The role of blood coagulation in cancer, inflammation and embryonic development
Bruggemann, L.W.

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Role of coagulation FVIII in septic peritonitis assessed in hemophilic mice


Center for Experimental and Molecular Medicine, Academic Medical Center Amsterdam, The Netherlands

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Inhibition of blood coagulation appears to be an important therapeutic strategy to improve the outcome in sepsis. However, the beneficial effect of anticoagulant treatment in sepsis is solely based on experimental data using inhibitors of the extrinsic coagulant pathway. The role of the intrinsic pathway of coagulation in the pathogenesis of sepsis has not been explored yet. In the current study, we contribute to determine the role of factor (F)VIII, the key player of the intrinsic coagulant pathway, on host defense against peritonitis. To this end, hemizygous FVIII-deficient mice and their wild-type littermates were challenged with $1 \times 10^4$ bacteria in a septic peritonitis model. The intraperitoneal injection of *E. coli* led to growth and dissemination of bacteria and provoked an inflammatory response as evident from elevated cytokine levels, increased cell influx into tissues, liver necrosis and endothelialitis resulting in mortality. The FVIII-deficient genotype slightly reduced bacterial outgrowth but had no effect on markers of inflammation and/or survival. In addition, FVIII-deficient mice showed profound activation of coagulation, thereby improving the hemophilic phenotype of FVIII-deficient mice. In conclusion, FVIII deficiency slightly modifies host defense in septic peritonitis in mice, but does not influence the final outcome of peritonitis. Therefore, we question the importance of the intrinsic coagulant pathway during sepsis.
Role of coagulation FVIII in septic peritonitis assessed in hemophilic mice

Introduction

Sepsis, a severe illness caused by overwhelming infection of the bloodstream by toxin-producing bacteria, is the most common cause of death among hospitalized patients in non-coronary intensive care units [1]. A characteristic hallmark of sepsis is an excessive disturbance in the balance between coagulation factors and their inhibitors [2, 3]. This imbalance leads to activation of procoagulant pathways, down-regulation of anticoagulant pathways and inhibition of fibrinolysis leading to disseminated intravascular coagulation (DIC), multiple organ failure and ultimately death.

Several lines of evidence support an important role of exacerbated coagulation in the pathogenesis of sepsis. Experimental animal studies using inhibitors of the coagulation cascade, such as anti-tissue factor (TF) antibodies [4], tissue factor pathway inhibitor (TFPI) [5,6] or active-site inhibited FVIIa (DEGR-FVIIa) [7] prevented DIC and increased survival in baboons intravenously injected with Escherichia (E.) coli. Furthermore, recombinant human activated protein C (APC) reduced mortality associated with sepsis in the recombinant human activated protein C worldwide evaluation in severe sepsis (PROWESS) study [8]. Finally, DIC has been shown to be an independent predictor of mortality in patients with sepsis and septic shock [9]. It is therefore generally accepted that manipulation of the coagulation cascade might be an important therapeutic strategy to improve the outcome in sepsis.

The proposed beneficial effect of preventing or reducing blood coagulation during sepsis is primarily built on experimental data using inhibitors of the extrinsic coagulant pathway. Whether inhibition of the intrinsic pathway also plays an important protective role in the pathogenesis of sepsis remains speculative but considering the detrimental role of blood coagulation one could envision a similar protective effect in sepsis. Indeed, FVIII and IX levels are dramatically increased during sepsis [10, 11] but changes of plasma FVIII levels were not related with the outcome of septic patients [12]. Alternative arguments against an important role of the intrinsic pathway in sepsis might be that some case reports describe hemophilic patients (genetic or acquired) with overt DIC in the course of sepsis [13-15]. In addition, several reports suggest that activation of coagulation in systemic inflammation is driven by a continuous and generalized release of tissue factor (TF) thereby questioning the importance of the intrinsic coagulant pathway [16-19]. However, there are no experimental data and/or clinical studies concerning the role of the intrinsic coagulant pathway in generalized inflammatory conditions and thus one can only speculate about its importance in sepsis. In the current study, therefore, we sought to determine the role of FVIII (the key player in the intrinsic pathway) in murine septic peritonitis to prove or refute the hypothesis that FVIII deficiency plays a protective role in sepsis.

