The role of blood coagulation in cancer, inflammation and embryonic development
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Role of the FV Leiden mutation in septic peritonitis assessed in FV Leiden transgenic mice


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Abstract

Objective: The factor V Leiden (FVL) mutation (Arg506Glu) results in the production of a FV protein that when activated, is relatively resistant to inactivation by activated protein C and thereby leads to predisposition to thrombosis. The rather high prevalence of the FVL mutation in the general population prompted speculation about a potential survival benefit for individuals carrying the FVL allele. Indeed, both clinical and experimental animal data suggest that a heterozygous FVL genotype might protect against the lethal consequences of sepsis. We sought to confirm the survival advantage of heterozygous FVL mice in septic disease.

Design: Controlled animal experiment.

Setting: Academic Research Laboratory.

Subjects: Wildtype, heterozygous and homozygous FVL mice subjected to $1\times10^4$ live bacteria as model for septic peritonitis.

Interventions: none

Measurements and Main Results: The intraperitoneal injection of *E. coli* led to growth and dissemination of bacteria and provoked an inflammatory response as evident from elevated cytokine levels (interleukins-6, interleukins-10 and tumor necrosis factor-α), induced thrombin-antithrombin complex levels, increased granulocyte influx into the peritoneal cavity, liver necrosis and adhesion of leukocytes to the vessel wall, resulting in approximately 50% mortality after 72 hours. The FVL genotype had no significant effect on bacterial outgrowth, markers of inflammation (i.e. tumor necrosis factor-α levels of 152 [96.2-200], 152 [99.7-1745] and 110 [99.7-177] pg/ml in peritoneal lavage fluid at t=20 hours for wildtype, heterozygous and homozygous FVL mice respectively), thrombin generation (i.e. thrombin-antithrombin complex levels of 19.9 [9.31-37.4], 10.4 [6.55-15.8] and 12.6 [8.24-29.0] ng/ml in peritoneal lavage fluid at t=6 hours for wildtype, heterozygous and homozygous FVL mice respectively) and survival (50%, 36% and 50% for wild-type, heterozygous and homozygous FVL mice respectively).

Conclusions: The FVL allele has no beneficial effect in mouse septic peritonitis and the general protective effect of FVL in sepsis needs further investigation.
Introduction

Factor V Leiden (FVL), an arginine to glutamine missense mutation in the factor (F)V gene at position 506 [1], is a major risk factor for venous thromboembolism [2]. The amino acid substitution in the activated protein C (APC) cleavage site of FV leads to decreased APC-mediated inactivation of FV and decreased FV cofactor activity for FVIIIa inactivation [3]. Despite an up to 80-fold increased risk for thrombosis in homozygous individuals [4], the FVL allele has a prevalence of about 5% in the Caucasian population [5]. This relative high prevalence provoked speculation about an associated survival advantage with the FVL mutation exerting positive selective pressure [6, 7]. As potential explanation for such a survival benefit, it has been proposed that female FVL carrier status might benefit from reduced blood loss during childbirth or that heterozygous carrier status might improve embryo implantation. FVL animal studies did however not provide evidence for the latter notion [8].

The interaction between blood coagulation and inflammation as part of the innate host defense mechanism has been firmly established [9]. In particular in the field of infectious disease the importance of this interaction has been recognized, since major complications of sepsis (i.e. disseminated intravascular coagulation (DIC) and multiple organ failure) are strongly linked with excessive disturbances in the balance between coagulation factors and their inhibitors [10, 11]. As activated coagulation factors induce inflammation whereas inflammation further enhances blood coagulation [12, 13], uncontrolled activation of either process might lead to a vicious circle ultimately leading to vascular injury, organ failure and death [14]. Indeed, anticoagulant treatment to dampen this vicious circle has particularly been successful with APC, which reduces mortality associated with sepsis [15-17].

