Chapter 6

Hemophilia and thrombophilia shape host defense during septic peritonitis in mice without affecting survival

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Submitted
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Abstract

The role of the coagulation system during sepsis has been established in experimental models using intravenous challenges with live bacteria or bacterial products, which show favorable effects of coagulation inhibition on both clinical parameters and on survival. To determine the effect on host-defense against peritonitis of an inherited predisposition to bleeding or thrombosis, FVIII deficient mice, FVLeiden mice, and their respective wildtype littermates received an intraperitoneal injection of live *Escherichia coli*. Peritonitis was associated with activation of coagulation, as reflected by elevated levels of thrombin-antithrombin (TAT) complexes in peritoneal fluid, and by increased fibrin deposition in liver and lungs. FVIII deficiency slightly reduced TAT levels, bacterial outgrowth and disseminated inflammation, but did not affect survival. Upon induction of peritonitis, FVLeiden mice showed increased TAT levels and an impaired host-defense as evident from increased bacterial outgrowth. However, like FVIII deficiency, FVLeiden did not influence sepsis-induced mortality. These data demonstrate that inherited tendencies to bleeding or thrombosis modify host-defense during septic peritonitis, but are of no importance for the final outcome of sepsis.

Introduction

*In vitro* and *in vivo* data have provided abundant evidence for a cross-talk between coagulation and inflammation. Inflammatory mediators influence the coagulation cascade through upregulation of coagulation factors like tissue factor (TF), thrombin and fibrin, and via inhibition of the fibrinolytic system. Endotoxemia studies in human volunteers and/or chimpanzees have demonstrated that endotoxin-induced activation of the extrinsic coagulation system appears to be mediated by pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6. TNF-α administration to healthy volunteers elicited rapid activation of coagulation which was similar to that evoked by endotoxin. Moreover, intervention with monoclonal IL-6 antibodies or IL-1 receptor antagonists attenuated endotoxin-induced coagulation. Evidence for the role of coagulation factors in inflammation is also derived from sepsis and/or endotoxemia models. For instance, inhibition of the TF/FVIIa complex with anti-TF antibodies, tissue factor pathway inhibitor (TFPI), or active-site inhibited FVIIa (DEGR-FVIIa) prevented disseminated intravascular coagulation (DIC) and increased survival in baboons intravenously injected with *Escherichia (E.) coli*. In addition, it was recently shown in a phase III study that adjuvant treatment with activated protein C (APC) decreased mortality in patients with severe sepsis. However, it is not yet clear whether the beneficial effects of APC administration stem from its anticoagulant function or from other properties of APC. For instance, APC has been suggested to have anti-inflammatory as well as anti-apoptotic effects on endothelial cells.
Altered coagulant phenotypes in murine peritonitis

Not only APC is considered to have functions outside of the coagulation cascade. Thrombin, for instance, has pro-inflammatory capacities, since it functioned as a chemotactic factor for neutrophils and was associated with an increase in adhesion molecule expression. In addition, TF may have important biological functions independent from its well-established role in blood coagulation. TF may be important for processes like embryogenesis, tumor progression and neovascularization, and chemotaxis. However, with regard to sepsis, TF’s proposed role in cell adhesion might be more relevant. During inflammation, mononuclear phagocytes cross the lymphatic endothelium in the basal-to-apical direction (i.e. reverse migration), a process dependent on the expression of TF on the surfaces of these cells. Taken together, these studies suggest that coagulation factors play an important role in sepsis.

In this study, we examined the consequences of an inherited predisposition to either bleeding or thrombosis on host-defense. We hypothesized that the prothrombotic phenotype of FVLeiden (FVL) mice would be disadvantageous in septic peritonitis, whereas hemophilic mice would be relatively protected against peritonitis.

Materials And Methods

Animals

The generation of FVIII deficient mice (exon 16 disrupted) was described in detail by Dr Bi and co-workers. The FVIII deficient mice (FVIII def) we used are direct descendents from a F1-cross, and thus genetically 50% C57Bl/6 and 50% 129Sv. FVL mice carrying a R504Q single amino acid mutation were described previously by Dr Cui and co-workers and are on a mixed genetic background of C57Bl/6 and 129Sv. To stay clear of an influence of differences in genetic background on the interpretation of the results, wildtype littermates were used as controls. 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. In brief, E.coli O18:K1 was cultured in Luria Bertani medium (LB; Difco, Detroit, MI) at 37°C, harvested at mid-log phase, and washed twice with sterile saline before injection to clear the bacteria of medium. Mice were injected intraperitoneally (i.p) with 10⁷ 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. Experiments with FVIII and FV mice were performed independently on
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separate days. Mice were either sacrificed 20 hours after induction of peritonitis or studied for survival time.

