Coagulation and anticoagulation in acute lung injury, pneumonia, and ventilator-associated lung injury

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Coagulation and Anticoagulation in Acute Lung Injury, Pneumonia, and Ventilator-Associated Lung Injury
Coagulation and Anticoagulation in Acute Lung Injury, Pneumonia, and Ventilator-Associated Lung Injury

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egeboren te Seoul, Zuid-Korea
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Faculteit der Geneeskunde
For my parents
부모님께
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General Introduction and Literature Review
General Introduction

Goda Choi
Marcus J. Schultz
Inflammation and coagulation are two important host defense mechanisms against bacterial pathogens. In response to intrusion of microorganisms, the immune system is immediately directed to the site of infection and initiates antimicrobial activity. At the same time, the coagulation network is activated: the formation of a clot limits tissue damage and prevents dissemination of the pathogen. Inflammation and coagulation, not surprisingly, require strict regulation, i.e., after containment of microorganisms the host response must be counterbalanced by deactivation or inhibition in order to restore homeostasis. Unfortunately, in severe disease states, these regulatory mechanisms may fail, resulting in an exaggerated and sustained host response.

Sepsis

The detrimental effects of uncontrolled host defense are best exemplified in the clinical syndrome of severe sepsis [1]: the hyperinflammatory response and the excessive coagulation activation lead to consumption of clotting factors and widespread depositions of fibrin, causing diffuse vascular damage, acute organ dysfunction, and eventually death [2]. Patients surviving this exaggerated inflammatory response, are furthermore vulnerable to secondary infections due to a state referred to as ‘immunoparalysis’ which is characterized by irresponsiveness or inability of the immune system to react to new threats [3]. Sepsis is associated with high mortality: although mortality has decreased over the past decennia [4-6], hospital mortality still exceeds 30% [5-11]. Treatment mostly relies on controlling the primary infection (e.g., antimicrobial therapy, drainage of abscesses or empyema), supporting failing organs (e.g., mechanical ventilation, renal replacement therapy, fluid therapy, and blood pressure regulation), and preventing secondary complications (e.g., glycemic control, deep venous thrombosis prophylaxis) [12]. In the search for specific treatment modalities for sepsis patients, many immunomodulatory drugs have been evaluated, however without much success [3].

Coagulation is a main target for treatment of sepsis. During sepsis there is increased expression of tissue factor by various cells. Binding and activation of factor VII to tissue factor leads to activation of the extrinsic coagulation pathway resulting in thrombin generation. Thrombin converts fibrinogen into fibrin and induces platelet aggregation, forming a clot (figure 1). Normally, the coagulation activation is regulated by the natural anticoagulants activated protein C (APC), antithrombin (AT), and tissue factor pathway inhibitor (TFPI) [13], while fibrin is eventually cleared by the fibrinolytic system (figure 2).
In sepsis however, in addition to the coagulation activation, systemic levels of the natural anticoagulants are decreased and fibrinolysis is impaired [14]. Therefore (recombinant) natural anticoagulants were investigated in the treatment of human sepsis. In fact, treatment with APC, AT, and TFPI was shown to reduce the coagulation response in the respective phase III trials [15-17]. However, both AT and TFPI failed to demonstrate beneficial effects on patient survival [15,17]. Treatment with recombinant human APC did lead to better patient outcome [16], still, without being completely clear what properties of APC – other than its systemic anticoagulant effects – are important for host protection.

**Acute Lung Injury and Pneumonia**

The lungs are involved in the disease process in the majority of sepsis patients. The most common site of primary infection leading to sepsis is the respiratory tract (the so-called,
‘pneumosepsis’) [7-9] (Figure 3). Vice versa, sepsis has been identified as the most important risk factor for acute lung injury [18-22]. Acute lung injury is a clinical syndrome that not only complicates the clinical course of sepsis, but affects many other critically ill patients, with a mortality of approximately 40%, but some report even higher mortality rates [18,20,22-27]. Acute lung injury is defined as “a syndrome of inflammation and increased permeability that is associated with a constellation of clinical, radiographic, and physiologic abnormalities that cannot be explained by, but may co-exists with, left atrial or pulmonary capillary hypertension,” and is confirmed by bilateral infiltrates on chest radiography and a PaO₂/FiO₂ ratio lower than 300 mmHg (40 kPa) [28]. More severe lung injury leading to a PaO₂/FiO₂ ratio lower than 200 mmHg (26.7 kPa) is referred to as the acute respiratory distress syndrome (ARDS) [29]. In community-acquired lung infection, *Streptococcus pneumoniae* is most frequently involved [30,31]. Gram-negative pathogens, such as *Pseudomonas aeruginosa*, are the leading cause of hospital-acquired pneumonia and ventilator-associated pneumonia [32-36].

With regard to the pathogenesis of sepsis and acute lung injury, there are many similarities. In the pulmonary compartment of patients with acute lung injury, there is a strong local proinflammatory response [37-48]. Also, there is increased procoagulant activity within the lungs [49-53]. A hallmark of ARDS is fibrin deposition within the airways; moreover, the amount of fibrosis and loss of lung function are important determinants for long-term survival [54-56]. For this reason, we hypothesized that anticoagulant therapy would be beneficial in acute lung injury and pneumonia [57,58]. Indeed, lung-protective effects of anticoagulant therapy with APC, AT, and TFPI have already been demonstrated, but most studies used animal models of endotoxin-induced acute lung injury [57,59]. Little is known about the role of natural anticoagulants during bacterial pneumonia.

Figure 3. Sites of primary infection in patients with sepsis. Data derived from [7-9].
Mechanical Ventilation

The majority of patients at the intensive care unit are intubated and mechanically ventilated at admission (e.g., after surgery) or require ventilatory support because of respiratory failure. Although mechanical ventilation certainly is a cornerstone of the intensive care management, mechanical ventilation puts patients at risk for nosocomial infections [60], while the ventilation itself may aggravate or even initiate lung injury. The latter has been referred to as ‘ventilator-associated lung injury’ (in patients) or ‘ventilator-induced lung injury’ (in experimental animal studies), and is believed to be caused by volutrauma and/or barotrauma (overdistension of lung tissue by high volumes and pressures), atelectotrauma (repeated recruitment and collapse of alveoli), and biotrauma (release of inflammatory mediators by epithelial and immune cells) [61,62]. In patients with ARDS it was shown that reducing tidal volumes and using positive end-expiratory pressures (so-called ‘lung-protective’ mechanical ventilation) limits biotrauma [63,64], thereby contributing to patient survival [65]. Currently, there is ongoing debate on whether patients without preexistent lung injury would also benefit from lung-protective mechanical ventilation. Furthermore, it is unknown what changes mechanical ventilation can induce in the pulmonary hemostatic balance.

Outline of the Dissertation

This dissertation focuses on hemostatic imbalances as seen in the bronchoalveolar compartment of critically ill patients, with regard to sepsis, acute lung injury, pneumonia, and mechanical ventilation. Research subjects included patients from the departments of intensive care medicine, internal medicine, and surgery, healthy volunteers, and laboratory animals. In chapter 2, the relationship between inflammation and coagulation in acute lung injury and pneumonia is reviewed. This chapter is followed by an observational study in which the use of APC was evaluated within our intensive care unit (chapter 3). Furthermore, the effects of intravenous treatment with APC, AT, and TFPI were investigated in an animal model of endotoxemia (chapter 4). In a separate part of this dissertation, pulmonary coagulation and fibrinolysis were investigated in patients with pneumonia, either ventilator-associated (chapters 5-8) or community-acquired (chapter 9). Also, the effects of intravenous anticoagulant treatment were studied in a rat model of nosocomial pneumonia (with P. aeruginosa, chapter 8) and community-acquired pneumonia (with S. pneumoniae, chapter 9). Finally, the last part of this dissertation focuses on mechanical ventilation. The results are presented from a randomized controlled trial in which surgical patients without lung injury were mechanically ventilated according to different ventilation strategies, using either higher...
tidal volumes with no positive end-expiratory pressure (PEEP) or lower tidal volumes with 10 cm H₂O PEEP. Effects of these ventilation strategies on pulmonary coagulation are described in chapter 10. In chapter 11 the effects of mechanical ventilation on pulmonary inflammation and apoptosis are discussed. The results from all parts are summarized and discussed in chapter 12.

References


Coagulation and Fibrinolysis in Acute Lung Injury and Pneumonia

*a review*

Goda Choi
Marcus J. Schultz
Tom van der Poll
Marcel Levi

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Injury to the lung leads to a number of sequelae, which include the production of proinflammatory cytokines, activation of coagulation, and inhibition of fibrinolysis [1,2]. Inflammation and coagulation consist of two different activation cascades, but research has demonstrated that there is extensive cross-talk, causing reciprocal activation and amplification [1-3]. Local activation of coagulation and inhibition of fibrinolysis have clearly been demonstrated in patients with acute respiratory distress syndrome (ARDS), in which intra- and extra-alveolar fibrin deposition is a histopathologic hallmark [4]. Pulmonary fibrin deposition is not limited to ARDS or acute lung injury (ALI), but activation of coagulation and inhibition of fibrinolysis may also occur in other pulmonary disorders, such as pneumonia [3].

In this review, coagulation and fibrinolysis are discussed in relationship to pulmonary cytokine production, particularly as seen in ALI/ARDS and pneumonia. We will highlight recent studies on pulmonary inflammation and the coagulation imbalance causing a procoagulant state in the lung. Furthermore, we will address possible therapeutic interventions aimed at restoring the coagulation balance during pulmonary inflammation.

Inflammation-Induced Production of Proinflammatory Cytokines

In the pulmonary compartment, host defense mechanisms consist of both cellular and humoral components [5]. Host defense cells such as epithelial cells, resident alveolar macrophages, and recruited polymorphonuclear cells must cooperate in mounting an effective inflammatory response against respiratory pathogens. Among the humoral components of inflammation, cytokines play a pivotal role in the communication between these cells. Cytokines are small glycoproteins that are produced by a large variety of cells. They have effects on many different cell types, and modulate local and systemic inflammation. Generally, cytokines are involved in the early phase of inflammation (e.g., proinflammatory cytokines, such as tumor necrosis factor-α (TNF) and interleukin (IL)-1), in the recruitment of immune cells to the site of inflammation (chemokines, such as IL-8), and in the activation of macrophages and phagocytes (e.g., IL-6) [5].

Acute Respiratory Distress Syndrome. Several studies have demonstrated that high levels of TNF and IL-1 are present in bronchoalveolar lavage fluids (BALF) and serum of patients with ARDS [6-8]. In most patients, ratios of BALF-to-serum cytokine concentrations were elevated, suggesting a pulmonary origin [6-9]. Concurrently, BALF levels of IL-6 are very high in patients at risk for ARDS, while remaining elevated throughout the course of established ARDS [6,10-12]. Also, relatively high levels of IL-8 have been measured in airways of ALI/ARDS patients [6,12-16]. Important is that elevated levels of cytokines have
been correlated with bad outcome. Meduri et al. [10] established that high levels of TNF, IL-1, IL-6, and IL-8 in bronchoalveolar lavage fluid (BALF) were associated with increased mortality (figure 1A), while others have confirmed the correlation of IL-8 with development and outcome of ARDS [15,16].

**Pneumonia.** During pneumonia, similarly to ARDS, proinflammatory cytokines and chemokines are produced at the site of infection, i.e., in the lung [12,17-19]. Dehoux and Boutten [17,18] measured levels of TNF, IL-1, IL-6, and IL-8 in patient with unilateral community-acquired pneumonia (CAP). Concentrations of these cytokines were significantly higher in BALF from the infected lung, as compared to the opposite, non-infected lung, or lungs of healthy controls. Recently, we reported on cytokine production in patients with ventilator-associated pneumonia (VAP) [20]. Compared to mechanically ventilated patients without pulmonary infection, patients with VAP had elevated levels of proinflammatory cytokines (TNF, IL-1, IL-6) and chemokines (IL-8) in BALF (figure 1B). Interestingly, cytokine levels increased before clinical diagnosis of VAP. Plasma levels of TNF, IL-1, IL-6, and IL-8 did not change in time or even declined, both in VAP patients and controls.