Materials and Methods

Animals

The generation of FVIII-deficient mice has been described in detail by dr. Bi et al. [20, 21].
Hemizygous FVIII-deficient mice and wild-type littermates were obtained by mating heterozygous FVIII-deficient females with hemizygous FVIII-deficient males. All mice were bred and maintained at the animal care facility at the Academic Medical Center according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. All mice were housed in the same temperature-controlled room with alternating 12h light/dark cycles. Male mice at an age of 8-10 weeks were used in the peritonitis model as described below.

**Induction of peritonitis**

Peritonitis was induced as described previously [22]. In brief, *E. coli* O18:K1 was cultured in Luria Bertani (LB; 10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract and 10 g/l NaCl) medium at 37 ºC, harvested at mid-log phase, and washed twice with sterile saline before injection. Mice were injected intraperitoneally (i.p.) with $10^4$ *E. coli* colony-forming units (CFU) in 200 μl sterile isotonic saline. The inoculum was plated on blood agar plates immediately after inoculation to determine viable counts. Mice were sacrificed 20 hours (n = 8 per group) after induction of peritonitis or studied for survival time (n = 8 per group).

**Collection of samples**

For measurements of bacterial outgrowth and host responses, animals were sacrificed at a time point shortly before mortality occurred (20 h). Mice were anaesthetized by inhalation of isoflurane (Forene, Abbott Laboratories Ltd, Maidenhead, UK) / O₂ (2% / 2l%) and a peritoneal lavage was performed with 5 ml sterile isotonic saline using an 18-gauge needle. The recovery of peritoneal lavage fluid was >90% in each experiment and did not differ between groups. After collection of peritoneal fluid, deeper anesthesia was induced by i.p. injection of 0.007 ml/g FFM mixture (Fentanyl (0.315 mg/ml), Fluanisone (10 mg/ml) (Janssen, Beersen, Belgium) and Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands)). Next, blood was drawn from the vena cava inferior with a sterile syringe, and transferred to tubes containing heparin (Becton-Dickinson, Franklin Lakes, NY, USA). Finally, tissues were removed for further analysis. Liver and kidney were used for assessment of bacterial load, histology and mRNA levels (kidney only), while lung was used for histology only.

**Enumeration of bacteria**

Liver and kidney sections were homogenized at 4°C in five volumes of sterile isotonic saline. Serial 10-fold dilutions in sterile isotonic saline were made from the homogenates, peritoneal lavage fluid and blood, and 50 μl were plated onto sheep-blood agar plates and incubated at 37°C. CFUs were counted after overnight culture.

**Cell counts**

Leukocyte counts in peritoneal lavage fluid were determined using a Coulter counter (Beckman Coulter, Fullerton, CA).
**Assays**

Thrombin-antithrombin complexes (TAT) were determined in plasma and peritoneal lavage fluid as a measurement of thrombin generation. TAT levels were measured with a mouse-specific, enzyme-linked immunosorbent assay (ELISA)-based method as described previously [23].

Cytokines were measured in plasma and in peritoneal lavage fluid by ELISAs according to the recommendations of the manufacturer (R&D Systems, Abingdon, U.K) (with detection limits in pg/ml), i.e. interleukin (IL)-6 (62.5), IL-10 (31.3), keratinocyte-derived chemokine (KC, also known as growth-regulated oncogene-α (GRO-α), a functional, murine analog of human IL-8) (24.7) and tumor necrosis factor (TNF)-α (62.5).

