Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 9

Inhibition of the tissue factor-factor VIIa pathway in murine pneumococcal pneumonia

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Abstract

The tissue factor-factor VIIa (TF-FVIIa) complex is essential for activation of blood coagulation during sepsis. Inhibition of TF-FVIIa in primate models of sepsis not only prevents disseminated intravascular coagulation, but also attenuates systemic and pulmonary inflammation, and improves survival. Streptococcus (S.) pneumoniae is the most important causative organism in community-acquired pneumonia (CAP) and a major cause of sepsis. In an attempt to define the role of TF-FVIIa in host defense against pneumococcal pneumonia, the following results were obtained: (1) patients with unilateral CAP demonstrated elevated concentrations of FVIIa, soluble TF and thrombin-antithrombin complexes (TATc) in broncho-alveolar lavage fluid (BALF) obtained from the infected site compared to the uninfected site; (2) mice with S. pneumoniae pneumonia displayed increased TF expression and fibrin deposits in lungs together with elevated TATc levels in BALF; (3) inhibition of TF-FVIIa by recombinant Nematode Anticoagulant Protein c2 attenuated the procoagulant response in the lung, but did not impact on host defense, as reflected by an unaltered outgrowth of pneumococci and an unchanged survival. These data suggest that TF-FVIIa activity contributes to activation of coagulation in the lung during pneumococcal pneumonia, but does not play an important role in the antibacterial host defense in this murine model.

Introduction

The blood coagulation system is activated during sepsis and inflammation, resulting in the deposition of fibrin within intra- and extravascular spaces (1-3). The proteins that are generated during activation of the coagulation system (i.e. factors VIIa (FVIIa), Xa (FXa), thrombin, fibrin) can provoke a pro-inflammatory response by their effects on chemotaxis, vascular permeability, inflammatory cell activation and cytokine release (4). The tissue factor (TF)-FVIIa dependent pathway is responsible for the initiation of coagulation in inflammatory diseases. Under physiological conditions, TF cannot be detected on vascular cells and only in very low quantities on circulating blood cells (5-8). However, TF can be induced on vascular cells and alveolar macrophages by several pro-inflammatory cytokines following a systemic inflammatory challenge such as bacterial infection (9). Several lines of evidence support the in vivo relevance of TF: 1) Infusion of TF in rabbits results in disseminated intravascular coagulation (DIC)(10) ; 2) Inhibition of TF-FVIIa in the setting of endotoxemia or bacteremia results in a reduced procoagulant response and/or prevention of DIC (10-18) ; 3) During clinical sepsis circulating levels of TF have been shown to be elevated compared to non-septic patients (19). Taken together, the TF-FVIIa complex appears an important component involved in the induction of coagulopathy in sepsis.Activation of the coagulation system has also been implicated in the pathogenesis of pulmonary bacterial infections resulting in the formation of fibrin in the interstitial and alveolar spaces of the lung (20, 21). Under physiologic conditions, fibrin deposition in the alveolar compartment is regulated by the fibrinolytic system where urokinase type plasminogen activator (uPA) and its
inhibitor, plasminogen activator inhibitor type-1 (PAI-1) function as the primary profibrinolytic and anti-fibrinolytic components, respectively (22). During the acute respiratory distress syndrome (ARDS), interstitial lung diseases and pneumonia, changes in the alveolar hemostatic balance in favor of a procoagulant response have been observed, characterized by an increase in TF, FVII and PAI-1, and a concurrent decrease in uPA activity (21, 23-27). When coagulation is blocked at the TF-FVIIa level during experimental sepsis or acute lung injury, the pulmonary inflammatory response is attenuated (28-33), suggesting that TF-FVIIa complex contributes to acute lung injury in sepsis.

Against this background the current study was undertaken to investigate the role of the TF-FVIIa complex in the host defense against bacterial pneumonia. For this, we first determined soluble TF, FVIIa and TAT complexes (TATc) in the infected lung of patients with unilateral community acquired pneumonia (CAP). Furthermore, we evaluated the role of the TF-FVIIa complex in host defense during pneumonia caused by Streptococcus pneumoniae, the most frequently found pathogen in CAP(34), by treating mice with recombinant Nematode Anticoagulant Protein c2 (rNAPc2), a potent and selective small protein inhibitor of the TF-FVIIa pathway (35, 36).