**Mechanical Ventilation.** Several studies in animals have suggested that mechanical ventilation can induce pulmonary inflammation as well [21,22]. Increasing evidence from preclinical and clinical studies suggests that mechanical ventilation may aggravate, or even initiate lung injury. Although questioned by some experts in the field [22], there is much evidence that locally produced proinflammatory cytokines and chemokines play a central role

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**Figure 1.** Bronchoalveolar cytokine levels in patients with acute respiratory distress syndrome (ARDS) and ventilator-associated pneumonia (VAP). (A) Non-survivors (closed bars) have significantly higher levels of proinflammatory cytokines and chemokines in bronchoalveolar lavage fluid, compared with survivors (open bars). Data are means. $^p < 0.01$, non-survivors versus survivors. (B) At the day VAP was diagnosed, pulmonary cytokine levels were significantly higher in VAP patients (closed bars) than in mechanically ventilated patients not having VAP (open bars). Data are means. $^* p < 0.05$, VAP versus non-VAP patients. Data adapted from [10,20].
in the so-called ‘ventilator-induced lung injury’ (VILI). Indeed, it has been demonstrated that mechanical ventilation using a ‘protective’ strategy (tidal volumes and/or sufficient positive end-expiratory pressures) can attenuate local cytokine release in patients with ARDS [23,24]. Indeed, Ranieri et al. [23] showed that ARDS patients who were ventilated according to the lung protective strategy, had a reduction of TNF, IL-1, IL-6, and IL-8 in BALF after 36 hours. Interestingly, and in accordance with another study on lung protective mechanical ventilation [25], a reduction was also seen for IL-6 in plasma of these patients [23]. Furthermore, in a study by Stuber et al. [24], in which ventilation settings were transiently changed from a lung protective setting to a more conventional - less protective - setting in patients with ALI, switching to the conventional strategy was associated with a marked increase of intraalveolar cytokine and plasma cytokine concentrations.

**Inflammation-Induced Coagulation Imbalance**

One of the major detrimental effects during the inflammatory response is the constitution of a procoagulant state. This coagulation imbalance is the net result of activation of the extrinsic coagulation pathway, suppressed fibrinolysis, and impaired activity of natural coagulation inhibitors (figures 2 and 3). The extrinsic coagulation pathway is initiated by tissue factor (TF), which is expressed at the surface of lung epithelial cells, lung fibroblasts, and alveolar macrophages in reaction to lung injury. IL-6 is the main cytokine involved in TF-expression [26-29]. TF forms complexes with factors VIIa (TF-FVIIa complex) and Xa, and stimulates generation of thrombin, which converts fibrinogen into fibrin (figure 3). Plasmin that cleaves fibrin into degradation products counterregulates formation of fibrin. In the lung, urokinase-type plasminogen activator (uPA) seems to be the predominant profibrinolytic factor, while tissue-type plasminogen activator (tPA) has a secondary role [30,31]. In patients with

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**Figure 2.** Coagulation imbalance in the pulmonary compartment. The procoagulant state is the net result of increased fibrin formation (due to activated coagulation and inhibition of physiological inhibitors of coagulation), and decreased fibrin degradation (due to impaired fibrinolysis).
Figure 3. Schematic view of pulmonary hemostasis. See chapter 1 for details. Ila, thrombin; APC, activated protein C; AT, antithrombin; FDP, fibrin degradation products; IL-6, interleukin-6; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TNF, tumor necrosis factor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

Acute Respiratory Distress Syndrome. Several studies have demonstrated that ARDS is characterized by an increased procoagulant state in the pulmonary compartment [34-36]. Fuchs-Buder et al. [34] sequentially analyzed procoagulant activity in BALF of patients developing ARDS and those at risk for ARDS. Early in the course of ARDS, procoagulant activity in BALF was significantly higher than in the patients at risk for ARDS. Similar changes were found by Idell et al. [36]. Günther et al. [30] analysed BALF of 25 patients with pulmonary inflammation however, fibrinolytic activity of uPA and tPA is impaired by increased expression of plasminogen activator inhibitor (PAI)-1 and PAI-2 [32]. Finally, regulation of the coagulation cascade is provided by physiologic anticoagulation factors, including activated protein C (APC), antithrombin (AT), and tissue factor pathway inhibitor (TFPI) [33].
ARDS and 35 healthy control subjects. In this study, total procoagulant activity was increased by nearly two orders of magnitude, as compared with healthy controls (figure 4). These studies all showed that activation of coagulation in ARDS was largely attributable to elevated local levels of TF.

During ARDS, in addition to activation of coagulation, fibrinolytic activity in the lungs is suppressed [30,35,36]. In the above-cited study by Idell et al. [36], fibrinolytic activity was undetectable in BALF at 3 days after onset of ARDS and remained depressed for up to 2 weeks. Depressed fibrinolytic activity was associated with increased PAI-1 levels, while PAI-2 concentrations approximated those of control samples and did not change over time [36]. In accordance, Günther et al. [30] also showed markedly reduced overall fibrinolytic capacity in BALF of patients with ARDS, with enhanced levels of PAI-1 (figure 4).

In a study on patients with sepsis and ARDS by Gando et al. [37], raised plasma levels of TF were not balanced by elevated levels of TFPI, i.e., the procoagulant activity was not adequately balanced by an anticoagulant activity. Probably high plasma levels of elastase were responsible for this imbalance, since elastase is able to degrade TFPI [37]. Unfortunately, these investigators did not measure levels of TF and TFPI in BALF of the studied patients. Although low levels of AT have been measured in BALF during sepsis-related lung injury in animals [32], no studies have been published that showed similar findings in humans with ARDS. Ware et al. [38] recently demonstrated decreased pulmonary levels of protein C in humans with ARDS. Compared with healthy individuals, patients with ARDS had lower levels of protein C in pulmonary edema fluid. It should be noted that Ware et al. did not measure APC, but its inactive form.

Pneumonia. Similar to ARDS, local activation of coagulation is seen during pneumonia. Günther et al. [30] showed increased procoagulant activity in BALF of patients with pneumonia, as compared with controls (figure 4). We recently demonstrated similar changes in pulmonary coagulation in patients that developed VAP: activation of coagulation was found
in non-directed bronchial lavages, while coagulation levels remained unchanged in lavage fluid from controls [39]. Activation of coagulation was attributable to elevated local levels of TF. In addition, we recently demonstrated that activation of coagulation is restricted to the site of infection, since in patients with unilateral community-acquired pneumonia [40], as well as in patients with unilateral VAP (chapter 5), activation of coagulation was only present in the affected lung. Levels of coagulation in the opposite, uninfected lung were similar to levels in non-ventilated lungs of healthy individuals.

In pneumonia, activation of coagulation is paralleled by inhibition of fibrinolysis. Günther et al. [30] have demonstrated less fibrinolytic activity in the lungs of patients with pneumonia. Alike the situation in ARDS, we found increased PAI-1 levels in the lungs of patients with CAP [41] and VAP [39]. Equally to activation of coagulation, inhibition of fibrinolysis is restricted to the site of infection, i.e., inhibition was only present in the infected lung. Interestingly, in VAP, inhibition of pulmonary fibrinolysis was already present before the diagnosis was made clinically.

Presently, it is unknown whether natural inhibitors of coagulation are downregulated in the pulmonary compartment during pneumonia. And, there are no studies on changes in pulmonary coagulation caused by mechanical ventilation.

Reciprocal Modulation of Inflammation and Coagulation

Inflammation and coagulation have tight interaction, i.e., they stimulate each other in both procoagulant and proinflammatory directions [3]. There are several ways in which coagulation is modulated by inflammatory mediators: (a) IL-6 stimulates TF expression [26,27,29]; (b) TNF indirectly stimulates TF expression by enhancing IL-6 production [42]; (c) TNF suppresses PAI-1 levels [43]; (d) TNF suppresses APC-activity [44]. Vice versa, coagulation factors are able to modulate the inflammatory response as well: (a) the TF-FVIIa complex stimulates release of various proinflammatory cytokines and chemokines, while factor Xa and thrombin have similar inflammatory effects [45-48]; (b) fibrin elicits an inflammatory response [1]; (c) decrease in APC-activity causes a rise in IL-6 levels, because of loss of inhibition of IL-6 release [49]; (d) natural coagulation inhibitors APC, AT, and TFPI all have distinct antiinflammatory effects [33].

Since there is reciprocal modulation of inflammation and coagulation, it can be speculated whether coagulation influences host defense during pneumonia. Our group has recently published some reports on the role of fibrinolysis factors in the pulmonary host defense. We showed that uPA receptor (uPAR) and uPA play an essential role in experimental murine
pneumonia [50]. uPAR deficiency was associated with reduced granulocyte recruitment in alveoli and lungs, more bacterial outgrowth, extensive dissemination of infection, and reduced survival. In contrast, uPA deficient mice showed enhanced host defense, associated with more neutrophil influx and less outgrowth of microorganisms. These results suggest that the function of uPAR in the response to infection is particularly pronounced when uPAR is unoccupied by uPA. The fact that these mechanisms are independent of fibrinolytic activity is illustrated by the fact that similar experiments in plasminogen or PAI-deficient mice showed no effect on the immune response to infection [41].

**Anticoagulant Therapy**

In recent years, anticoagulant strategies have extensively been evaluated in the treatment of sepsis to attenuate the detrimental effects of the procoagulant state. Encouraging results from animal models have stimulated human studies, but most could not demonstrate any treatment benefit. Indeed, most anticoagulant strategies, including AT [51] and TFPI [52], were not beneficial in the treatment of sepsis, while increasing the risk of bleeding [49,51,52]. Nevertheless, the PROWESS trial did show a beneficial effect of APC in sepsis, as reflected in an increase in survival rate while mildly attenuating the proinflammatory response [49].

Several cellular mechanisms have been proposed to clarify these *in vivo* effects. APC exerts many antiinflammatory effects *in vitro*, e.g., inhibition of TNF production by monocytes and macrophages, suppression of NF-κB expression, inhibition of cytokine

**Figure 5.** Proposed mechanisms of protease-activated receptor (PAR)-1 mediated regulation of vascular integrity. Thrombin (FIIa) disrupts the vascular barrier by PAR-1 cleavage (left). Activated protein C (APC) binds to the endothelial protein C receptor (EPCR) and has barrier protective effects either by direct sphingosine 1-phosphate receptor-1 (SIP1) cross-activation or indirectly via PAR-1 (right). Models are as proposed in [58, 59].
signalling, interference with cytokine-induced upregulation of cell surface leukocyte adhesion molecules and genes related to inflammation [53-56]. Riewald et al. proposed that PAR-1 was a major target of APC signalling [57], but it remained unclear how the same signalling receptor could possess both pro- and antiinflammatory effects, dependent of the protease involved. New studies suggest that APC bound to endothelial protein C receptor may exert protective effects on the vascular barrier via sphingosine 1-phosphate receptor-1 cross-activation (figure 5); either directly or via PAR-1 [58,59]. However, the \textit{in vivo} relevance of APC-mediated PAR-1 activation has been challenged [60], since the described cellular effects of APC \textit{in vitro} are at concentrations much higher than achieved during the treatment of severe sepsis. Compared to thrombin, approximately a thousand- to ten thousand-fold higher concentrations of APC is needed for PAR-1 activation [61]. Indeed, the antiinflammatory effects of APC in patients with sepsis were shown to be very modest [49,62].

Sepsis is a most important predecessor of ALI/ARDS [4], and as demonstrated in the most recent large sepsis trials, nearly half of the patients had disease involvement of the pulmonary compartment [49,51,52]. Sepsis and ARDS share many aspects of derangements of coagulation and inflammation. Therefore, encouraging results from the PROWESS trial have inspired several research groups to evaluate the role of anticoagulation in inflammatory lung disease. It is tempting to speculate whether the physiological inhibitors of coagulation can be beneficial for lung injury. APC has already proven beneficial in bleomycin-induced murine lung injury [63] and endotoxin-induced pulmonary vascular injury in rats [64]. Furthermore, APC prevented lung injury during bacteremia and endotoxiaemia in various animal models [64,65]. Currently, clinical trials are being set up to treat patients with pneumonia and ALI/ARDS with APC, and also with TFPI.