Collection of samples

For measurements of bacterial outgrowth and host responses, animals were sacrificed at a time point shortly before mice started dying (20h). At the time of sacrifice, mice were anaesthetized by inhalation of isoflurane (Abbott Laboratories Ltd., Kent, UK) / O₂ (2% / 21%). A peritoneal lavage was then performed with 5 mL sterile isotonic saline using an 18-gauge needle, and peritoneal lavage fluid was collected in sterile tubes (Plastipack; Becton-Dickinson, Mountain View, CA). 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 0.07 mL/10 g FFM mixture (Fentanyl (0.315 mg/mL)-Fluanisone (10 mg/mL) (Janssen, Beersen, Belgium), Midazolam (5 mg/mL) (Roche, Mijdrecht, The Netherlands)). Next, blood was drawn out of the vena cava inferior with a sterile syringe, and transferred to tubes containing heparin (Becton-Dickinson).

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, ELISA-based method as described previously. Cytokines were measured by ELISAs according to the recommendations of the manufacturer, i.e. interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α (all R&D Systems, Abingdon, U.K).

Histological examination

Directly after sacrificing the mice, samples from the liver and the lung 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 scored without knowledge of the type 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 an immunohistochemical staining for fibrin. Fibrin staining was performed on paraffin slides after deparaffinization and rehydration using standard immunohistochemical procedures. Formaldehyde induced cross-linking was disrupted by boiling the slides in 0.1 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 EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (v/v) Tween 20, pH 8.0). As primary antibody biotinylated goat anti-mouse fibrinogen Ab (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) was used. ABC solution (DAKO) was used as staining enzyme. 0.03% H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) 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 25 x was counted.

**Enumeration of bacteria**

The number of *E. coli* CFU was determined in peritoneal fluid, blood and liver homogenates. For this, livers were harvested and homogenized at 4°C in five volumes of sterile isotonic saline. Serial 10-fold dilutions in sterile isotonic saline were made from these homogenates, peritoneal lavage fluid and blood, and 50-μl volumes were plated onto sheep-blood agar plates and incubated at 37°C and 5% CO₂. CFU were counted after o/n culture.

**Cell counts**

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

**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 and FVL mice is altered**

FVIII-def mice have previously been described as mice with a bleeding tendency.²¹,²²,²₆ To demonstrate that the FVIII-def mice in our breeding colony are indeed hemophilic, we measured FVIII activity in citrated plasma. As is shown in figure 1A, no FVIII activity could be detected in plasma of hemophilic mice, as compared to 100% FVIII activity in wildtype littermates. FVL mice are known to have a strong prothrombotic phenotype characterized by spontaneous fibrin deposition in several tissues.²³ To demonstrate that the FVL mice in our breeding colony indeed show a prothrombotic phenotype, we measured plasma TAT levels. As is shown in figure 1B, TAT levels in plasma of untreated FVL mice are significantly increased as compared to wildtype littermates (8.3 ± 1.2 ng/mL vs. 1.1 ± 0.1 ng/mL).
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Figure 1: Hemophilic mice and FVL mice show an altered coagulant phenotype.
A. Factor VIII deficient mice show no factor VIII activity. Mean ± SEM of factor VIII activity in citrated plasma of 8 wildtype and 8 hemophilic mice. B. TAT levels in plasma of FVL mice are elevated as compared to wildtype mice. *P < 0.05 vs. wildtype mice.

Bacterial outgrowth is influenced by an altered coagulant phenotype

To obtain insight into the role of coagulation in antibacterial defense during peritonitis, we compared the number of E. coli CFU 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 (to evaluate to which extent the infection was disseminated) of respectively FVL mice and their wildtype littermates and of FVIII def mice and their wildtype littermates (Figure 2). FVIII def mice showed lower bacterial outgrowth at all three body sites (in PF 3.2*10^{10} ± 5.1*10^9 CFU/mL for wt mice vs. 4.8*10^9 ± 1.6*10^9 CFU/mL for FVIII def mice, in blood 1.3*10^{10} ± 6.2*10^9 CFU/mL vs. 3.9*10^8 ± 1.3*10^8 CFU/mL, and in liver 2.1*10^{10} ± 8.8*10^9 CFU/mL vs. 1.9*10^{10} ± 6.9*10^9 CFU/mL), while FVL mice showed an increased bacterial outgrowth as compared to wildtype

Figure 2: Bacterial outgrowth is influenced by an altered coagulant phenotype. Bacterial outgrowth (expressed as mean ± SEM of CFU/mL) in peritoneal lavage fluid (left panel), blood (middle panel) and liver (right panel) 20h after infection. FVIII wildtype (black bars), factor VIII deficient (open bars), FV wt (spotted bars), and FVL mice (dashed bars) were i.p. infected with 10^8 CFU E. coli at t=0h. *P < 0.05 vs. wildtype controls.
littermates (in PF $8.2 \times 10^7 \pm 3.6 \times 10^7$ CFU/mL for wt mice vs. $2.3 \times 10^9 \pm 2.0 \times 10^9$ CFU/mL for FVL mice, in blood $2.5 \times 10^6 \pm 1.5 \times 10^5$ CFU/mL vs. $4.6 \times 10^6 \pm 2.8 \times 10^5$ CFU/mL, and in liver $4.8 \times 10^5 \pm 2.3 \times 10^5$ CFU/mL vs. $4.1 \times 10^6 \pm 1.7 \times 10^6$ CFU/mL).