**Histological examination**

Samples from liver, lung and kidney were removed, fixed in 4% formalin, and embedded in paraffin for routine histology. Sections of 4 μm thickness were stained with hematoxylin and eosin. Slides were coded and analyzed without knowledge of the genotype of mice. Inflammation was characterized by the influx of leukocytes and by the presence of endothelialitis (i.e. sticking of leukocytes to the vessel wall). The degree of endothelialitis was rated 0 if absent, 1 if seen once or twice, 2 if seen in all vessels, or 3 if seen massively in most vessels. The degree of influx of leukocytes was rated 0 if absent, 1 if seen occasionally, 2 if seen regularly, 3 if omnipresent, or 4 if omnipresent and resulting in dense infiltrates in the intra-alveolar septa.

Coagulation activation was assessed using immunohistochemical staining for fibrin on paraffin slides after deparaffinization and rehydration using standard immunohistochemical procedures. Formaldehyde-induced cross-linking was disrupted by boiling the slides in 0.01 M citrate buffer (pH 6.0). Next, endogenous peroxidase activity was quenched using 0.3% H₂O₂ in methanol and non-specific binding was blocked with TENG-T (10 mM Tris, 5 mM ethylenediaminetetraacetic acid, 0.15 M NaCl, 0.25% gelatin, 0.05% (v/v) Tween 20, pH 8.0). As primary antibody biotinylated goat anti-mouse fibrinogen antibody (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) was used. ABC solution (DAKO, Glostrup, Denmark) was used as staining enzyme; 0.03% H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) in 0.05 M Tris pH 7.6 was used as substrate. To compare the degree of fibrin deposition between the individual mice, the number of positively stained vessels in 10 fields at a magnification of 20x was counted.

**RNA isolation**

Total RNA was isolated from snap frozen tissue using guanidine isothiocyanate (Trizol®; Gibco, Carlsbad, CA, USA) / chloroform extraction followed by precipitation with 2-propanol. After washing with 80% ethanol, the isolated RNA was dissolved in RNase free water and stored at -80°C until usage. cDNA was made by reverse transcription from total RNA using random hexamer primers (Life Technologies, Cergy Pointoise, France) and Superscript II reverse transcriptase (Life Technologies).
TF and TFPI mRNA analysis

TF and TFPI mRNA levels were measured by a quantitative real-time reverse transcription-polymerase chain reaction using Light Cycler technology (Roche Molecular Biochemicals, Alameda, CA, USA) with SYBR Green II detection using standard software for quantification as recommended by the manufacturer. Primer pairs used were 5'-GGAAAGGCTCAAGCAGGAAA-3' and 5'-CAGGAATTCCATTGCTCTGTG-3' for TF and 5'-TGTCTGAGGACTGATGAC-3' and 5'-GGTTCTCCTTTCCCATCAGT-3' for TFPI. Expression levels were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The following conditions were used: 40 cycles at 95°C for 15 seconds, 60°C for 5 seconds, and 72°C for 20 seconds.

Statistical analysis

Data were analyzed using the SPSS statistical package. Data are expressed as means ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann-Whitney U-test in case of histology data and using the Student’s t-test in case of the other data. Survival curves were compared by log-rank test. A value of p<0.05 was considered to represent a statistically significant difference.

Results

Coagulant phenotype of hemophilia A mice

FVIII-deficient mice have previously been described as mice with a bleeding tendency [20,21,24]. To demonstrate that the FVIII-deficient mice in our breeding colony are indeed hemophilic, we measured FVIII activity in citrated plasma. No FVIII activity could be detected in plasma of hemophilic mice, as compared to 100% FVIII activity in wild-type littermates (data not shown).