**Conclusion**

Extensive research has increased our understanding of the pathologic processes involved in inflammatory lung disease. Inflammation and coagulation should not be considered as separate independent processes, because they are closely related. In various disorders affecting the lung, proinflammatory cytokines are initiated by a local inflammatory response. Pulmonary inflammation contributes to a strong profibrotic state within the pulmonary compartment, resulting from the activation of coagulation and inhibition of both anticoagulant and fibrinolytic systems. Reciprocal activation of coagulation and inflammation leads to ongoing fibrosis. Anticoagulant strategies have been demonstrated to attenuate both coagulation and inflammation, and may be of clinical benefit in inflammatory lung disease.
References


Sepsis and Sepsis-Induced Acute Lung Injury
Timing of Drotrecogin alfa (activated) Treatment in Severe Sepsis

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Abstract

**Background.** Drotrecogin alfa (activated) (DrotAA) is currently the only registered therapy for the treatment of severe sepsis, but its clinical value has continuously been challenged. Early treatment, i.e., within 24 hours of appearance of first organ dysfunction, was recently shown to be associated with decreased mortality.

**Methods.** Forty patients with sepsis treated with commercial DrotAA were included, and dichotomized to early treatment (within 24 hours of first organ dysfunction) and late treatment (after 24 hours). Baseline characteristics and hospital mortality were compared between the groups.

**Results.** Twenty-nine patients were treated with DrotAA within 24 hours of first organ dysfunction, and 11 patients thereafter. Earlier treated patients were younger, and more often had pneumosepsis (45% versus 9%, \( p = 0.03 \)). There were no differences in hospital mortality rates (38% versus 36%, \( p = 0.93 \)).

**Conclusion.** This small observational study investigating the commercial use of DrotAA demonstrated that early treatment was not beneficial to patients with severe sepsis. Further studies are warranted to identify the most ideal candidates for DrotAA treatment.
Drotrecogin alfa (activated) (DrotAA), also known as recombinant human activated protein C (rhAPC), is currently the only US Food and Drug Administration (FDA)-approved therapy for the treatment of severe sepsis. Recently, the effect of timing of DrotAA treatment on outcome of severe sepsis was evaluated, using the integrated clinical trial database (INDEPTH), demonstrating an association between early treatment (i.e., within 24 hours of appearance of first organ dysfunction) and lower mortality [1]. We assessed timing of DrotAA treatment at our own (mixed) intensive care unit (ICU) to critically evaluate our own clinical experience with DrotAA.

Methods
This retrospective analysis covers a 3-year period since the commercial availability of DrotAA in the Netherlands. All DrotAA treated patients were selected from the Patient Data Management System (MetaVision, iMDsoft) from the Academic Medical Center, University of Amsterdam (Amsterdam, The Netherlands). DrotAA was prescribed in accordance with the national guideline [2]. The study protocol was approved by our medical ethical committee, informed consent was not required.

Baseline characteristics were collected, and time from first organ dysfunction to start of treatment was calculated. The whole patient group was dichotomized in patients who were treated within 24 hours from first organ dysfunction to start of DrotAA treatment (early treatment), and those who were treated after 24 hours (late treatment). Data was analyzed using Student’s t test or $\chi^2$ test, as appropriate (SPSS 12.0, SPSS).

Results
Forty patients were treated with DrotAA, 29 patients were treated within 24 hours and 11 patients thereafter. As shown in table 1, patients treated within 24 hours were younger, and more often had pneumosepsis (45% versus 9%, $p = 0.03$). In these patients, pneumonia was diagnosed as a community-acquired pneumonia in 12 of 14 cases (86%). Streptococcus pneumoniae was the most frequently involved pathogen in these cases of pneumonia (7 of 12 cases, 58%). Hospital mortality rates were comparable between early and late treatment (38% versus 36%, $p = 0.93$).
Discussion

In this small retrospective study evaluating DrotAA treatment practice in our ICU, early treated patients were younger and more often had community-acquired pneumonia. Notably, and in contrast to the analysis of the INDEPTH data, there were no differences in mortality between early and late treated patients. This may be due to the relatively small number of treated patients, but may also exemplify the fact that local practices do not necessarily match published data from large randomized trials.

Given that patients with a community-acquired pneumonia seem to benefit most from DrotAA treatment [3], it would have been interesting to identify differences in primary sites of infection between early and late treatment within the INDEPTH database. We could not investigate why our intensivists would initiate DrotAA treatment more rapidly in younger patients and patients with community-acquired pneumonia. The latter is particularly interesting because of the evidence in the literature suggesting that lung-specific effects of DrotAA contribute to better outcome in sepsis patients. First, DrotAA treatment has been

<table>
<thead>
<tr>
<th>Table 1. Baseline patient characteristics based on time to treatment</th>
<th>0 – 24 hrs (n = 29)</th>
<th>&gt;24 hrs (n = 11)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean ± SD)</td>
<td>51.5 ± 17.6</td>
<td>67.4 ± 9.9</td>
<td>0.008*</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>13 (45%)</td>
<td>3 (27%)</td>
<td>0.31*</td>
</tr>
<tr>
<td>APACHE II score (mean ± SD)</td>
<td>23.9 ± 5.5</td>
<td>26.9 ± 9.8</td>
<td>0.22*</td>
</tr>
<tr>
<td>Time from first organ dysfunction to start of treatment, hours (mean ± SD)</td>
<td>12.2 ± 6.8</td>
<td>45.7 ± 21.8</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Number of organ dysfunctions</td>
<td>3.6 ± 1.2</td>
<td>3.3 ± 1.3</td>
<td>0.55*</td>
</tr>
<tr>
<td>Mechanical ventilation, n (%)</td>
<td>25 (86%)</td>
<td>11 (100%)</td>
<td>0.19*</td>
</tr>
<tr>
<td>Vasopressors, n (%)</td>
<td>28 (97%)</td>
<td>10 (91%)</td>
<td>0.47*</td>
</tr>
<tr>
<td>Recent surgery, n (%)</td>
<td>8 (28%)</td>
<td>6 (55%)</td>
<td>0.13*</td>
</tr>
<tr>
<td>Primary site of infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory, n (%)</td>
<td>13 (45%)</td>
<td>1 (9%)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Abdominal, n (%)</td>
<td>8 (28%)</td>
<td>5 (45%)</td>
<td>0.28*</td>
</tr>
<tr>
<td>Urogenital, n (%)</td>
<td>2 (7%)</td>
<td>2 (18%)</td>
<td>0.29*</td>
</tr>
<tr>
<td>Other, n (%)</td>
<td>6 (2%)</td>
<td>3 (27%)</td>
<td>0.66*</td>
</tr>
</tbody>
</table>

APACHE II, Acute Physiology and Chronic Health Evaluation II; *Student’s t test; \(\chi^2\) test.

40
associated with a more rapid recovery from respiratory failure [4]. Second, sepsis patients with community-acquired pneumonia seemed to benefit most from DrotAA treatment [3]. And finally, several experimental studies point out that there were significant beneficial effects of DrotAA in the bronchoalveolar compartment [5,6].

The controversial FDA-approval of DrotAA in 2001 was largely based on the phase III randomized controlled trial PROWESS (Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) study, in which patients with severe sepsis had a 25% 28-day mortality rate with DrotAA versus 31% with placebo ($p = 0.005$) [7]. The single arm ENHANCE-US (Extended Evaluation of Recombinant Human Activated Protein C [United States]) study and the international ENHANCE study confirmed a 25 to 26% 28-day mortality rate with DrotAA treatment [8,9]. Yet the efficacy and safety of DrotAA have continuously been challenged [10]. In the PROWESS study a 3.5% serious bleeding risk was reported in the DrotAA treated patients versus 2.0% in the placebo group ($p = 0.06$) [7]. In the ENHANCE trials, the risk of serious bleeding was 4.0% and 6.5% respectively [8,9]. And furthermore, the ADDRESS (Administration of Drotrecogin Alfa [Activated] in Early Stage Severe Sepsis) study demonstrated that mortality of low-grade sepsis was 19% with DrotAA versus 17% without ($p = 0.34$), with increased bleeding risk: 3.9% versus 2.2% ($p = 0.01$). Currently, critical care experts agree that DrotAA should only be given to patients with high risk of death from severe sepsis [11].

In conclusion, in this small observational study investigating the commercial use of DrotAA we demonstrated that early treatment was not particularly beneficial to patients with severe sepsis. In times when prescription rates of DrotAA seem to be correlated more with reimbursement than the number of sepsis patients [12], additional studies are needed to identify the most ideal candidates for DrotAA treatment. More specifically, studies are warranted to investigate what mechanisms are involved in the lung-protective effects of DrotAA during acute lung injury and pneumonia.
References

Natural Anticoagulants Limit LPS-induced Pulmonary Coagulation but not Inflammation

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submitted for publication
Abstract

Background. Pulmonary coagulopathy and hyperinflammation may contribute to an adverse outcome in sepsis. We sought to determine the effects of natural inhibitors of coagulation on bronchoalveolar hemostasis and inflammation in a rat model of endotoxemia.

Methods. Male Sprague-Dawley rats were randomized to treatment with normal saline, recombinant human activated protein C (APC), plasma-derived antithrombin (AT), recombinant human tissue factor pathway inhibitor (TFPI), heparin, or recombinant tissue-type plasminogen activator (tPA). Rats were intravenously injected with lipopolysaccharide (LPS), inducing a systemic inflammatory response and pulmonary inflammation.

Results. At 4 and 16 hours after LPS injection, blood and bronchoalveolar lavage were obtained and markers of coagulation and inflammation were measured. LPS injection caused an increase in levels of thrombin-antithrombin complexes, whereas plasminogen activator activity was attenuated, both systemically and within the bronchoalveolar compartment. Administration of APC, AT, and TFPI significantly limited LPS-induced generation of thrombin-antithrombin complexes in the lungs, and tPA stimulated pulmonary fibrinolytic activity. Yet, none of the agents had significant effects on the production of pulmonary cytokines, chemokines, neutrophil influx, and myeloperoxidase activity.

Conclusion. Natural inhibitors of coagulation prevent bronchoalveolar activation of coagulation, but do not induce major alterations of the pulmonary inflammatory response in rat endotoxemia.
Severe sepsis is a clinical syndrome characterized by a systemic inflammatory response and activation of coagulation, potentially leading to intravascular depositions of fibrin and microvascular thrombosis, or bleeding related to uncontrolled consumption of coagulation factors [1]. Under physiological circumstances, activation of coagulation is regulated by natural inhibitors of coagulation, i.e., activated protein C (APC), antithrombin (AT), and tissue factor pathway inhibitor (TFPI). In sepsis, these anticoagulant systems are impaired, most likely due to massive consumption and downregulation by inflammatory mediators; which forms the rationale for therapeutic restoration of these natural anticoagulant pathways [2]. Unfortunately, both plasma-derived AT [3] and recombinant human (rh)TFPI [4] have failed to reduce patient mortality in severe sepsis. The pivotal phase III clinical trial with rhAPC did show a significant increase in patient survival [5], but there is ongoing debate on the exact mechanisms by which APC prevents death.

One of the proposed mechanisms in which APC exerts its protective effects relates to pathways involving the lungs. The lungs are the most frequently involved organ system in sepsis-related multiple organ failure. Notably, APC treatment causes more rapid resolution of respiratory failure during sepsis [6] and limits both coagulation and influx of neutrophils in the pulmonary compartment in experimental lung injury [7,8]. In a relatively limited number of patients with sepsis, lung-protective effects have also been suggested during treatment with AT [9] and TFPI [10], but these were not confirmed in larger phase III clinical trials [3,4]. In the current study, using a rat model of endotoxemia, it was hypothesized that APC, AT, and TFPI would have differential effects on pulmonary coagulation and inflammation, potentially explaining different outcomes in human sepsis.