Coagulation activation during peritonitis in mice with an altered coagulant phenotype

To establish the role of hemophilia A and FVL in the coagulant response to peritonitis, we measured TAT levels in plasma and peritoneal lavage fluid obtained 20h after infection (Figure 3). TAT levels of wildtype mice are strongly elevated upon peritonitis as compared to the TAT level of 1 ng/mL in untreated wildtypes. FVIII def mice showed significantly lower TAT levels in plasma and peritoneal lavage fluid than wildtype littermates, but to our surprise, these levels were about 4 times higher than in untreated wildtypes. Peritonitis induced TAT levels of FVL mice did not differ from the levels of their wildtype littermates. Strikingly, TAT levels of FVIII wt and FV wt mice are rather different ($4.6 \pm 0.4$ ng/mL vs. $12 \pm 1.8$ ng/mL for peritoneal fluid and $8.2 \pm 2.0$ ng/mL vs. $2.2 \pm 0.8$ ng/mL for blood, respectively).

We also analyzed tissue sections for histological signs of coagulation activation upon induction of peritonitis. As is shown in figure 4K, the number of vessels positively stained for fibrin did not differ between wildtype and FVIII def mice or between FVL and wildtype mice 20 hours after induction of peritonitis.

An altered coagulant phenotype influences the inflammatory response to peritonitis

Coagulation and inflammation are supposed to be strongly linked. To determine whether the capacity to generate thrombin influences the inflammatory response to E. coli peritonitis several parameters were evaluated. As is shown in table I, the release of IL-6, TNF-α and IL-10 into the peritoneal lavage fluid 20 hours after induction of peritonitis was tended to be lower in FVIII def mice than in wildtype littermates. The cytokine levels in peritoneal lavage fluid of the prothrombotic FVL mice were tended to be lower than in wildtype littermates.
Table I. Chemokine and cytokine levels in peritoneal lavage fluid

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>FVIII wt</th>
<th>FVIII def.</th>
<th>FV wt</th>
<th>FVL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (ng/mL)</td>
<td>7.4 ± 1.7</td>
<td>6.6 ± 1.5</td>
<td>2.2 ± 1.3</td>
<td>1.0 ± 0.9</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>200 ± 41</td>
<td>150 ± 28</td>
<td>99 ± 59</td>
<td>&lt; 62 *</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>94 ± 18</td>
<td>&lt; 62 *</td>
<td>126 ± 62</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 (10⁴ CFU). * P<0.05 vs. matching wildtypes.

Upon histopathological examination, all mice displayed foci of liver necrosis associated with thrombus formation (Fig 4A-D). The extent of liver necrosis did not differ between the genotypes (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 and lung did not differ between the different genotypes and their wildtype littermates (Fig 4A-J).

An altered coagulant phenotype does not influence survival

To investigate the role of either a prothrombotic or a hemophilic phenotype in the outcome of peritonitis, we performed survival studies. No difference was seen in mortality between FVL mice and their wildtype littermates or between the FVIII def mice and their wildtype littermates (Figure 5). Please note, however, that survival between FVIII wt en FV wt mice was very different.

Figure 5: Survival is not influenced by an altered coagulant phenotype. Survival after i.p. infection with 10⁴ CFU E. coli in FVIII wt (closed squares), FVIII def. (open squares), FV wt (closed circles), and FVL mice (open circles). Differences between FVIII def and FVIII wt and between FV wt and FVL were not significant. Please note that experiments with FVIII and FV mice were performed as two separate experiments.