Figure 1. Bacterial outgrowth is influenced by FVIII deficiency. Bacterial outgrowth (expressed as mean ± SEM of CFU/ml) in peritoneal lavage fluid (PF), blood, liver and kidney 20h after infection. Wild-type (black bars) mice and FVIII deficient mice (open bars) were i.p. infected with 10^4 CFU E. coli at t=0h. *p<0.05 vs. wild-type controls.
To obtain insight into the role of coagulation in antibacterial defense during peritonitis, we compared the number of *E. coli* CFUs 20h after infection in peritoneal lavage fluid (the site of the infection), blood (to evaluate to what extent the infection became systemic), and in liver and kidney (to evaluate to which extent the infection was disseminated) of FVIII-deficient mice and their wild-type littermates. FVIII-deficient mice showed modestly reduced bacterial outgrowth in peritoneal lavage fluid and blood compared to wild-type mice (figure 1), whereas bacterial outgrowth in liver and kidney did not differ between the two groups (for peritoneal lavage fluid, $3.2 \cdot 10^{10} \pm 5.1 \cdot 10^9$ CFU/ml for wild-type mice vs. $4.8 \cdot 10^9 \pm 1.6 \cdot 10^9$ CFU/ml for FVIII deficient mice, for blood $1.3 \cdot 10^{10} \pm 6.2 \cdot 10^9$ CFU/ml vs. $3.9 \cdot 10^8 \pm 1.3 \cdot 10^8$ CFU/ml, for liver $2.1 \cdot 10^{10} \pm 8.8 \cdot 10^9$ CFU/ml vs. $1.9 \cdot 10^9 \pm 6.9 \cdot 10^9$ CFU/ml, and for kidney $2.4 \cdot 10^{10} \pm 5.3 \cdot 10^9$ vs $2.9 \cdot 10^{10} \pm 6.0 \cdot 10^9$ CFU/ml).

Coagulation activation during peritonitis is only partly dependent on the FVIII genotype

To establish the role of FVIII deficiency in the coagulant response to peritonitis, we measured TAT levels in plasma and peritoneal lavage fluid obtained 20h after infection (figure 2). TAT levels of wild-type mice are strongly elevated upon peritonitis as compared to the TAT level of 1 ng/ml in untreated wild-types. Septic FVIII-deficient mice showed significantly lower TAT levels in plasma and peritoneal lavage fluid than wild-type littermates, but these levels were about 4 times higher than in untreated wild-types. Histological analysis of both liver and lung slides confirmed coagulation activation upon induction of peritonitis. However, as shown in figure 3B, the number of vessels positively stained for fibrin did not differ between wild-type and FVIII deficient mice 20 hours after induction of peritonitis.
Chapter 7

FVIII deficiency does not influence the inflammatory response to peritonitis
To determine the role of FVIII in the inflammatory response to peritonitis, we evaluated cytokine release and cell influx into the peritoneal cavity. As given in table 1, the release of TNF-α into the peritoneal cavity 20 hours after induction of peritonitis was slightly lower in FVIII-deficient mice than in wild-type littermates, while IL-6, IL-10 and KC levels did not differ between the two groups. The influx of leukocytes into the peritoneal cavity did not differ between the two groups as well (1.6-10^6 ± 2.7-10^5 cells/mL for FVIII-deficient mice vs. 1.0-10^6 ± 2.1-10^5 cells/mL for wild-type mice).

Upon histopathologic examination, all mice displayed foci of liver necrosis associated with thrombus formation. The extent of liver necrosis did not differ between mice with different FVIII genotype (data not shown). Inflammation, as characterized by adhesion of leukocytes to the

**Figure 3.** FVIII deficiency does not influence peritonitis-induced changes in liver, lung and kidney histology. Graphical representation of the degree of inflammation (A) in liver, lung and kidney and of fibrin deposition (B) in liver and lung 20 hours after i.p. infection with 10^4 CFU E. coli in wild-type (black bars) and FVIII deficient mice (open bars). Fibrin deposition is shown as the number of positively stained vessels observed in 10 microscopy fields at a magnification of 25x. The degree of inflammation is shown according to the scoring system described in the materials and methods section. Differences between the two groups were not significant.