Material and Methods

Rats

Male Sprague-Dawley rats (200-250 g) were purchased from Harlan (The Hague, The Netherlands). The rats were allowed to acclimatize to laboratory conditions for at least 7 days (12:12 hrs day-night cycle at 22°C). The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.
Study Design

Endotoxemia was induced by 7.5 mg/kg lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO), administered into the penile vein under isoflurane (3%) anesthesia. Rats were randomized to placebo (normal saline) or treatment with one of the natural anticoagulants: APC, AT, or TFPI (n = 8 per group). Additionally, heparin and tissue-type plasminogen activator (tPA) were involved in the randomization process. Four healthy rats were used as controls without endotoxemia. All agents were administered in bolus injections of 2 mL/kg, 30 minutes before injection of LPS. Therapeutic doses were determined using data from previous studies [4,10-14]. All used agents and doses are described in Table 1. Considering the plasma clearance, rats sacrificed at 16 hrs after LPS injection received additional injections of APC, TFPI, heparin, or tPA at 6 and 12 hrs; all rats were administered the same volume of fluid (2 mL/kg) at 6 and 12 hrs.

---

**Table 1. Pharmaceutical agents and used doses**

<table>
<thead>
<tr>
<th>Biological equivalent</th>
<th>Abbr.</th>
<th>Agent</th>
<th>Company</th>
<th>Dose</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated protein C</td>
<td>APC</td>
<td>Drotrecogin alfa (activated)</td>
<td>Eli Lilly (Indianapolis, IN)</td>
<td>500 mg/kg</td>
<td>[12]</td>
</tr>
<tr>
<td>Antithrombin (III)</td>
<td>AT</td>
<td>Plasma-derived antithrombin III</td>
<td>Baxter (Vienna, Austria)</td>
<td>250 U/kg</td>
<td>[12,13]</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor</td>
<td>TFPI</td>
<td>Tifacogin</td>
<td>Chiron (Emeryville, CA)</td>
<td>1.5 mg/kg</td>
<td>[4,10,11]</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>heparin</td>
<td>Unfractionated heparin</td>
<td>Leo Pharma (Ballerup, Denmark)</td>
<td>300 U/kg</td>
<td>[12]</td>
</tr>
<tr>
<td>Tissue-type plasminogen activator</td>
<td>tPA</td>
<td>Tenecteplase</td>
<td>Boehringer Ingelheim (Ingelheim, Germany)</td>
<td>1.25 mg/kg</td>
<td>[14]</td>
</tr>
</tbody>
</table>

Abbr. = abbreviation. Refs. = references used for estimating therapeutic doses.

At 4 and 16 hrs after LPS injection, rats were sacrificed with intraperitoneal injections of ketamine 80 mg/kg (Eurovet, Bladel, The Netherlands) and medetomidine 0.5 mg/kg (Novartis, Arnhem, The Netherlands). Blood was collected from the inferior *vena cava* in citrated (0.109 M) vacutainer tubes. The right lung was ligated, and the left lung was lavaged three times with 2 mL ice-cold normal saline. Right lungs were weighed and homogenized in 4 volumes (i.e., 4 × lung weight [mg] in mL) of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Total cell number in each lavage sample were
determined by an automated cell counter (Coulter Counter, Coulter Electronics, Hialeah, FL). Neutrophil counts in lavage fluids were performed on Giemsa-stained cytospin preparations.

For coagulation assays, plasma and cell-free supernatants from bronchoalveolar lavage were used. For cytokine and chemokine measurements in lungs, supernatants were used from lung homogenates diluted 1:1 in lysis buffer, containing 150 mM NaCl, 15 mM Tris, 1 mM MgCl₂, 1 mM CaCl₂, 1% Triton, and pepstatin A, leupeptin, and aprotinin (all 20 ng/mL).

**Assays**

Thrombin-antithrombin complexes (TATc; Behring, Marburg, Germany) and fibrin degradation products (FDP; Asserachrom D-Di, Diagnostica Stago, Asnières-sur-Seine, France) were measured using ELISA. Antithrombin (AT), plasminogen activator activity (PAA), and plasminogen activator inhibitor (PAI)-1 activity were measured by automated amidolytic assays [15-17]. Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant (CINC)-3 were measured using ELISA (R&D Systems, Abingdon, United Kingdom). Myeloperoxidase (MPO) activity was determined by measuring the H₂O₂-dependent oxidation of 3,3',5',5' tetramethylbenzidine, and was expressed as activity per gram lung tissue [18].

**Statistical analysis**

Comparisons between the experimental groups and saline-treated placebo group were performed using one-way ANOVA or Kruskal-Wallis test, followed by post-hoc Dunnett’s or Dunn’s tests. A p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL) and Prism 4.0 (GraphPad Software, San Diego, CA).

**Results**

**Coagulation and Fibrinolysis**

Compared to controls which were not injected with LPS, endotoxemia caused increased generation of TATc (figure 1), an effect which was attenuated by APC, AT, and TFPI at both 4 and 16 hrs after LPS injection (figure 1A). Plasma PAA was significantly decreased after LPS injection; tPA increased PAA to levels exceeding assay maximum at both timepoints.
Figure 1. The effects of anticoagulants on plasma levels of thrombin-antithrombin complexes (A, TATc) and systemic plasminogen activator activity (B, PAA), 4 and 16 hrs after injection of lipopolysaccharide (LPS, Escherichia coli O111:B4).

Data represent mean ± SEM. Dunnett’s test: *p < 0.05, †p < 0.01, ‡p < 0.001 all versus saline.

Figure 2. The effects of anticoagulants on levels of thrombin-antithrombin complexes (A, TATc), antithrombin activity (B, AT in international units [IU]), and fibrin degradation products (C, FDP) in bronchoalveolar lavage fluid (BALF), 4 and 16 hrs after injection of lipopolysaccharide (LPS, Escherichia coli O111:B4). Data represent mean ± SEM. Dunnett’s test: *p < 0.05, †p < 0.01, ‡p < 0.001, all versus saline.

Figure 3. The effects of anticoagulants on plasminogen activator activity (A, PAA) and levels of plasminogen activator inhibitor type 1 (B, PAI-1) in bronchoalveolar lavage fluid (BALF), 4 and 16 hrs after injection of lipopolysaccharide (LPS, E. coli O111:B4).

Data represent mean ± SEM. Dunnett’s test: ¹p < 0.01, ‡p < 0.001, all versus saline.
With AT treatment, plasma PAA was also significantly higher than with saline at both timepoints (figure 1B).

Moreover, bronchoalveolar levels of TATc were increased by endotoxemia (figure 2). APC, AT, and TFPI limited LPS-induced generation of bronchoalveolar TATc (figure 2A), also preventing a decrease in AT activity and limiting FDP generation (figure 2B-C). Heparin-treated rats also showed less bronchoalveolar TATc, but only at 4 hrs (figure 2A), while AT activity was not different from saline-treated rats (figure 2B). Pulmonary PAA in lungs was significantly reduced by endotoxemia, with concurrently enhanced PAI-1 activity in lungs (figure 3). tPA increased bronchoalveolar PAA and FDP levels (figure 3A and 2C), while abolishing enhanced PAI-1 activity (figure 3B).

**Inflammatory Response**

During rat endotoxemia, TFPI treatment enhanced IL-6 generation in lungs at 16 hrs (figure 4B); all other agents did not induce significant changes in pulmonary levels of TNF-α, IL-6, and CINC-3. Inflammatory cell influx into the lungs was not observed at 4 hrs after LPS injection. There was an increase in neutrophil counts in the lungs during endotoxemia at 16 hrs, but there were no differences in the number of neutrophils between the anticoagulant groups and the saline group (table 2). Finally, myeloperoxidase (MPO) in lung tissue was not influenced by any treatment (table 2).
We have shown that the application of systemic anticoagulant agents alters bronchoalveolar coagulation during endotoxemia. APC, AT, and TFPI had major effects on limiting thrombin generation, both systemically and in the bronchoalveolar compartment. However, the various agents had very limited effects on pulmonary inflammation, suggesting that the inflammatory response is not significantly directed by changes in pulmonary coagulation itself, at least not in this rat model of endotoxemia. The question what mechanisms account for differences in clinical outcome with various anticoagulant strategies remains unresolved.

In the pathogenesis of endotoxin-induced lung injury, an important role has been attributed to neutrophils. Uchiba et al. demonstrated that endotoxin-induced pulmonary vascular injury in Wistar rats is mainly mediated by activated neutrophils [19]. Using this model, it was shown that APC [20], AT with or without heparin co-administration [21], and TFPI [22] all had inhibitory effects on activated neutrophils, thereby limiting neutrophil accumulation, MPO activity, and cytokine generation in the lungs in the first hour after endotoxin administration. Also, in healthy volunteers challenged with intrapulmonary endotoxin, it was shown that neutrophil influx is significantly inhibited by rhAPC infusion [7]. In our rat model, neutrophil accumulation and MPO activity in lungs were not significantly altered by any of the administered agents. This discrepancy with previous studies may have been caused by

### Table 2. Total cell and neutrophil counts in bronchoalveolar lavage fluid / MPO activity in lungs

<table>
<thead>
<tr>
<th></th>
<th>Total cells</th>
<th>Neutrophils</th>
<th>MPO (units / g lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>18.2 (15.4-20.5)</td>
<td>0</td>
<td>1.8 (1.1-2.8)</td>
</tr>
<tr>
<td>Endotoxemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n = 12)</td>
<td>38.2 (27.7-55.4)*</td>
<td>2.7 (0.1-20.2)</td>
<td>14.0 (11.7-16.2)</td>
</tr>
<tr>
<td>APC (n = 8)</td>
<td>32.0 (26.3-47.3)</td>
<td>8.9 (4.0-37.2)</td>
<td>11.5 (8.5-14.7)</td>
</tr>
<tr>
<td>AT (n = 8)</td>
<td>50.1 (32.9-71.9)</td>
<td>4.3 (3.8-23.8)</td>
<td>12.0 (10.4-13.9)</td>
</tr>
<tr>
<td>TFPI (n = 8)</td>
<td>39.1 (36.9-46.7)</td>
<td>6.1 (4.1-24.1)</td>
<td>14.6 (13.2-14.8)</td>
</tr>
<tr>
<td>Heparin (n = 8)</td>
<td>22.5 (20.5-41.9)</td>
<td>7.4 (2.1-14.6)</td>
<td>13.4 (12.3-18.8)</td>
</tr>
<tr>
<td>tPA (n = 8)</td>
<td>21.5 (18.1-28.9)</td>
<td>5.3 (1.3-11.6)</td>
<td>14.2 (12.4-15.7)</td>
</tr>
</tbody>
</table>

Cell counts 16 hrs after injection with lipopolysaccharide (Escherichia coli O111:B4). Controls are uninfected rats (n = 4). Data are expressed as median (interquartile range) × 10⁴ per milliliter of bronchoalveolar lavage fluid. MPO, myeloperoxidase.

*Kruskal-Wallis test, \( p = 0.008 \); Dunn’s post-tests \( p > 0.05 \), all versus saline.

### Discussion

We have shown that the application of systemic anticoagulant agents alters bronchoalveolar coagulation during endotoxemia. APC, AT, and TFPI had major effects on limiting thrombin generation, both systemically and in the bronchoalveolar compartment. However, the various agents had very limited effects on pulmonary inflammation, suggesting that the inflammatory response is not significantly directed by changes in pulmonary coagulation itself, at least not in this rat model of endotoxemia. The question what mechanisms account for differences in clinical outcome with various anticoagulant strategies remains unresolved.

In the pathogenesis of endotoxin-induced lung injury, an important role has been attributed to neutrophils. Uchiba et al. demonstrated that endotoxin-induced pulmonary vascular injury in Wistar rats is mainly mediated by activated neutrophils [19]. Using this model, it was shown that APC [20], AT with or without heparin co-administration [21], and TFPI [22] all had inhibitory effects on activated neutrophils, thereby limiting neutrophil accumulation, MPO activity, and cytokine generation in the lungs in the first hour after endotoxin administration. Also, in healthy volunteers challenged with intrapulmonary endotoxin, it was shown that neutrophil influx is significantly inhibited by rhAPC infusion [7]. In our rat model, neutrophil accumulation and MPO activity in lungs were not significantly altered by any of the administered agents. This discrepancy with previous studies may have been caused by
differences in endotoxin delivery, species, investigated timepoints, and the subsequent different roles for neutrophil involvement in the experimental models.