Genetic analysis of FVL carriership in the PROWESS patient population suggests that heterozygous FVL carriers might be protected against sepsis [18]; a notion later confirmed combining the PROWESS and ENHANCE studies [19]. However, FVL has been associated with increased morbidity in childhood meningococcal disease [20]. In a recent population-based study [21], it has been suggested that the FVL mutation might be associated with infectious disease susceptibility and an increased risk of mortality from sepsis. Thus, the effect of the FVL mutation on infectious disease susceptibility and outcome is controversial. To add to the controversy, heterozygous FVL mice are clearly protected against endotoxin-induced sepsis but surprisingly homozygous FVL mice show a similar survival rate as wildtype non FVL carriers [8]. Due to the lack of sufficient homozygous FVL patients in both the PROWESS and ENHANCE studies, clinical data on the susceptibility of FVL homozygotes to sepsis are not available.

In the current study, we sought to confirm the survival advantage of heterozygous FVL mice in septic disease by challenging wild-type, heterozygous and homozygous FVL mice with live bacteria in a well-established Escherichia (E). coli septic peritonitis model.
Materials and Methods

Animals

FVL mice carrying a R504Q single amino acid mutation have been described previously by Dr Cui and co-workers [22]. R504Q mice were backcrossed to C57BL/6J mice for 4 generations (N4), and N4 R504Q heterozygous mice were intercrossed to produce homozygous, heterozygous and wildtype offspring. 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 subsequently.

Induction of peritonitis

Sepsis was induced using the well-established model of E.coli-induced peritonitis as described previously [23]. In brief, E. coli O18:K1 was cultured in Luria Bertani medium (LB; Difco, Detroit, MI) 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 either sacrificed 6 (9 mice per genotype) or 20 (11 mice per genotype) hours after induction of peritonitis or studied for survival time (10 mice for wildtype and homozygous FVL mice and n=11 for heterozygous FVL mice).

Collection of samples

For measurements of bacterial outgrowth and host responses, animals were killed at an early time point (6 hours) and at a time point directly before mortality occurred (20 hours). At the time of kill, mice were anaesthetized by inhalation of isoflurane (Forene, Abbott Laboratories Ltd., Kent, UK) / O₂ (2% / 21%). A peritoneal lavage was then performed with 5 ml of 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 0.007 ml/g FFM mixture, which consisted of 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 out of the vena cava inferior with a sterile syringe, and transferred to tubes containing heparin (Becton-Dickinson). Finally, tissues were removed for further analysis. Liver, lung, and kidney were used for assessment of bacterial load and histology, while spleen was used for bacterial load only.

Enumeration of bacteria

The number of E. coli CFUs was determined in peritoneal fluid, blood, liver, kidney and spleen
homogenates. For this, livers, lungs, kidneys and spleens were harvested and homogenized at 4°C in five volumes of sterile isotonic saline. Serial ten-fold dilutions in sterile isotonic saline were made from the homogenates, peritoneal lavage fluid and blood, and 50-μl volumes were plated onto sheep-blood agar plates and incubated at 37°C. CFUs were counted after overnight (16 hours) culture.

**Cell counts**

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

**Assays**

Protein levels in peritoneal lavage fluid was determined by murine-specific enzyme-linked immunosorbent assays (ELISA) according to the recommendations of the manufacturer (R&D Systems, Abingdon, U.K), with detection limits between 62.5 and 5000 pg/ml and coefficients of variance between 4 and 10%. As we only obtained small amounts of plasma, protein levels of interleukin (IL)-6, IL-10, IL-12p70, TNFα, monocyte chemotactic protein-1 (MCP-1) and interferon-γ (IFN-γ) were determined in plasma by BD™ Cytometric Bead Array according to manufacturer's protocol with detection limits between 20 and 5000 pg/ml and coefficients of variance between 2 and 10%.

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 [24].