**Table 1.** Chemokine and cytokine levels in peritoneal lavage fluid 20h after administration of E. coli.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>wild-type</th>
<th>FVIII deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (ng/ml)</td>
<td>7.4 ± 1.7</td>
<td>6.6 ± 1.5</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>200 ± 41</td>
<td>150 ± 28</td>
</tr>
<tr>
<td>KC (ng/ml)</td>
<td>10.4 ± 1.1</td>
<td>8.1 ± 1.5</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>94 ± 18</td>
<td>&lt;62 *</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (n = 8 mice per group) at 20h after i.p. administration of E. coli (10^4 CFU). * P<0.05 vs. matching wild-types.

**FVIII deficiency does not influence the inflammatory response to peritonitis**
To determine the role of FVIII in the inflammatory response to peritonitis, we evaluated cytokine release and cell influx into the peritoneal cavity. As given in table 1, the release of TNF-α into the peritoneal cavity 20 hours after induction of peritonitis was slightly lower in FVIII-deficient mice than in wild-type littermates, while IL-6, IL-10 and KC levels did not differ between the two groups. The influx of leukocytes into the peritoneal cavity did not differ between the two groups as well (1.6-10^6 ± 2.7-10^5 cells/mL for FVIII-deficient mice vs. 1.0-10^6 ± 2.1-10^5 cells/mL for wild-type mice).

Upon histopathologic examination, all mice displayed foci of liver necrosis associated with thrombus formation. The extent of liver necrosis did not differ between mice with different FVIII genotype (data not shown). Inflammation, as characterized by adhesion of leukocytes to the
vessel wall (i.e. endothelialitis) and by influx of leukocytes, was evidently induced by peritonitis in all mice. The degree of inflammation in liver, lung and kidney did not differ between the different genotypes and their wild-type littermates (Figure 3A).

**Figure 4.** Septic peritonitis-induced mortality is not influenced by FVIII deficiency. Survival after i.p. infection with $10^4$ CFU *E. coli* in wild-type mice (closed squares; n=8) and FVIII deficient mice (open squares; n=8). Differences between the two groups were not statistically significant.

**Figure 5.** TF/TFPI mRNA balance is disturbed 20 hours upon *E. coli* administration. Mean ± SEM of TF (A) and TFPI (B) mRNA levels and of the ratio between TF and TFPI (C) levels in kidney. Wild-type (black bars) mice and FVIII deficient mice (open bars) were i.p. infected with $10^4$ CFU *E. coli* at t=0h. At none of the time points studied differences between the two groups were statistically significant.
**FVIII deficiency does not influence survival**

To investigate the role of FVIII deficiency on the outcome of peritonitis, we performed survival studies. No difference in mortality was seen between FVIII-deficient mice and their wild-type littermates (Figure 4).

**Disturbed balance between TF and TFPI mRNA suggests on-going initiation of coagulation during peritonitis**

To prove or refute the hypothesis that coagulation activation in FVIII deficient mice is dependent on continuous initiation of the coagulation cascade due to a disturbed balance between TF and TFPI levels, we measured TF and TFPI mRNA levels in kidney samples. As shown in Figure 5, administration of *E. coli* resulted in an increase in TF mRNA levels (9.4 ± 2.2 fold in wild-type mice 20 hours after administration of *E. coli*) and in decreased TFPI expression (1.6 ± 0.06 fold in wild-type mice 20 hours after administration of *E. coli*) thus increasing the TF/TFPI ratio 15.4 ± 3.9 fold. The increase in TF (7.8 ± 2.8 fold) and decrease in TFPI (1.5 ± 0.07 fold) mRNA levels in FVIII-deficient mice was similar to that in wild-type mice leading to a comparable increased TF/TFPI ratio (16.0 ± 7.7 fold) upon induction of septic peritonitis.