Heparin had been shown to exert lung protective effects during endotoxemia in sheep [23], and low-molecular weight heparin had been able to limit lung injury in endotoxemic swine [24] and mice [25]. In our experiment, heparin had a mild anticoagulant effect and a modest antifibrinolytic effect: pulmonary TATc and systemic PAA were decreased at 4 hrs after LPS injection, while pulmonary PAI-1 activity was increased after 16 hrs; this in contrast to in vitro data suggesting that heparins have profibrinolytic effects [26]. Notably, treatment with heparin did not result in altered inflammatory response within the lungs. Opposing the anticoagulant strategies, tPA was used to stimulate fibrinolysis. Increased fibrinolytic activity did not affect neutrophil influx into the lungs, nor did it alter generation of inflammatory mediators.

A most important limitation of the study is the rat model of endotoxemia. Lung injury models with either direct endotracheal instillation of endotoxin or via endotoxemia have routinely been used in experimental studies because of its relative ease and good reproducibility. It should be noted that endotoxin-induced lung injury is a simplified model of patients with pneumonia or sepsis with acute lung injury. If acute lung injury is related to infectious processes, interference with coagulation could theoretically limit containment of the primary infection, promoting microbial dissemination [27].

In conclusion, in a rat model of endotoxemia, we have demonstrated that systemic application of natural anticoagulants significantly inhibits bronchoalveolar activation of coagulation, but does not lead to distinct effects on cytokine and chemokine production or neutrophil migration and activity. It remains uncertain if controlled pulmonary coagulation significantly contributes to patient outcome, and needs to be established in appropriate clinical trials.

Acknowledgments

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References

Pneumonia
Disturbed Alveolar Fibrin Turnover during Pneumonia is Restricted to the Site of Infection

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J.W. Olivier van Till
Paul Bresser
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Marcel Levi
Tom van der Poll

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Abstract

Background. Severe infection is associated with profound alterations in the systemic hemostatic balance, with activation of coagulation and suppressed fibrinolysis. Within the alveolar compartment, similar disturbances have been described during pulmonary inflammation. We investigated whether local hemostasis is influenced during ventilator-associated pneumonia (VAP).

Methods. In 5 patients with unilateral VAP, bronchoalveolar lavage fluid was obtained from both the infected site (as identified on chest radiography) and the contralateral non-infected lung (with no clinical or radiographic abnormalities). Markers for coagulation and fibrinolysis were compared between infected and non-infected lungs. Ten healthy volunteers and 10 mechanically ventilated patients without pneumonia served as controls.

Results. Strong activation of coagulation (high levels of thrombin-antithrombin complexes, soluble tissue factor, factor VIIa) was detected in lavage fluid from infected lungs, compared to that from non-infected lungs and controls. Furthermore, in infected lungs fibrinolysis was depressed, with high levels of plasminogen activator inhibitor type 1.

Conclusion. VAP is characterized by a hypercoagulant state, mainly restricted to the site of infection.
Sepsis in critically ill patients is almost invariably associated with a disturbed hemostatic balance: increased expression of tissue factor (TF) promotes activation of the coagulation pathway; concordantly, fibrinolytic activity is suppressed, mainly by upregulation of plasminogen activator inhibitor type 1 (PAI-1), the main inhibitor of plasminogen activation [1]. The most severe clinical manifestation of these alterations is a syndrome known as disseminated intravascular coagulation, characteristically associated with intravascular deposition of fibrin [2]. Remarkably similar alterations in fibrin turnover have been demonstrated on a local level during pulmonary bacterial infections, leading to fibrin deposits in the interstitial and alveolar spaces of the lung [1,3-5]. We recently described the clinical course of ventilator-associated pneumonia (VAP), in which procoagulant activity was increased within the pulmonary compartment, and fibrinolysis was decreased [3]. While this longitudinal study examined hemostatic alterations in fluid obtained from the airways in a non-directed way, in the present study we sought to determine whether the disturbed fibrin turnover is restricted to the site of infection. For this, bilateral bronchoalveolar lavage (BAL) was performed on patients with unilateral VAP, and markers of coagulation and fibrinolysis in BAL fluid (BALF) from the infected lung were compared with that from the contralateral non-infected lung. Healthy volunteers and mechanically ventilated patients without pneumonia served as controls.

Material and Methods

Patients

The study protocol was reviewed and approved by the Medical Ethics Committee of the University of Amsterdam. Written informed consent was obtained from all subjects or their legal representatives. Five consecutive patients with unilateral VAP were investigated, fulfilling the following criteria: fever or hypothermia (< 35.0 or > 37.7 °C), leukocytosis or leukopenia (< 3 or > 10 × 10⁹/L), worsening of PaO₂/FiO₂-ratios, and a novel unilateral infiltrate on chest radiography. The diagnosis had to be supported by the results of microbiological culture of the BALF, or a clinical course consistent with VAP. For safety reasons, patients were excluded if PaO₂ ≤ 10 kPa while FiO₂ ≤ 0.60. Ten mechanically ventilated patients without any signs of pulmonary infection, and 10 healthy individuals (mean ± SEM, 32 ± 8 years old) served as controls.
Sampling and Specimen Processing

BALF was obtained in a standardized fashion according to the guidelines of the American Thoracic Society, using a flexible fiberoptic videobronchoscope. Seven successive 20 ml aliquots of prewarmed 0.9% saline were instilled in a subsegment of the lung and each aspirated immediately with low suction (general recovery: 10-15 mL). BAL was initiated at the non-infected lung in a subsegment of the middle lobe or lingula, followed by a lavage of a subsegment of the infected lobe, as localized on chest radiography. Recovery from the infected and non-infected sites did not differ. In all control subjects, BALF was obtained from the right middle lobe. Blood samples were drawn into sterile vacutainer tubes containing citrate, prior to lavage. The first aliquot was discarded; the second and third BALF recoveries from both sites were sent for microbial culture and virus isolation. The remaining BALF was centrifuged at 3000 rpm for 15 minutes at 4 °C, as well as the blood samples. Supernatants were stored at –80 °C until assays were performed.

Measurements

Thrombin-antithrombin complex (TATc), soluble TF (sTF), PAI-1, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 concentrations were measured using specific commercially available ELISAs according to the manufacturer’s instructions (TATc: Behringwerke AG, Marburg, Germany; sTF: American Diagnostics, Greenwich, CT; PAI-1: TintElize PAI-1, Biopool, Umea, Sweden; TNF-α and IL-6: Sanquin Diagnostics, Amsterdam, Netherlands). Levels of factor VIIa (FVIIa) were determined by an enzyme capture assay for determining FVIIa activity, urokinase-type plasminogen activator (uPA) levels by means of ELISA, and plasminogen activator activity (PAA) using an amidolytic assay, all as described before [3].

Statistical Analysis

Wilcoxon signed-rank test was used to compare paired BALF-samples from infected and non-infected lungs. Differences between patients with VAP and controls were analyzed using Mann-Whitney U test. BALF and plasma levels are expressed as median with interquartile range (IQR), unless otherwise stated. A $p$-value of 0.05 or less was considered statistically significant.
Results

Patient characteristics are presented in table 1. All patients with VAP underwent two-sided BAL within 12 hrs of fulfilling the inclusion criteria, and recovered uneventfully with antibiotic therapy. All patients received low molecular heparin for deep venous thrombosis-prophylaxis. No other anticoagulant medication was given. Mechanically ventilated controls, matched in age, ventilation duration, and mechanical ventilator settings, showed no signs of pulmonary infection throughout their clinical course. Mechanical ventilation settings were as follows: in all patients tidal volumes were kept between 6 and 8 mL/kg using a pressure controlled or pressure support mode; positive end-expiratory pressure (PEEP) levels were set according to a strict protocol, in which optimal PEEP was defined as the lowest level of PEEP with maximum PaO₂. Levels of PEEP were 9.0 (8.0-11.5) cm H₂O in patients with VAP, and 8.0 (7.3-13.0) cm H₂O in mechanically ventilated controls ($p = 0.9$).

Table 1. Clinical and biochemical parameters of mechanically ventilated patients

<table>
<thead>
<tr>
<th>VAP patients</th>
<th>Sex</th>
<th>Age</th>
<th>Duration of MV (days)</th>
<th>Site of infiltrate</th>
<th>BALF-culture</th>
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<tr>
<td>1</td>
<td>M</td>
<td>51</td>
<td>11</td>
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<td><em>Klebsiella pneumoniae, E. coli</em></td>
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<td>75</td>
<td>6</td>
<td>Left lower lobe</td>
<td>Polymicrobial flora</td>
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</table>

Mechanically ventilated control patients

<table>
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<th>Site of infiltrate</th>
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</tbody>
</table>

BALF, bronchoalveolar lavage fluid; MV, mechanical ventilation.
*a*site of infiltrate was determined by chest radiography.
Figure 1. Left panels. Concentrations of procoagulant markers in bronchoalveolar lavage fluid (BALF): thrombin-antithrombin complex (TATc), soluble tissue factor (sTF), and factor VIIa (FVIIa). Right panels. BALF levels of fibrinolytic markers: plasminogen activator activity (PAA), plasminogen activator inhibitor-1 (PAI-1), and urokinase-type plasminogen activator (uPA).

Comparisons between infected and non-infected lungs (interconnected for the same patient) were performed with Wilcoxon signed-rank test for paired samples; differences between VAP and control patients were analyzed using Mann-Whitney $U$ test: *$p < 0.05$; †$p < 0.01$; ‡$p < 0.001$.

Some symbols may not be entirely visible due to data overlap.
Pulmonary Levels of Coagulation and Fibrinolysis Activity

BALF levels of coagulation and fibrinolysis activity are presented in figure 1. In VAP patients, levels of TATc, sTF, and FVIIa were significantly higher in BALF from the infected lung, as compared to BALF levels from the contralateral non-infected lung. Levels of coagulation in the non-infected lungs were higher than in BALF from control subjects. In VAP patients, PAA was significantly lower in infected lungs, while concentrations of PAI-1 and uPA were significantly higher. In non-infected lungs from VAP patients PAA was lower than in BALF from control subjects. PAI-1 and uPA concentrations were significantly lower in controls, as compared with levels from non-infected lungs in VAP patients.

Systemic Activity of Coagulation and Fibrinolysis

In plasma, TATc levels were similar in patients with VAP and mechanically ventilated controls (9.2 [8.0-9.8] and 7.1 [6.3-9.2] mU/mL, respectively), but levels were significantly higher than in healthy subjects (4.7 [3.0-5.2] mU/mL, both p < 0.01). Plasma levels of PAA were equal in all groups (median 101%).

Pulmonary Inflammatory Mediators

Concentrations of TNF-α were very low or undetectable in BALF from all patients and healthy subjects (no difference between groups). IL-6 levels in BALF showed a large variation; the highest concentrations were found in BALF from infected lungs (574 [102-1399] pg/mL); in BALF from non-infected lungs, levels were 83.4 (20.1-559) (p < 0.05 versus infected lungs), from mechanically ventilated controls, 153 (75.0-314) pg/mL (p = 0.44 versus infected lungs), and from healthy individuals, 20.3 (16.7-73.4) pg/mL (p = 0.05 versus infected lungs).

Discussion

In the present study, patients with VAP had a dramatic increase in local procoagulant activity, and a simultaneous depression of fibrinolysis. The procoagulant shift of the hemostatic balance occurred in a compartmentalized fashion, i.e., disturbed fibrin turnover was restricted to the site of infection. Although our study involved only 5 patients with unilateral VAP, the data derived from lavages of the infected site showed no overlap with that from lavages of the uninfected lung or control subjects. While BALF obtained from the non-infected lung of
patients with VAP displayed modest coagulation disturbances, compared to BALF from control patients, spill of BALF from the infected to the non-infected site was unlikely, since the twosided BAL was consistently started with lavage of the non-infected lung. Hence, these data indicate that a unilateral VAP results in moderate coagulation disturbances in the non-infected lung mirroring the more pronounced hypercoagulant state in the contralateral infected lung.