Material and methods

Patient study

Design A bilateral bronchoalveolar lavage (BAL) was performed in four patients with a unilateral CAP. Inclusion criteria were fever (>37.7°C), new unilateral infiltrate on chest roentgenogram within two days after admission, no antibiotic pre-treatment and PaO₂ >7.5 kPa while breathing room air. Ten healthy volunteers (mean age 32±8 yr) served as controls. Details of the patients and the procedure of BAL have been published previously (37). The protocol was approved by the institutional Medical Ethics Committee and written informed consent was obtained from all subjects.

Assays

TATc and soluble TF were measured using commercially available ELISAs according to the manufacturers recommendations, i.e. TATc (Behringwerke AG, Marburg, Germany), and soluble TF (American Diagnostics, Greenwich, CT). FVIIa was measured by an enzyme capture assay as described in detail previously (38).

Mouse studies

Animals

All experiments were approved by the institutional Animal Care and Use Committee. For all experiments 10 to 12 week old female BALB/c mice were used (Harlan Sprague Dawley Inc., Horst, the Netherlands).
Experimental design

Pneumonia was induced by intranasal inoculation with \textit{S. pneumoniae} (ATCC 6303; Rockville, MD) \((5 \times 10^4\) CFU in 50 \(\mu l\)) as described previously (37, 39, 40). rNAPc2 (Corvas International, Inc, San Diego, California, USA) (36) was given subcutaneously every 6 h after induction of pneumonia at a dose of 10mg/kg of body weight (in 100 \(\mu l\) sterile phosphate buffered saline, PBS), starting at the time of inoculation. This treatment schedule was based on kinetic studies in mice in which a single subcutaneous rNAPc2 dose of 10 mg/kg was found to induce a two fold prolongation of the activated prothrombin time and a four fold prolongation of the activated partial thromboplastin time at 0.5h after rNAPc2 administration, with a gradual return to baseline values at 6h postinjection (41). Controls received PBS (100 \(\mu l\)) subcutaneously every 6 h. At 30 or 42h after infection, BAL fluid (BALF), lungs and blood were harvested, and lung homogenates prepared, according to previously described methods (37, 39, 40).

Histologic examination

After 24h fixation of lungs in 10\% formalin e and embedding in paraffin, 4 \(\mu m\) thick sections were stained with hematoxylin and eosin. Immunohistochemical staining for TF and fibrin were performed on paraffin slides exactly as described previously (37, 41).

Assays

TATc levels were measured in BALF by ELISA (37, 41). Cytokine and chemokine levels were measured using commercially available ELISAs, in accordance with the manufacturers recommendations: Tumor necrosis factor \(\alpha\) (TNF), Interleukin 6 (IL-6) (Pharmingen, San Diego, CA), Macrophage inflammatory protein 2 (MIP-2) and KC (R\&D systems, Abingdon, United Kingdom). Detection limits were 50 pg/ml (TNF), 37pg/ml (IL-6), 47 pg/ml (MIP-2), 12 pg/ml (KC), respectively.

Statistical analysis

Data are expressed as means \(\pm\) SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann Whitney U test. Survival curves were compared by log-rank test. In the clinical study infected lung BALF was compared to non-infected lung BALF and healthy controls using the Mann Whitney U test. P< 0.05 was considered to represent a statistically significant difference.
Figure 1 Coagulation markers in patients with community-acquired pneumonia Coagulation markers (sTF, FVIIa, TAT complexes) were measured in BALF from 4 patients with CAP with a unilateral infiltrate on the chest roentgenogram, from the area of the infiltrate (A) and from the uninfected site (B). 10 healthy volunteers served as controls (C). Horizontal lines express medians.

Results

Alveolar coagulation activation in pneumonia patients

Patient characteristics have been presented in an earlier report (42), and are summarized in Table I. All patients underwent bilateral BAL within 12h after admission, first at the uninfected site followed by the area with the infiltrate determined from the chest roentgenogram. BALF from healthy subjects served as control. Soluble TF and FVIIa levels were increased in BALF of infected lungs compared with the levels in BALF from uninfected lungs and healthy controls (Figure 1).