**Histologic examination**

Directly after the mice were killed, 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 used for immunohistochemical staining for the presence of granulocytes. After deparaffinization and rehydration of the paraffin slides using standard immunohistochemical procedures, the slides were digested in a solution of 0.25% pepsin (Sigma) in 0.01 M HCl. Endogenous peroxidase activity was quenched using 1.5% H2O2 in phosphate-buffered saline. A fluorescein isothiocyanate-labeled goat anti-mouse Ly6-G antibody (Pharmingen, San Diego, CA) was used as primary antibody and a biotinylated rabbit anti-fluorescein isothiocyanate antibody (DAKO, Glostrup, Denmark) was used as secondary antibody. Staining was developed using ABC solution (DAKO) as staining enzyme and 0.03% H2O2 and 3,3’-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris pH 7.6 as substrate. Quantification of positively stained cells was performed on coded samples by counting the number of positively stained cells in 10 fields at a magnification of 25x.

Coagulation activation was assessed using immunohistochemical staining for fibrin on paraffin slides after deparaffinization and rehydration as described previously [25]. In short, 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% H$_2$O$_2$ 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, Glostrup, Denmark) was used as staining enzyme. We used 0.03% H$_2$O$_2$ and 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, USA) in 0.05 M Tris pH 7.6 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 25x was counted.

**Statistical analysis**

Data are expressed as median (25$^{th}$ percentile-75$^{th}$ percentile). Groups were compared using Kruskal-Wallis test. Survival curves were compared by log-rank test. A value of p < 0.05 was considered to represent a statistically significant difference.

**Results**

**Bacterial outgrowth is slightly influenced by the FVL genotype**

To obtain insight into the role of the FVL genotype in antibacterial defense during peritonitis, we compared the number of *E. coli* CFUs 6 and 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, lung, kidney and spleen (to evaluate to which extent the infection was disseminated) of homozygous and heterozygous FVL mice and their wildtype littermates (Figure 1). Six hours after administration of *E. coli*, bacterial outgrowth was higher in peritoneal lavage fluid of heterozygous mice than of wildtype or homozygous FVL mice (4.1 [2.9-5.3], 2.5 [1.6-2.9] and 1.9 [1.35-2.4] 10$^6$ CFU/ml for heterozygous, wildtype and homozygous FVL mice, respectively). At other body sites no differences were found between the three genotypes 6 hours after induction of peritonitis. Twenty hours after administration of *E. coli*, bacterial outgrowth in peritoneal lavage fluid was higher in mice carrying the FVL mutation than in wildtype mice (1.6 [0.91-2.9], 2.9 [2.6-4.6] and 3.0 [2.7-4.7] 10$^9$CFU/ml for wildtype, heterozygous and homozygous FVL mice, respectively). In blood, liver, lung, kidney and spleen bacterial outgrowth 20 hours after administration of *E. coli* was not influenced by the FVL genotype.

**FVL marginally influences the inflammatory response to peritonitis**

To determine whether the FVL genotype influences the peritonitis-induced inflammatory response, we determined leukocyte infiltration and cytokine release into the peritoneal cavity. As shown in Table 1, IL-6, IL-10 and TNF-α levels increased after *E. coli* administration. At both 6 hours and at 20 hours after induction of peritonitis the cytokine levels were not dependent on the FVL genotype. The influx of leukocytes into the peritoneal cavity did not depend on the FVL genotype at either time point (Table 1).
As *E. coli* peritonitis results in systemic inflammation, we determined the influence of the FVL genotype on levels of circulating cytokines. As shown in Table 2, IL-6, IL-10, IL-12p70, TNF-α, MCP-1 and IFN-γ levels were not significantly different dependent on the genotype.