Mechanical ventilation by itself may initiate – or aggravate – pulmonary inflammation [6]. However, in mechanically ventilated patients without pneumonia there was no activation of coagulation and inhibition of fibrinolysis, while being ventilated with the same mechanical ventilation mode and settings. Furthermore, similar changes in fibrin turnover have been found in spontaneously breathing patients with unilateral community-acquired pneumonia [4]. Therefore, we favor the explanation that enhanced fibrin formation was triggered by infection, and not mechanical ventilation.

The TF-FVIIa pathway is essential for activation of the blood coagulation system during systemic bacterial infection [2,7]. TF has also been implicated in the procoagulant response in the lungs, being present at multiple sites in the airways, including the vascular adventitia, epithelial cells, and alveolar macrophages. Inhibition of the TF-FVIIa pathway completely prevented intrapulmonary fibrin deposition and diminished lung inflammation after intratracheal delivery of endotoxin in rats [8]. In addition, TF inhibition attenuated lung inflammation and injury in experimental endotoxemia and bacteremia [9]. We here demonstrated that activation of the TF-FVIIa pathway was detected in patients with VAP at the site of the infection. These findings suggest that local activation of the TF-FVIIa pathway contributes to activation of coagulation at the site of the infection during VAP, and that inhibition of TF-FVIIa pathway could potentially be beneficial to patients with pulmonary inflammatory disease.

There has been much interest in the interaction between coagulation and inflammation in the lung: several reports have been published on disturbed fibrin formation in both patients with VAP, community acquired pneumonia, and acute lung injury [3-5]. Proinflammatory cytokines increase expression of TF, activating the coagulation cascade, while fibrin itself has been shown to enhance the inflammatory response [1]. We chose to measure IL-6 and TNF-α because they play an important role in activation of coagulation and attenuation of fibrinolysis respectively during sepsis [7]. However, in the present study, TNF-α levels in BALF were either not detectable or very low, and varied widely, as previously demonstrated in both developing VAP and established CAP [10,11]. Likewise, concentrations of IL-6 showed a
large variation. Although differences between infected and uninfected lungs in patients with VAP were statistically significant, local IL-6 levels in the infected lungs were not different from levels in mechanically ventilated controls without pneumonia. Apparently, during pulmonary inflammation IL-6 and TNF-α are not the major determinants in activation of coagulation and depression of fibrinolysis within the pulmonary compartment.

In conclusion, VAP is characterized by a local procoagulant shift in the hemostatic balance of the airways. The imbalance is mainly restricted to the infected pulmonary compartment, and is the net result of increased TF-FVIIa mediated coagulation activity, together with inhibition of fibrinolysis, related to high PAI-1 levels. Future studies are warranted to examine whether inhibition of the hypercoagulant response in the lung during pneumonia has a beneficial effect.

References

Clinical and Hemostatic Responses to Treatment in Ventilator-Associated Pneumonia: Role of Bacterial Pathogens

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Goda Choi
Marcus J. Schultz
Lilibeth A. Pineda
Corey Mankowski

Crit Care Med 2007; 35: 490-6
Abstract

Objective. To determine pathogen-specific kinetic changes in the alveolar procoagulant activity, tissue factor (TF), and tissue factor pathway inhibitor (TFPI) expression during the course of ventilator-associated pneumonia (VAP) and to assess the relationship between clinical resolution, intraalveolar bacterial eradication, and restoration of hemostatic balance.

Methods. Thirty-five patients with microbiologically documented VAP who received adequate antimicrobial coverage and 13 controls were included. Non-directed bronchoalveolar lavage was performed at the onset of VAP, and on days 4 and 8 after initiation of antibiotic therapy. Samples were assayed for procoagulant activity, TF, TFPI, and thrombin-antithrombin complex (TATc). The corresponding Clinical Pulmonary Infection Score (CPIS) was collected simultaneously.

Results. Isolated pathogens included Pseudomonas aeruginosa (n = 13), methicillin-resistant Staphylococcus aureus (MRSA) (n = 8), methicillin-sensitive S. aureus (MSSA) (n = 7), and Escherichia coli (n = 7). Although procoagulant activity and TF were increased among the various pathogens at the onset of VAP, the levels of those with P. aeruginosa remained elevated at end of treatment compared with controls and other etiologic agents. TFPI levels were elevated for the duration of the study for all pathogens. A universal increase in TATc was noted at the onset of VAP but the difference among the group of pathogens was significant at day 4 and 8 post therapy. Despite the persisting hemostatic imbalance and incomplete intraalveolar eradication of P. aeruginosa at end of therapy, the CPIS fell comparably at each time point irrespective of the etiologic agents.

Conclusion. Alveolar activation of the TF-dependent pathway may be species-specific in VAP and may not be adequately balanced by TFPI. The disparity between clinical response and eradication of P. aeruginosa from the intraalveolar space suggests the need for biological markers to guide response to therapy.
Intraalveolar fibrin deposition is a characteristic histopathologic feature of a variety of human lung diseases [1] and experimental animal models of inflammatory pulmonary syndromes [2,3]. Under physiologic conditions, the alveolar space has net fibrinolytic activity due to the presence of urokinase-type plasminogen activator [4]. However, during acute lung injury and chronic inflammatory lung disorders, the balance between coagulation and fibrinolysis is perturbed in favor of an increased local procoagulant activity resulting in intraalveolar fibrin deposits [5,6]. These fibrin deposits enhance inflammatory response by mediating the release of proinflammatory cytokines, enhancing vascular permeability, and promoting alveolar infiltration of inflammatory cells [7,8].

Several studies have focused on the coagulation imbalance in patients with ventilator-associated pneumonia (VAP) [9-11]. The coagulation activation which is primarily mediated by the extrinsic pathway has been shown to coincide with a reduced fibrinolytic capacity. Both tissue factor (TF) and factor VIIa levels are increased in the bronchoalveolar lavage (BAL) fluid of patients with VAP while the fibrinolytic processes are inhibited via a simultaneous rise in plasminogen activator inhibitor type 1 levels [10]. Furthermore, thrombin antithrombin complexes (TATc) are more pronounced in VAP patients who receive inadequate antimicrobial therapy compared with those who develop VAP caused by organisms initially susceptible to the antibiotic regimens prescribed [12]. Given the fact that TF pathway is modulated by a balance between TF and tissue factor pathway inhibitor (TFPI), increased levels of TFPI have been reported in plasma and BAL of patients with acute respiratory distress syndrome (ARDS) [13,14] and bacterial pneumonia [15]. The systemic activation of TF-dependent pathway was found in these cases to be inadequately counterbalanced by the TFPI levels. In patients with VAP, the intrinsic phenotype of bacterial pathogens may alter the balance of the coagulation cascade by modulating TF and TFPI expression independently. Until now, no study has evaluated systematically the potential role of individual pathogens in altering the coagulative balance inside the alveolar space following antimicrobial therapy, nor has any investigation tackled the relationship between clinical improvement and restoration of hemostatic balance in function of bacterial isolates.

The current study was undertaken to characterize the temporal changes in the alveolar procoagulant activity, TF, and TFPI expression caused by specific pathogens commonly isolated during the course of VAP and to investigate the relationship between clinical resolution, intraalveolar bacterial eradication, and restoration of hemostatic balance.
**Material and Methods**

**Study Population**

A prospective case-control study was conducted in the medical intensive care units of 2 university-affiliated tertiary care centers between 2003 and 2005. Some of the subjects included in this trial were part of previous investigations [10,12]. Inclusion criteria included mechanically ventilated patients ≥ 18 yrs of age and older for ≥ 72 hrs and a clinical picture compatible with pneumonia. VAP was suspected when a new or progressive infiltrate was present along with at least two of the following signs and symptoms: (a) purulent respiratory secretions; (b) fever, defined as body temperature ≥ 38 °C or hypothermia defined as body temperature ≤ 35 °C; and/or (c) leukocytosis defined as white blood cell count ≥ 10,000 /mm³ or leukopenia with total white blood cell count < 4,500 /mm³ or > 15 % immature neutrophils (bands) regardless of total peripheral white blood cell count [16]. Exclusion criteria included immunocompromised patients (neutropenia, AIDS, lymphoma, or anticipated chemotherapy), change or introduction of antibiotics within the last 24 hrs prior to enrollment, inadequate antimicrobial therapy, polymicrobial infection, concomitant bacteremia or fungemia, use of anticoagulant therapy (excluding the use of unfractionated heparin administered as 5000 units subcutaneously every 8 hrs for deep vein thrombosis prophylaxis) or activated protein C, and presence of underlying ARDS. Patients who were admitted with the diagnosis of community-acquired pneumonia were not considered eligible for participation unless the underlying infection had resolved clinically and they were off antibiotics for ≥ 72 hrs. A control group of patients who were intubated for airway protection on hospital admission were also studied. The study was approved by the Institutional Review Board and an informed consent was obtained from all eligible participants or their next of kin.

**Data Collection**

Demographic and clinical data were recorded on study enrollment and included age, gender, reasons for mechanical ventilation, duration of mechanical ventilation before study onset, prior antibiotic therapy, temperature, leukocyte count, ratio of PaO₂/FiO₂, radiologic score [17], Acute Physiology and Chronic Health Evaluation II score [18], and the Clinical Pulmonary Infection Score (CPIS) [19]. The radiologic score was obtained from a frontal chest radiograph. Each lung was divided into two quadrants, and each quadrant was scored either 0 or 1, depending on whether alveolar consolidation was absent or present. Adequate antimicrobial therapy was defined as the use of at least one antibiotic to which an isolate
recovered from BAL fluid was susceptible *in vitro*. Following discontinuation of antibiotic therapy, patients were monitored daily for new onset fever, purulent tracheal secretions, and new pulmonary infiltrate. ARDS was defined according to the criteria of the North American-European Consensus Conference requiring the presence of generalized pulmonary infiltrate, \( \text{PaO}_2/\text{FiO}_2 < 200 \), and the absence of clinical evidence of left atrial hypertension [20]. Shock was defined as systolic arterial pressure < 90 mmHg with signs of peripheral hypoperfusion or need for continuous infusion of vasopressor or inotropic agents [21].

### Samplings and Processing of Respiratory Secretions

A non-directed BAL (Ballard Medical Products, Draper, UT) was performed on control patients within 12 hrs of intubation. Patients suspected of having VAP underwent a non-directed BAL before initiation of antimicrobial coverage. A repeat non-directed BAL was performed on these patients on day 4 and day 8 after onset of VAP. Four aliquots (20 mL each) of sterile saline were instilled and aspirated. The first 20 mL recovered was discarded. One half the amount was sent for microbiology processing, and the rest was filtered through two layers of sterile gauze and centrifuged at 1500 \( \times g \) for 10 min at 4 °C. The cell free supernatant was stored in small aliquots at –70 °C for hemostatic assays. Cells were resuspended in phosphate-buffered saline and counted by means of a Neubaeur chamber as described elsewhere [22]. The viability of the cells was assessed by trypan blue. Differential cell counts were performed on cytopsin preparations stained with a modified Giemsa-based Diff-Quick stain (Baxter Scientific Products, McGraw Park, IL).

### Diagnosis of VAP

Microbiological specimens were processed as described previously [23]. Both Gram and Wright stains were carried out on cytocentrifuge preparations. Patients were considered to have VAP when the following criteria were present: \( \geq 2 \% \) of the cells in the cytocentrifuge preparations from BAL contained intracellular bacteria and at least one bacterial species grew at a concentration of \( \geq 10^4 \) colony forming unit (CFU)/mL from the BAL sample on semi-quantitative culture. Patients were considered to have a relapse of VAP when a second episode of VAP occurred by a bacterial strain of similar serotype to the first VAP episode; otherwise, it was considered a superinfection.
**Assays**

Procoagulant activity was measured on BAL fluid using a modified one-stage recalcification assay as described previously [24]. In brief, the clotting assay was conducted by incubating 0.1 mL of BAL fluid supernatant with 0.1 mL of citrated human plasma for 2 mins at 37 °C. After the addition of 0.1 mL of 20 mM CaCl₂, clotting time was determined by the manual tilt method. A standard curve was derived by using serial dilution of thromboplastin (Fisher Scientific, Fair Lawn, NJ). A 1:10000 dilution of thromboplastin was arbitrarily assigned a value of 1 unit/mL. Specific markers for TF, TFPI, and TATc were assessed by using commercially available ELISAs (American Diagnostica, Greenwich, CT and Hemex Laboratories, Phoenix, AZ).