Table 1. Clinical and biochemical parameters of CAP patients.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Fever (°C)</th>
<th>X-Ray</th>
<th>BALF culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>42</td>
<td>38.6</td>
<td>Right middle lobe</td>
<td>S. pneumoniae</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>47</td>
<td>38.9</td>
<td>Right middle lobe</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>40</td>
<td>39.1</td>
<td>Right upper lobe</td>
<td>S. pneumoniae</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>36</td>
<td>37.8</td>
<td>Right middle and lower lobe</td>
<td>-</td>
</tr>
</tbody>
</table>

Clinical and biochemical parameters of 4 CAP patients with a unilateral infiltrate on chest X-ray. X-Ray shows the site of the infiltrate on chest roentgenogram.
Figure 2. Increased TF expression in lungs of mice during pneumococcal pneumonia. TF immunostaining of lung of saline inoculated mice (A), 42h after inoculation with *S. pneumoniae* in PBS treated mice (B), and 42h after inoculation with *S. pneumoniae* in rNAPc2 treated mice (C). Magnification x 40. Representative slides are shown from a total of five mice per group.

In addition, soluble TF and FVIIa concentrations were higher in BALF from uninfected lungs of CAP patients than in BALF from healthy controls. Elevated soluble TF and FVIIa levels were associated with increased alveolar TATc concentrations, which were higher in infected lungs of patients compared to uninfected lungs and healthy individuals. TATc concentrations in BALF were 5 fold higher in *S. pneumoniae* infected mice compared to non-infected mice, demonstrating that the development of pneumococcal pneumonia is associated with alveolar thrombin generation in this model (Figure 3). Treatment with rNAPc2 significantly decreased BALF TATc levels during pneumonia but not completely back to the levels in uninfected animals (P < 0.05 vs. PBS treated mice with pneumonia [Figure 3]).

Figure 3. rNAPc2 decreases generation of TATc in the alveolar compartment during murine pneumococcal pneumonia. TATc in BALF 42h after intranasal administration of *S. pneumoniae*. rNAPc2 (closed bar) was given subcutaneously every 6h after induction of pneumonia at a dose of 10 mg/kg of body weight starting at timepoint 0. The control group (open bar) received PBS subcutaneously every 6h. Uninfected mice were intranasally inoculated with saline (stripes). Data are mean ± SE of 6 mice per group. * P < 0.05, vs. control.
Histopathology

Both groups of mice displayed similar lung inflammation characterized by interstitial inflammation, small areas of pneumonia, and pleuritis 30 and 42h following infection (Figure 4 A and B). In the lungs of rNAPc2 treated mice, less fibrin(ogen) formation was observed than in control treated mice (Figure 4 C and D).

![Histologic sections of lungs](image)

**Figure 4** rNAPc2 does not affect lung inflammation
Histologic sections of lungs of control treated (A) and rNAPc2 treated mice (B) 42h after inoculation with *S. pneumoniae* (H&E staining) showing a similar inflammatory infiltrate. Immunostaining for fibrin(ogen) showed less fibrin deposition in rNAPc2 treated mice (D) than in control treated mice (C) 42h after infection with *S. pneumoniae*. Magnification x20. Slides are representative of five mice per group.

Mortality

To investigate the role of TF-factor VIIa in the outcome of pneumococcal pneumonia, we performed a survival study. Mice were treated with rNAPc2 or PBS every 6h until 42h after infection. No difference was seen in mortality after rNAPc2 treatment compared with control treatment (Figure 5).
Bacterial outgrowth
To assess the impact of inhibition of TF-FVIIa at earlier phases of the infection, we determined the outgrowth of S. pneumoniae in lungs 30 and 42h after infection. At both time-points rNAPc2 and PBS treated mice contained the same number of S. pneumoniae CFU's in their lungs (Figure 6). At 30h postinfection 5/8 (62.5%) of rNAPc2 treated mice had a positive blood culture after 30h, whilst at that time point blood cultures displayed no growth of pneumococci in PBS treated mice. After 42h all mice in both groups had positive blood cultures (non significant).