Upon histopathologic examination, all mice displayed foci of liver necrosis associated with thrombus formation. 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...
### Table 1: Cytokine levels in peritoneal lavage fluid, TAT complexes and cell influx into peritoneal cavity in mice after intraperitoneal injection of *E. coli*.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>t = 6 hours</th>
<th>t = 20 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>FVL +/-</td>
</tr>
<tr>
<td>IL-6 (ng/mL)</td>
<td>0.19[0.11-0.31]</td>
<td>0.11[0.07-0.32]</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>&lt; 62.5</td>
<td>&lt; 62.5</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>113[67.2-147]</td>
<td>69.7[49.8-105]</td>
</tr>
</tbody>
</table>

Data are median [25th percentile-75th percentile] at 6 (n=9 mice per genotype) or 20h (n=11 mice per genotype) after i.p. administration of *E. coli* (10⁴ CFU). Differences between the groups were not statistically significant.

### Table 2: Cytokine levels in plasma in mice after intraperitoneal injection of *E. coli*.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>t = 6 hours</th>
<th>t = 20 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>FVL +/-</td>
</tr>
<tr>
<td>IL-12p70 (pg/mL)</td>
<td>2.75[0.43-4.60]</td>
<td>4.87[4.87-14.57]</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>172.8[23.6-392.9]</td>
<td>285.6[218.7-322.6]</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>365.4[718.8-5412]</td>
<td>771.9[7467-823.6]</td>
</tr>
<tr>
<td>TAT (ng/mL)</td>
<td>59.2[54.3-64.1]</td>
<td>64.9[61.9-69.9]</td>
</tr>
</tbody>
</table>

Data are median [25th percentile-75th percentile] at 6 (n=9 mice per genotype) or 20h (n=11 mice per genotype) after i.p. administration of *E. coli* (10⁴ CFU). Differences between the groups were not statistically significant.
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and by influx of leukocytes, was evidently induced by peritonitis in all mice. The degree of inflammation in liver and kidney did not differ between the different genotypes and their wildtype littermates (Figure 2). At 6 hours after induction of peritonitis, homozygous FVL mice showed reduced granulocyte influx into their lungs, whereas at 20 hours no differences in granulocyte influx were evident.

FVL does not influence coagulant response to peritonitis

To determine whether the FVL genotype influences the peritonitis-induced coagulant response, we determined TAT complexes in both PF and plasma. As shown in Table 1 and 2, there were no differences between the different genotypes. In addition, fibrin deposition in the organs (liver, lung and kidney) was, although omnipresent especially in lung, not significantly different between the FVL genotypes.
To investigate the role of the FVL genotype on the outcome of peritonitis, we performed survival studies. No difference in mortality rate was seen between the three genotypes (Figure 3).

**Discussion**

Genetic analysis of FVL carriership in septic patients of the PROWESS and ENHANCE study combined with animal data from a FVL endotoxemia model suggested that heterozygous carriers of the FVL allele would be protected against sepsis [17-19]. Our data, however, do not confirm these findings and question the general beneficial effect of FVL in sepsis.

In the current study, we investigated whether the murine FVL allele influences septic peritonitis by challenging wildtype and FVL (either heterozygous or homozygous) mice with live *E. coli*. Overall, the FVL genotype had no effect on bacterial outgrowth, markers of inflammation, thrombin generation and survival. Small differences between the groups were observed (i.e. increased bacterial outgrowth in peritoneal lavage fluid of heterozygous mice at 6 hours, lower bacterial outgrowth in peritoneal lavage fluid of wildtype mice at 20 hours, and reduced granulocyte influx into the lungs of homozygous FVL mice at 6 hours); however, these small differences are not consistent over time and importantly did not influence survival. Whether these differences are merely the result of chance findings or reflect actual delicate differences in the response of FVL genotypes to septic peritonitis remains elusive.