**Statistical Analysis**

Results are expressed as mean values ± SD. Means were compared using the Student’s t test when normally distributed and the Mann-Whitney U test otherwise. Proportions were compared using the χ² test with Yates correction or Fisher’s exact test when necessary. Analysis of variance for repeated measures was carried out for sequential measurements and post hoc tests (Bonferroni/Dunn analysis) were used for comparison of all pairs of columns. Correlation between hemostatic response and bacterial load was assessed using Spearman’s rank test. Statistical significance was defined as \( p < 0.05 \). All statistical analyses were performed using SPSS version 12.0 (SPSS, Chicago, IL).

**Results**

**Study Population**

A total of 48 patients were enrolled during the study period. Thirty-five were diagnosed with VAP and 13 were identified as controls. The mean age of participants was 61.9 ± 14.4 for patients with VAP and 57.2 ± 15.6 for controls (\( p = 0.35 \)). Community-acquired pneumonia and congestive heart failure accounted for 23% and 20% of the causes for intensive care unit admission in the VAP group while drug overdose and cerebrovascular accident were responsible for 46% and 31% of intensive care unit admissions in the control subjects. Univariate analysis of the baseline clinical variables revealed a higher Acute Physiology and Chronic Health Evaluation II score (26.3 ± 5.8) and worse radiographic score (3.3 ± 0.7) in those who developed VAP compared with controls (15.6 ± 3.4 and 0, respectively; \( p < 0.001 \)).
VAP complicated the course of eight patients with community-acquired pneumonia. The etiology of community-acquired pneumonia was documented in five patients (three had *Streptococcus pneumoniae*, one had *Klebsiella pneumoniae*, and one had an influenza virus). The etiology of VAP in these cases was completely different from that determined in community-acquired pneumonia. The bacterial pathogens isolated from respiratory secretions included *Pseudomonas aeruginosa* (n = 13), methicillin-resistant *Staphylococcus aureus* (n = 8), methicillin-sensitive *Staphylococcus aureus* (MSSA, n = 7), and *Escherichia coli* (n = 7).

Table 1 displays the clinical characteristics of the study cohort according to the etiology of VAP. There was no significant difference in age, gender, or severity of illness among the various groups (p = 0.4, p = 0.6, and p = 0.2; respectively). Only the length of mechanical ventilation prior to VAP was shorter for those with MSSA and *E. coli* compared with those with MRSA and *P. aeruginosa*. All patients with VAP were eventually treated with an appropriate antibiotic regimen that was active against the bacteria isolated from their respiratory secretions. The regimens consisted of either a combination of vancomycin plus cefipime (n = 12) or vancomycin plus piperacillin/tazobactam (n = 23). All antibiotics were discontinued automatically after 7 days of therapy.

**Table 1. Characteristics of the study population**

<table>
<thead>
<tr>
<th></th>
<th>MSSA (n = 7)</th>
<th>MRSA (n = 8)</th>
<th><em>E. coli</em> (n = 7)</th>
<th><em>P. aeruginosa</em> (n = 13)</th>
<th>Control (n = 13)</th>
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<tr>
<td>Age, years</td>
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<td>Days of MV prior to VAP</td>
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<td>15.3 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6 ± 6.7</td>
<td>16.9 ± 5.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Radiologic score</td>
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<td>APACHE II</td>
<td>22.4 ± 5.4</td>
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<td>24.1 ± 6.0</td>
<td>25.9 ± 4.7</td>
<td>15.6 ± 3.4&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; MV, mechanical ventilation; TCA, tricyclic antidepressant; SSRI, selective serotonin reuptake inhibitor; VAP, ventilator-associated pneumonia; APACHE, Acute Physiology and Chronic Health Evaluation. <sup>a</sup>Compared with MSSA and *E. coli*, p = 0.001 and p = 0.04, respectively; <sup>b</sup>compared with MSSA and *E. coli*, p < 0.001 and p = 0.01, respectively; <sup>c</sup>compared with MSSA, MRSA, *E. coli*, and *P. aeruginosa*, p < 0.001.
Figure 1. Serial bronchoalveolar lavage fluid procoagulant activity at onset of ventilator-associated pneumonia (VAP) and at day 4 and day 8 after onset of VAP caused by *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and methicillin-sensitive *Staphylococcus aureus* (MSSA) compared with controls (C). *p < 0.05 compared with controls; † p < 0.05 compared with MRSA; ‡ p < 0.05 compared with *Escherichia coli*. Analysis of variance results among the four groups for days 0, 4, and 8 are 0.7, < 0.001, and < 0.001, respectively.

Figure 2. Serial bronchoalveolar lavage fluid tissue factor (TF) antigen at onset of ventilator-associated pneumonia (VAP) and at day 4 and 8 after onset of VAP caused by *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and methicillin-sensitive *S. aureus* (MSSA) compared with controls (C). *p < 0.05 compared with controls; † p < 0.05 compared with MRSA; ‡ p < 0.05 compared with *Escherichia coli*. Analysis of variance results among the four groups for days 0, 4, and 8 are < 0.001, < 0.001, and < 0.001, respectively.
Procoagulant Activity

As shown in figure 1, procoagulant activity was significantly higher at VAP onset compared with controls ($p < 0.001$) but declined subsequently from day 4 onward. Although the procoagulant activity was comparable between the various pathogens at the onset of VAP (59.1 ± 14.8 units/mL for MRSA, 62.1 ± 13.1 units/mL for P. aeruginosa, 62.0 ± 15.1 units/mL for E. coli, and 54.3 ± 12.4 units/mL for MSSA, $p = 0.7$), the decline was not uniform. While the procoagulant activity of patients with VAP caused by E. coli and MSSA was not significantly different from controls at end of treatment (6.7 ± 3.9 vs. 2.8 ± 3.8 units/mL; $p = 0.07$, and 5.7 ± 3.2 vs. 2.8 ± 3.8 units/mL; $p = 0.2$, respectively), the procoagulant activity of patients with VAP caused by P. aeruginosa showed elevated activity at end of treatment.

Figure 3. Serial bronchoalveolar lavage fluid tissue factor pathway inhibitor (TFPI) at onset of ventilator-associated pneumonia (VAP) and at day 4 and 8 after onset of VAP caused by Pseudomonas aeruginosa, methicillin-resistant S. aureus (MRSA), Escherichia coli, and methicillin-sensitive S. aureus (MSSA) compared with controls (C).

* $p < 0.05$ compared with controls.
Analysis of variance results among the four groups for days 0, 4, and 8 are 0.9, 0.4, and 0.005, respectively.

Figure 4. Serial bronchoalveolar lavage fluid thrombin-antithrombin (TAT) complex at onset of ventilator-associated pneumonia (VAP) and at day 4 and day 8 after onset of VAP caused by Pseudomonas aeruginosa, methicillin-resistant S. aureus (MRSA), Escherichia coli, and methicillin-sensitive S. aureus (MSSA) compared with controls (C).

* $p < 0.05$ compared with controls; † $p < 0.05$ compared with MRSA. Analysis of variance results among the four groups for days 0, 4, and 8 are 0.1, 0.02, and < 0.001, respectively.
compared with controls (17.5 ± 6.8 vs. 2.8 ± 3.8 units/mL; $p < 0.001$) and other etiologic agents ($p < 0.001$). Although there was no correlation between hemostatic response and total bacterial load (CFU/mL) at any specific time point, there was a significant association between procoagulant activity and total bacterial load (CFU/mL) over the study period for each of the organisms involved ($r = 0.8, p < 0.001$).

Pathogen-Specific Intraalveolar TF, TFPI, and TAT Levels

Figure 2 shows the temporal changes in BAL fluid TF antigen among patients with P. aeruginosa, MRSA, E. coli and MSSA at the onset of VAP, on day 4, and at end of treatment. TF levels were significantly higher than those in the control group for all pathogens at the onset of VAP ($p < 0.001$). Furthermore, the TF levels in those VAP caused by P. aeruginosa showed a greater elevation than those observed in the other infectious categories and continued to be elevated for the duration of the study. Similarly, TF values for MRSA VAP remained elevated at all time points compared with controls but to a lesser extent than those observed with P. aeruginosa. Only patients infected with E. coli and MSSA showed TF levels comparable to controls at end of treatment. Of interest, the TF levels of patients with P. aeruginosa and MRSA were both higher than those with E. coli and MSSA on day 8. In contrast to the changes in the levels of TF, there were no significant differences in the time course of TFPI levels from VAP onset to the end of treatment among the four groups of pathogens (figure 3). The TFPI levels were, however, significantly elevated compared with controls irrespective of the causative agents and remained elevated for the duration of the study. At each time point, the difference between TFPI levels among the various groups were not significantly different except at end of treatment when patients with P. aeruginosa VAP demonstrated higher TFPI levels in relation to E. coli and MSSA ($p = 0.02$ and $p = 0.03$; respectively). To assess the balance of TF and TFPI on overall fibrin deposition, we measured the levels of TATc in BAL across all time points (figure 4). TATc levels increased significantly for all infectious categories after the onset of VAP and remained elevated at the end of treatment compared with controls. In particular, patients with VAP caused by P. aeruginosa showed a trend for a larger increase in TATc compared with the other patients with VAP caused by MRSA, E. coli, and MSSA at VAP onset but the difference became significant only at day 4 and day 8 posttherapy ($p = 0.02$ and $p < 0.001$; respectively).
Clinical Response

Resolution of the clinical parameters as depicted by the CPIS was comparable in all participants irrespective of the etiology of VAP (Figure 5) despite lack of complete sterility of the alveolar compartment. The bacterial count of causative pathogens dropped significantly after the introduction of antimicrobial therapy for all organisms; however, we were still able to recover colonies of *P. aeruginosa* (n = 5) and MRSA (n = 2) from BAL cultures at end of treatment (Figure 6). Of interest, relapse of VAP occurred in 3 out of the 5 patients who had persistence *P. aeruginosa* in BAL cultures. The CPIS of those patients with persistent infection at end of therapy was not significantly different from those in whom eradication was achieved (median [range], 4 [3-5] and 3.5 [3-4], respectively, \( p = 0.3 \)). Two other patients with negative cultures at end of treatment developed superinfection, one with *P. aeruginosa* and the other with MRSA.