![Figure 5 rNAPc2 does not influence mortality. Survival after S. pneumoniae inoculation in control treated (open circles) and rNAPc2 treated mice (closed squares). Mortality was assessed twice daily.](image)

![Figure 6 rNAPc2 does not influence bacterial outgrowth in lungs. S. pneumoniae CFU's in lungs of control treated (open bars) and rNAPc2 treated mice (closed bars) 30 (A) and 42h (B) after inoculation. Data are mean ± SEM. N =8 per group per time point.](image)

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Immune responses

Cell recruitment to the site of infection is an important part of host defense against pneumococcal pneumonia. For this reason we compared cell influx in the alveolar spaces of rNAPc2 and PBS treated mice after inoculation with *S. pneumoniae*. The number of recruited granulocytes in the BALF did not differ between groups 42h after inoculation (Table II). Cytokines and chemokines are involved in the early immune responses against infection (43, 44) and proteins produced during coagulation activation affect production of these mediators (45-47). Locally produced cytokine levels were not altered after treatment with rNAPc2 compared to PBS treated mice (data not shown). However, CXC chemokine (KC and MIP-2) levels were both increased 30h after infection in rNAPc2 treated mice compared to PBS treated mice. Chemokine levels in lung homogenates were similar 42h following infection (Figure 7).

**Table II. Effect of rNAPc2 on cellular composition in BALF.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>rNAPc2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cells</strong></td>
<td>15.4 ± 6.6</td>
<td>11.5 ± 3.4</td>
</tr>
<tr>
<td><strong>Granulocytes</strong></td>
<td>5.9 ± 2.4</td>
<td>8.8 ± 2.6</td>
</tr>
<tr>
<td><strong>Alveolar macrophages</strong></td>
<td>9.2 ±6.2</td>
<td>2.5 ±0.8</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td>0.3 ±0.2</td>
<td>0.2 ±0.1</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (x 10^4 /ml BALF) of 6 mice per group, 42h after inoculation with *S. pneumoniae* CFU. Differences between groups were not significant.
Discussion

The TF-FVIIa pathway plays an essential role in the activation of the blood coagulation system during inflammatory challenges such as systemic bacterial infection. Evidence indicates that TF also is important for a procoagulant response in the pulmonary compartment. Indeed, TF is present at multiple sites in the lung, including the vascular adventitia, on airway epithelial cells and on alveolar macrophages, and inhibition of the TF-FVIIa pathway completely abrogated intrapulmonary fibrin deposition and diminished lung inflammation after intratracheal delivery of endotoxin in rats (20, 29). In addition, TF inhibition has been shown to reduce lung inflammation and injury in experimental endotoxemia and bacteremia (32, 33). To the best of our knowledge, the current investigation is the first to address the role of TF in the host response to pneumonia. We here demonstrate that activation of the TF-FVIIa pathway can be detected in patients with CAP at the site of the infection. In accordance, TF expression and local coagulation activation could be demonstrated in the lungs of mice with experimentally induced pneumococcal pneumonia. Inhibition of the TF-FVIIa complex with rNAPc2 reduced activation of coagulation in the lungs, but did not influence antibacterial defense mechanisms. These findings suggest that rNAPc2 functions primarily as an anticoagulant during murine pneumococcal pneumonia.

Our findings in patients with CAP confirm and extend earlier studies (26, 48). In a recent study, patients with severe pneumonia were reported to display increased procoagulant activity in their BALF, which was attributable to tissue factor activity (26). In this report, we have demonstrated increased levels of soluble TF, FVIIa and TATcc in BALF obtained from the site of the infection. Comparison with BALF from the uninfected side clearly established that activation of the coagulation system occurs in a compartmentalized fashion during CAP. Although our study involved only 4 patients, overlap with the data obtained from BALF-derived from the uninfected side or from lungs of healthy volunteers did not occur. Thus, our human data, together with the demonstration of TF in mouse lungs during pneumococcal pneumonia indicate that coagulation is activated locally in the lung during lower respiratory tract infection.

In the present study rNAPc2 was used to inhibit the TF-FVIIa pathway. This small protein was originally isolated from the hematophagous nematode hookworm *Ancylostoma caninum*, and subsequently produced in recombinant form using the yeast *Pichia pastoris* (36). rNAPc2 inhibits TF-FVIIa mediated coagulation by high affinity binding to zymogen factor X or factor Xa prior to the formation of an inhibitory complex with TF-FVIIa (35). This contrasts with the mechanism of action of TF pathway inhibitor (TFPI), which binds only to factor Xa at its catalytic center, followed by the formation of the quaternary TFPI/FXa – TF/FVIIa complex. The utilization of zymogen FX as an inhibitory scaffold by rNAPc2 obviates the need for forming FXa prior to the inhibition of the TF-FVIIa complex. The efficacy of rNAPc2 in attenuating coagulation *in vivo* has been demonstrated in several earlier investigations, where rNAPc2 completely prevented endotoxin-induced coagulation
activation in chimpanzees (18), and strongly reduced the incidence of acute deep vein thrombosis in patients undergoing unilateral knee arthroplasty compared to the best current prophylactic regimens (49).