Discussion

The role of the coagulation system during sepsis has been firmly established in experimental models using intravenous challenges of live bacteria or bacterial products such as endotoxin, showing beneficial effects of coagulation inhibition on both clinical parameters, such as bacterial outgrowth and inflammatory state, and on survival. For instance, inhibition of the TF/FVIIa pathways using
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active-site inhibited FVIIa, TFPI, or antibodies against TF not only prevented disseminated intravascular coagulation (DIC) but also lethality in baboons infused with high doses of *E. coli.*

In the present study we explored the consequences of genetically altered coagulation profiles in the host coagulant, inflammatory and anti-bacterial responses to intra-abdominal sepsis induced by i.p. injection of *E. coli,* by comparing FVIII deficient and FVL mice with their wildtype littermates. We demonstrate that in a murine model of septic peritonitis, an altered coagulant phenotype influences host-defense, as was shown by reduced bacterial outgrowth in FVIII deficient mice and by increased bacterial outgrowth in FVL mice when compared to wildtype littermates. In addition, inflammation and coagulation

Figure 4: Histology of liver and lung is not influenced by an altered coagulant phenotype. Representative H&E staining of liver (A-D) and lung (E-H), graphical representation of the degree of inflammation in liver (I) and lung (J), and graphical representation of fibrin deposition in liver and lung (K) 20 hours after i.p. infection with 10^4 CFU *E. coli* in FVIII wildtype (A, E, black bars), factor VIII deficient (B, F, open bars), FV wt (C, G spotted bars), and FVL mice (D, H, dashed bars) Fibrin deposition is shown as the number of positively stained vessels observed in 10 microscopy fields at a magnification of 20x. The degree of inflammation is shown according to the scoring system described above.
activation in the peritoneal cavity of FVIII deficient mice was decreased. Although coagulation activation in the peritoneal cavity of FVL mice did not differ from wildtype littermates upon septic peritonitis, cytokine levels in the peritoneal lavage fluid tended to be decreased. Nevertheless, inflammation and fibrin deposition in liver and lung and the outcome of septic peritonitis (i.e. survival) was not influenced by a genetically altered coagulant phenotype. These findings suggest that genetic modifications causing either hemophilia or thrombophilia modify host-defense during septic peritonitis in some extent, but are of no importance for the final outcome of sepsis.

A more detailed look at our data reveals several remarkable observations. First, the degree of coagulation activation we observed in FVIII deficient mice was unexpectedly strong. In the current view of the blood coagulation system, FVIII is of major importance during the propagation phase of coagulation, which predicts decreased TAT levels and fibrin deposition in FVIII deficiency. The presence of significant TAT levels in blood and peritoneal lavage fluid of FVIII deficient mice challenged with E. coli questions the importance of FVIII in the propagation phase. However, one might argue that the observed TAT levels are the consequence of continuing initiation of coagulation via the TF/VIIa complex. This hypothesis is supported by the fact that under normal circumstances tissue factor pathways inhibitor (TFPI) immediately inhibits the TF/VIIa complex, but during sepsis the balance between TFPI and TF is disturbed, which might result in a hypercoagulable state primarily driven by TF/VIIa, with relatively little support from FVIII dependent processes.

A second remarkable observation is that upon induction of septic peritonitis TAT levels of FVL mice are comparable to the levels observed in wildtype mice and even lower than in unchallenged FVL mice. Whether increased consumption of coagulation factors resulting in a rapid decline in TAT levels in FVL mice explains this observation remains untested. However, the fact that during sepsis disseminated intravascular coagulation (DIC) frequently occurs, which results in consumption of coagulation factors leading to a bleeding tendency, supports this hypothesis.

Contrary to our prior hypothesis that FVL mice would react more to septic peritonitis than wildtype littermates, FVL tended to show lower cytokine levels in the peritoneal lavage fluid than their wildtype littermates. As patients with sepsis benefit from administration of APC, probably due to its direct anti-inflammatory properties, it might well be that increased APC levels in FVL mice (upon induction of peritonitis) are responsible for the diminished cytokine production. Indeed, recently Kerlin et al. reported elevated levels of APC in FVL mice upon endotoxin administration.

Comparison of FVIII wildtypes and FV wildtypes reveals several discrepancies. Upon induction of peritonitis FVIII wt mice have 2.6 times higher TAT levels in blood than FV wt mice, while TAT levels in peritoneal lavage fluid are 3.7 times lower. Bacterial outgrowth in PF, blood and liver of FVIII wildtypes is 400-40000 times higher than in FV wt mice, while survival of FVIII wt mice is remarkably diminished as compared to FV wt mice. These discrepancies between different wildtype mice once more stress that the genetic background is a substantial factor in animal models of sepsis. This point gets even more relevance when realizing
that FVIII mice are on a 50% C57Bl/6 / 50% Sv129 background, while FVL mice are not viable on this background.\textsuperscript{23}

In summary, we investigated whether an altered coagulant genotype influences the outcome of septic peritonitis in mice. Our results clearly show that genetic predispositions to either a severe hemophilic or a profound prothrombotic phenotype modify host-defense to some extent, but are of minor importance for the final outcome of sepsis.

Acknowledgements

FVIII deficient mice are a generous gift of Dr. M. Neerman-Arbez. FVL mice are a generous gift from Dr. R.J. Westrick. We would like to thank Angelique Groot, Ingvild Kop and Joost Daalhuijsen for their expert technical assistance.

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2000;11:591-598.