As indicated above, the suggestion that heterozygous carriers of the FVL allele would be protected against sepsis is largely based on the PROWESS study [17]. In this study, 32 FVL
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carriers and 768 non-carriers were placebo treated and of these patients 5 FVL carriers and 243 non-carriers died within the study interval of 28 days, leading to a relative risk of 0.41 (95% confidence interval, 0.16-1.08) in favor of FVL carriers [18]. Including both placebo and APC treated patients, thereby increasing sample size, marginally affects the relative risk but significantly improves confidence (0.42; 95% confidence interval 0.20-0.48). In order to confirm these data, the ENHANCE study determined the relative risk of FVL carriership for sepsis showing a similar trend [19]. Pooling the PROWESS and ENHANCE data to further increase samples size results in a relative risk of 0.82 (95% confidence interval, 0.57-1.17). Overall these data indeed show a trend towards increased survival of FVL carriers in septic disease; however, the numbers of FVL carriers are rather small and the effect is not statistically significant [19].

In a recent population-based study, the FVL mutation has been suggested to be associated with infectious disease susceptibility and an increased risk of mortality from sepsis [21]. This suggestion is based on the notion that FVL carriers had more skin infections and increased 28-day mortality when suffering from sepsis. However, FVL carriership did not influence the risk for several other types of infection (such as pneumonia and viral infections), whereas it increased the risk of urinary tract infections. Future large epidemiologic studies would therefore be needed to prove or refute the association between FVL carriership and sepsis although our murine study does not provide a rationale for such studies.

Of course, many differences between murine experiments as the ones described in this article and clinical studies might explain the discrepancy between the results. For instance, the murine study was performed on healthy, relatively young mice, while the average age of patients in human population was 63 years [17]. More important, we used an inbred mouse strain in which we induced sepsis by peritoneal infection, while the patients included in the clinical studies formed a very heterogeneous group both with regard to ethnic background, gender; pre-existing medical conditions like diabetes, malignancies, hypertension and lung disease; and underlying cause of sepsis (both site and type of infection). Finally, all patients received extensive medical treatment, including antibiotics, whereas the mice were obviously not treated.

Kerlin and colleagues [18] studied the effect of the FVL allele during mouse endotoxemia and quite surprisingly showed a beneficial effect of heterozygous FVL mice (but not homozygous FVL mice) on survival. Remarkably, the beneficial effect of a single FVL allele was only evident at doses of endotoxin producing death in approximately 50% of the animals [8]. At higher or lower doses of endotoxin the survival advantage of FVL was abolished. The discrepancy between the endotoxemia study and our study might reflect intrinsic differences of the models used. The endotoxemia model employs part of the outer membrane of Gram-negative bacteria whereas our model employs live bacteria. If the FVL allele (partly) prevents endotoxin-induced inflammation but does not affect outgrowth and/or dissemination of bacteria, the beneficial effect might be lost in a septic peritonitis model.

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 [24]. Therefore, our model results in early systemic infection, mimicking the condition of severe abdominal sepsis. As a consequence, we cannot generally conclude that FVL does not influence the outcome of peritonitis. For this, the effect of FVL should also be investigated in other models of abdominal infection such as that induced by cecal ligation and puncture or by local instillation of an infected clot. Second, bacteria were administered in the absence of concurrent antibiotic therapy, and, therefore, our data do not provide insight into the effects of FVL in septic mice treated with antibiotics. Future studies of FVL mice that induce sepsis originating at different body sites or induced by different pathogens (either in the presence or absence of antibiotics) should help elucidating the actual benefit of a FVL allele.

Conclusions

We sought to confirm the survival advantage in sepsis conferred by a FVL allele by subjecting wild-type, heterozygous, and homozygous FVL mice to septic peritonitis. Our data, however, do not confirm a beneficial role of the FVL allele and question the general beneficial effect of FVL in sepsis.

Acknowledgements

FVL mice are a generous gift from Dr. R.J. Westrick. We would like to thank Ingvild Kop and Joost Daalhuijsen for their expert technical assistance. This study was supported by grants from the Netherlands Heart Foundation to L.W. Brüggemann (99.197) and S.H.H.F. Schoenmakers (98.159).
References