The importance of TF in the pathogenesis of sepsis has been well recognized. Treatment with DEGR-FXa (an inhibitor of thrombin generation) strongly reduced the coagulopathy related to experimental sepsis in baboons, but did not increase survival (50). In contrast, in the same primate model of sepsis, inhibition of the TF-FVIIa complex by either a monoclonal antibody against TF (11), or recombinant TF pathway inhibitor (rTFPI)(14, 17) or active site inactivated FVIIa (51, 52) was associated with both anti-coagulant and anti-inflammatory effects, and an increased survival. Furthermore, blocking TF-mediated coagulation in experimental sepsis or acute lung injury models attenuated the inflammatory response in the lung, including neutrophil infiltration, and edema formation (29, 30, 32, 33). In addition, co-infusion of rTFPI together with TF into rabbits, suppressed the TF mediated fibrin deposition in the lung (53). Together, these findings indicate that the TF-FVIIa complex plays a prominent role in the procoagulant and inflammatory response of the lung to sepsis. In contrast, the present study did not show a role for TF in the pulmonary inflammatory response during bacterial pneumonia when inhibited by rNAPc2 in this model. In line with this, we recently showed that although intravenous infusion of rTFPI attenuates coagulation activation following low dose endotoxin administration to volunteers, it did not influence inflammatory pathways (16, 54). However, there are several instances where TF has been shown to have a direct inflammatory effect in vivo. These include: 1) recombinant human TF injected intra-articularly induced morphological signs of arthritis and infiltration of mainly mononuclear cells in synovia (55); 2) leukocyte infiltration was reduced after treatment of rabbits with anti-TF antibody in an acute myocardial injury model (56); treatment with anti-TF antibody or TFPI diminished glomerular inflammation and glomerular fibrin deposition in experimental models of glomerulonephritis (57, 58); and spinal cord ischemia (59). Taken together, these studies suggest a role for TF in the regulation of inflammation in addition to its procoagulant function and that this role may be dependent on the experimental model and/or inflammatory challenge or target organ.

The potential role of TF-mediated signalling in leukocyte activation and augmented cytokine production has been demonstrated in several earlier investigations (14, 52, 60). On the contrary we found modestly elevated chemokine concentrations in the lungs of rNAPc2 treated mice at 30 h post inoculation, but not at later time points during the infection. The relatively elevated chemokine concentrations at 30 h after the infection could be related to the modestly (albeit not significantly) higher bacterial loads in rNAPc2 treated mice. In addition, it should be noted that in vitro studies have suggested that rNAPc2 may facilitate activation of protease-activated receptors 1 and 2 by FXa by stabilizing the ternary TF-FVIIa-FXa complex (61). In this respect the mechanism of action of rNAPc2 differs from that of TFPI: whereas rNAPc2 inhibits FVIIa but not FXa in this complex, TFPI inhibits both. Interestingly, at this early time point only rNAPc2 treated mice displayed positive blood cultures, suggesting that local coagulation contributes to the containment of the infection.
The present data are in line with a recent study from our laboratory that reported on the effect of rNAPc2 in a murine model of abdominal sepsis caused by intraperitoneal injection with *Escherichia coli* (41). In that investigation, rNAPc2 also inhibited local and systemic activation of coagulation, as measured by TATc concentrations in peritoneal lavage fluid and plasma and fibrin deposits in organs, while not influencing inflammatory and antimicrobial responses or mortality (41).

Elimination of TF activity has gained interest because of its potential beneficial effects in severe bacterial sepsis based on a number of experimental studies. In addition, TF has been implicated in the pathogenesis of lung injury during severe bacterial sepsis and after pulmonary exposure to endotoxin. Recently, treatment with recombinant TFPI was reported to inhibit activation of coagulation in patients with severe sepsis, with respiratory tract infection as the main source of sepsis, while not influencing mortality (62). Our present findings using a murine model of pneumococcal pneumonia suggest that TF mediated coagulation activation is present in the lung, but that the role of TF-FVIIa complex in antibacterial host defense may be limited.

References


inflammatory or antibacterial response to abdominal sepsis induced by Escherichia coli in mice. *J. Infect. Dis* in press.


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