF (fig.1) (probably due to an acute phase response elicited by signals generated in response to surgical intervention), could delay the clearance of factor VIII and, consequently, could cause elevation of factor VIII. Again, the slightly increased factor VIII half-life is not sufficient to explain the increase of plasma factor VIII concentration.

We also considered the possibility that increased plasma factor VIII levels reflect elevated concentrations of activated factor VIII (factor VIIIa), rather than factor VIII. Factor VIIIa could be generated as a consequence as intravascular thrombin formation due to surgical trauma and liver failure (9). This would lead to spurious high factor VIII levels when measured with a one-stage clotting assay (28). However, no significant differences were observed between factor VIII levels as measured with the chromogenic (two-stage) assay and levels measured with a one-stage clotting assay (data not shown).
To shed further light on extra-hepatic factor VIII synthesis or storage we initiated immunohistochemical studies aimed at identifying the cellular origin of extra-hepatic factor VIII. This study revealed that notably the spleen and the kidney are potential candidates. These tissues display intense staining for factor VIII, notably following hepatectomy. Particularly in reticular cells of the spleen factor VIII was clearly detectable (fig.5). The number of factor VIII-positive cells seemed to increase after hepatectomy (fig.5D). This picture might reflect increased factor VIII synthesis. Also endothelial cells of the glomerulus expressed factor VIII (fig.3). Our finding that in the kidney of the mouse factor VIII mRNA is primarily detected in glomeruli is consistent with this observation (6). Enhanced factor VIII staining in the kidney after hepatectomy may also reflect increased uptake of factor VIII, e.g. mediated by megalin, an endocytotic factor VIII receptor (29). This would compensate for the loss of catabolism of factor VIII by the liver.

Surprisingly, double staining revealed colocalization of factor VIII and VWF in the glomerulus, suggesting endothelial cell-selective expression of factor VIII. It can not be ruled out that also this staining pattern reflects internalization of factor VIII, however. We also identified clusters of stromal cells that only expressed factor VIII. We were unable to further identify these cells. In addition, tubular epithelial cells displayed intense factor VIII staining, notably after hepatectomy. Taken together, these findings support that both spleen and kidney are involved in regulating plasma factor VIII. Previous immunohistochemical studies on the cellular distribution of factor VIII in spleen and kidney were less conclusive in this respect (30). It is possible that the different antibodies employed (monoclonal and polyclonal antibodies), or the different species examined in part explain the different results.

In attempts to identify the mechanisms underlying the increase of factor VIII, we also investigated whether factor VIII mRNA previously shown to be abundantly present in various organs, was increased after hepatectomy. This would indicate either a stimulation of the synthesis or an increased stability of the factor VIII mRNA. This appeared not to be the case. We did not see an increase in factor VIII mRNA levels in biopsies of different organs, including kidney, lung and spleen (table 2), rather a decrease in factor VIII mRNA levels. We can only speculate about the cause of the hepatectomy-mediated increase of factor VIII levels. A plausible hypothesis based on our results is that during the anhepatic state signals are released that play a role at the translational or post-translational level.

In conclusion, the present study demonstrates significant increase in plasma factor VIII levels after total hepatectomy, showing unambiguously the presence of extra-hepatic factor VIII synthesis or release from previously stored factor VIII.

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REFERENCE