**Discussion**

The central aim of this study was to proof or refute the hypothesis that FVIII deficiency plays a protective role in sepsis. In theory, FVIII deficiency could limit the pathogenesis of sepsis by reducing the formation of activated coagulation factors, which play a detrimental role in inflammatory disease [4-7, 25-27]. However, the current study seems to refute this hypothesis and excludes an important role of FVIII in sepsis.

In the present study, we determined the effect of FVIII deficiency on host coagulant, inflammatory and anti-bacterial responses to intra-abdominal sepsis. Septic peritonitis was induced by i.p. injection of *E. coli* in hemizygous FVIII-deficient males and their wild-type littermates. Although FVIII deficiency did influence host defense to some extent, as evident from reduced bacterial outgrowth in blood and peritoneal lavage fluid, it did not protect against sepsis-induced mortality. In agreement, the degree of inflammation, as visualized by leukocyte influx into the peritoneal cavity, cytokine and chemokine release into the peritoneal lavage fluid and leukocyte influx in liver, lung and kidney was not dependent on the FVIII genotype.

As expected, induction of peritonitis led to coagulation activation in wild-type mice, as visualized by increased TAT levels in plasma and peritoneal lavage fluid and by enhanced fibrin deposition in liver and lung. Remarkably, FVIII-deficient mice showed profound coagulation activation as well, indicating that the intrinsic coagulant pathway is overshadowed by the extrinsic pathway and is thus of minor importance in sepsis. Indeed, several reports already suggested that coagulation activation in sepsis is the consequence of continuous initiation via the TF/FVIIa complex [16-19]. TF production not balanced by TFPI promoted poor prognosis
associated with DIC and organ dysfunction in patients with sepsis and/or trauma [18-19]. In fact, we show that upon induction of septic peritonitis the balance between TF and TFPI mRNA shifted towards the TF side. This shift was equally prominent in wild-type mice as in FVIII-deficient mice, suggesting that continuous activation of the extrinsic coagulant pathway is responsible for the observed coagulant activity in FVIII-deficient mice.

The overall effect of FVIII deficiency on septic peritonitis is rather limited, most likely because sepsis improves the hemophilic phenotype of FVIII-deficient mice. As bacterial outgrowth is clearly reduced in peritoneal lavage fluid and in blood of FVIII-deficient mice, we cannot rule out the possibility that FVIII (or actually the intrinsic coagulant pathway) slightly exacerbates the progress of sepsis. However, there are no clinical studies assessing the occurrence of sepsis in hemophilic patients. Some case reports describe hemophilic patients with septic disease [13-15, 28] but from these individual cases it is impossible to conclude whether hemophilia would protect against sepsis. The risk of acquiring infections from transfusion of blood and blood products [29] further complicates a sound evaluation of septic risk in hemophilia patients compared to the general population.

Several issues should be kept in mind when interpreting our data. First, in the present study we used a virulent, invasive *E. coli* strain that, after intraperitoneal injection, rapidly enters the circulation [23]. Therefore, our model results in early systemic infection, mimicking the condition of severe abdominal sepsis. Secondly, bacteria were administered in the absence of concurrent antibiotic therapy, and, therefore, our data do not provide insight into the effects of FVIII in septic mice treated with antibiotics. Thirdly, one should realize that our study does not include a careful time course analysis and one might argue that FVIII could play a role early on in peritonitis. However, such a role would be of minor importance, as it does not affect the final outcome of peritonitis. Finally, differences in bacterial outgrowth in blood and peritoneal lavage fluid, observed 20 hours after induction of peritonitis, are absent in distant organs and at this moment we do not have a valid explanation for these apparent discrepant results.

In conclusion, FVIII deficiency slightly modifies host-defense during septic peritonitis in mice, but does not influence the final outcome of peritonitis. Therefore, we question an important role for the intrinsic coagulant pathway during septic peritonitis and provide further evidence for a deleterious role of continuous activation of the extrinsic coagulant pathway in sepsis.

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