Discussion

The current study demonstrates the following observations: (a) Temporal hemostatic resolution is not uniform and varies according to the bacterial species responsible for VAP; (b) the enhanced intraalveolar procoagulant activity is driven by a rise in TF expression and is not counterbalanced by TFPI; and (c) clinical response precedes normalization of hemostatic balance and may not always indicate intraalveolar bacterial eradication.
Our investigation supports previous studies in finding increased procoagulant activity in the alveolar compartment of patients with VAP by showing a uniform increase in procoagulant activity at the onset of VAP compared with controls [9]. This increase has been attributed to the activation of the tissue factor pathway on the surface of alveolar macrophages acting in concert with factor VIIa [25]. The increased levels of tissue factor expression may even precede the onset of VAP by few days in tandem with a depression of the local fibrinolytic activity [10]. Under physiologic condition, TFPI acts to prevent excessive fibrin deposition by inhibiting activated factor Xa in a concentration-dependent manner [26]. The TFPI-Xa complex binds to TF-VIIa resulting in the inhibition of TF-VIIa activity [27]. An anticipated rise in TFPI levels would be the appropriate response to the observed procoagulant activity in such instance. Indeed, we have observed a significant rise in the TFPI levels at the onset of VAP when compared with controls, but the persistent elevation in procoagulant activity on subsequent days despite the initiation of adequate antibiotic therapy coupled with continuous TATc deposit would suggest that the increased levels of TFPI in VAP patients were not adequate to prevent the coagulation disturbance precipitated by TF. These results coincide with similar studies in patients with other inflammatory pulmonary syndromes. Sabharwal *et al.* [13] reported a substantial increase in BAL levels of TFPI in those at risk (seven-fold) and in those with established ARDS (20-fold) compared with controls. Similarly, de Moerloose *et al.* [15] demonstrated that the BAL TFPI levels in patients with bacterial and *Pneumocystis carinii* pneumonia were increased compared with controls. Although TF levels in BAL were not measured in both studies, the authors argued that the increased coagulant activity was not balanced by adequate increases in TFPI. To explain the apparent discrepancy, we have to consider the following possibilities. First, during the process of thrombin activation, TFPI inhibits the TF-VIIa activity *only* after binding to Xa. Hence, TFPI would not be able to halt the coagulation process when TF generation continues [28]. Second, the abundant intraalveolar inflammatory cell leukocyte-derived metalloproteinases in patients with hospital-acquired pneumonia [29] inactivate TFPI but not TF, factor VIIa, or factor Xa [30], which was shown to lead to considerable loss of biologic activity namely anti-Xa activity. Third, neutrophil elastase is known to cleave TFPI between Thr$_{87}$ and Thr$_{88}$, which is within the polypeptide that links the first and second kunitz domains [31]. Having a significantly elevated neutrophil elastase, the ability of TFPI to neutralize both factors Xa and TF-VIIa is greatly impaired. Hence, the persistent synthesis of TF coupled with inactivation and cleavage of TFPI would likely underline the imbalance of coagulation in patients with VAP. One of the potential concerns of the study is the possibility that increased alveolar-
capillary barrier permeability in VAP could induce a potential bias through the leakage of coagulation factors from the vascular compartment into the alveoli. However, we favor the opinion that activation of coagulation is a local process for the following reasons: (a) Ample evidence from previous trials [9,13] suggests that the hemostatic disturbance in the alveolar compartment is a localized phenomenon and would not explain these observations; and (b) our laboratory previously demonstrated local production of plasminogen activator inhibitor-1 and TF within the pulmonary compartment during experimentally induced pneumonia in mice [32]. A potential limitation of our study is the use of blinded non-directed BAL in retrieving alveolar fluid, which could attenuate the observed disturbances in coagulation variables if the fluid was obtained from non-infected area [11]. However, the potential error would have been distributed randomly across the species in question, thus minimizing the possibility of bias. Residual effects of prior antimicrobial therapy might also alter the expression of these factors and therefore should be taken into consideration when interpreting the results of the study.

Clinical and experimental studies have provided temporal clues on the progression of hemostatic disturbance in patients with VAP. However, to our knowledge, no studies have examined the effect of specific bacterial pathogens on the degree of coagulation disturbance, let alone the restoration of hemostatic balance following therapy. In the present study, we have demonstrated that infection with *P. aeruginosa* not only leads to a larger procoagulant propensity but is also responsible for a delayed restoration of hemostatic balance compared with the other pathogens. Both TF and TATc levels remained significantly elevated 4 days after therapy and at end of treatment in comparison to MRSA, *E. coli* and MSSA. We hypothesized that these observations could be explained by either a high load of bacteria or an exuberant host response to invading pathogens. Given the bacterial load was comparable to those with MRSA VAP on day 4 of therapy, further investigations will be needed to determine whether the response is related to a specific virulence factor or an intrinsic host immune reaction [33].

Our findings may have significant clinical implications. Previous studies have indicated that clinical and microbiological response may parallel one another [34,35] and that serial measurements of CPIS may be a good indicator of response to therapy [36]. None of these investigations, however, has obtained evidence of intraalveolar bacterial eradication at end of therapy. Despite the improvement of the CPIS of our patients following a full course of antibiotic therapy, sterilization of the alveolar space was not completely achieved in patients with *P. aeruginosa*. Similarly, Dennesen *et al.* [37] found persistent colonization of *P. aeruginosa* in the endotracheal aspirates of patients with VAP following treatment whereas
bacterial colonization with \textit{S. aureus}, \textit{Haemophilus influenzae}, and \textit{S. pneumoniae} completely disappeared. Whether the lack of eradication is related to an excessive fibrin deposition or antibiotic tolerance [37,38], the duration of treatment for VAP caused by \textit{P. aeruginosa} may need to be extended. In a prospective, double-blind clinical trial comparing 8 to 15 days of antibiotic therapy in patients with microbiologically proven VAP, a higher rate of recurrence was observed in non-fermenting Gram-negative bacilli when antimicrobial therapy was administered for only 8 days compared to 15 [39]. In our case, 23\% of patients with \textit{P. aeruginosa} had a relapse. Guidelines for the duration of treatment in these cases remain controversial [40] and in the absence of adequate biological markers of resolution, it remains an arbitrary practice based on interplay between the physician comfort levels, clinical defervescence, and the host immune response.

In conclusion, the temporal resolution of the hemostatic disturbance following treatment may be affected by the etiologic agents responsible for VAP. Following treatment, clinical improvement precedes the restoration of hemostatic balance. The disparity between clinical response and eradication of \textit{P. aeruginosa} from the intraalveolar space suggests the need for biological markers to guide response to therapy.

\textbf{References}

Local Attenuation of the Protein C Pathway in Patients Developing Pneumonia

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Summary

Bronchoalveolar fibrin deposition is a hallmark of acute inflammatory lung disease, and results from activation of coagulation and inhibition of fibrinolysis. The role of the anticoagulant protein C system in the pathogenesis of pneumonia is unknown.

We investigated the relationship in time between alterations in the anticoagulant protein C pathway and ventilator-associated pneumonia. In mechanically ventilated patients developing pneumonia, local pulmonary levels of protein C and activated protein C were measured, as well as soluble thrombomodulin.

Whereas patients without pneumonia did not have any changes in pulmonary levels of protein C, activated protein C, and soluble thrombomodulin, increased levels of soluble thrombomodulin were measured in lavage fluids from patients developing pneumonia, accompanied by suppressed levels of protein C and activated protein C.

Apparently, the protein C system does not compensate for increased coagulation activation in the pathogenesis of pneumonia. The protein C system may be a target for therapeutic interventions.
Pneumonia is characterized by a disturbed alveolar fibrin turnover, which is the net result of activation of coagulation and attenuation of fibrinolysis [1]. We recently demonstrated in patients developing ventilator-associated pneumonia (VAP), that suppression of fibrinolysis preceded the clinical diagnosis, while procoagulant effects were predominantly seen thereafter [2]. Presently, we extended these findings by investigating the relationship in time between alterations in the anticoagulant protein C (PC) pathway and VAP.

Methods

Patients. Subjects included in the present study are identical to those enrolled in an investigation in which the local disturbances in hemostatic balance and expression of cytokines were evaluated [2,3]. Critically ill patients, expected to be in need of mechanical ventilation for more than 5 days, were enrolled into the study. The use of immunosuppressive drugs such as corticosteroids was a reason for exclusion. The protocol was reviewed and approved by the Central Oxford Regional Ethics Committee.

Diagnosis of Pneumonia. The diagnosis of pneumonia required a combination of the clinical features of VAP together with microbiology confirmation [4]. Serial chest radiography were assessed by a specialist chest physician who was unaware of clinical features or scores. To standardise the reporting of the clinical features of VAP, the clinical variables were scored daily as a clinical pulmonary infection score. The clinical diagnosis had to be supported by the results of microbiological culture of the non-directed bronchial lavage fluid. Diagnosis of VAP required either a significant growth ($\geq 10^4$ colony forming units [CFU]/mL) on semi-quantitative culture of the lavage fluid or growth of a lower colony count ($10^3$ CFU/mL) associated with isolation of the pathogen from culture of blood. If the study investigators agreed that the clinical, radiological and microbiological data were consistent with a diagnosis of VAP, then the diagnosis was considered to be confirmed. The day of onset of VAP was then taken to be the day that the attending clinicians started antibiotic treatment.

Non-directed Bronchial Lavage Technique. A non-directed bronchial lavage was performed on admission and on alternate days throughout the period of mechanical ventilation. The lavage was performed, by instilling 20 mL sterile 0.9% saline via a standard 50 cm, 14 gauge tracheal suction catheter, as described previously [4]. In short, the distal end of the catheter was introduced via the endotracheal tube and advanced until significant resistance was encountered. Immediately after instillation over 10-15 seconds, fluid was aspirated before withdrawal of the catheter. Generally 5-10 mL of fluid was recovered.
Specimen Processing. Lavage fluid and blood samples were kept at 4 °C until processing, which was performed within one hour. An aliquot of the supernatant was sent to the microbiology department for semi-quantitative culture as described previously. Cell-free supernatants were stored at –80 °C until assays were performed.

Assays. We measured levels of PC, activated PC (APC), and soluble thrombomodulin (sTM) in non-directed bronchial lavage fluids collected every other day from critically ill patients during mechanical ventilation. APC was measured with an enzyme capture assay, using monoclonal antibody HAPC 1555 and chromogenic substrate Spectrozyme PCa (American Diagnostica, Greenwich, CT) [5]. PC activity was determined with an amidolytic assay using chromogenic substrate S2366 (Chromogenix, Milan, Italy). sTM was measured with ELISA (Diagnostica Stago, Asnières-sur-Seine, France). Serial data from patients were evaluated using analysis of repeated measures with a linear mixed model, as described before [2]. Data are presented as medians (interquartile ranges).

Results

The patient population was as previously described [2]. In short, specimen collection was initiated in 60 consecutive patients; 28 patients were selected for final analysis, which required at least 3 sample sets (i.e., a minimum ventilation duration of ≥ 5 days). Nine patients developed VAP, 19 patients did not show any signs of pulmonary infection during the clinical course. There were no significant differences between patients developing VAP and patients without VAP with regard to baseline characteristics and mechanical ventilation settings.

Baseline levels of PC, APC, and sTM were not different for patients without and with VAP. Baseline concentrations for patients without and with VAP are respectively: PC, 0.78 (0.69-0.89) versus 0.82 (0.72-0.90) U/mL; APC, 0.52 (0.44-0.59) versus 0.47 (0.36-0.59) ng/mL; sTM, 102 (79-123) versus 98 (75-131) ng/mL (all *p* > 0.05).

In patients developing VAP, the clinical diagnosis was preceded by a decrease in pulmonary PC levels, as measured in lavage fluids. PC levels dropped from 0.69 (0.45-0.86) before VAP to 0.47 (0.24-0.56) U/mL at the day of diagnosis of VAP (*p* < 0.0001, figure 1). In patients not developing VAP, pulmonary PC levels remained unchanged (*p* = 0.07).

The decline in PC levels in the infected lungs was accompanied by a decrease in levels of APC. APC levels dropped from 0.40 (0.25-0.46) before VAP to 0.21 (0.21-0.37) ng/mL at the day of diagnosis (*p* < 0.01, figure 1). The suppression of APC occurred before the clinical diagnosis of VAP. Furthermore, in patients who developed a VAP a significant increase in sTM levels was observed.
Local levels of sTM increased from 95 (120-151) before VAP to 214 (186-312) ng/mL on the day of diagnosis ($p < 0.0001$, figure 1), thereafter increasing to 274 (311-362) 8 days after VAP was diagnosed. To a lesser extent, in patients who did not develop VAP during the study, sTM increased from 102 (79-123) at admission to 130 (102-156) ng/mL at day 10 of mechanical ventilation ($p < 0.01$, figure 1).

**Figure 1.** Levels of protein C (PC), activated protein C (APC), and soluble thrombomodulin (sTM) in non-directed bronchial lavage fluid, prospectively collected in mechanically ventilated patients. 
*Left panels.* Patients who did not develop pneumonia; day 0 denotes start of mechanical ventilation. 
*Right panels.* Patients who developed a ventilator-associated pneumonia (VAP); day 0 corresponds with the day at which VAP was clinically diagnosed. Data represent medians with interquartile ranges.
Discussion

In sepsis, low levels of APC contribute, at least in part, to the systemic procoagulant shift of the hemostatic balance, potentially evolving into disseminated intravascular coagulation [6]. Correction of the systemic hemostatic balance has been acknowledged as a pivotal goal in the treatment of patients with sepsis. Indeed, treatment with recombinant human APC was shown to reduce mortality in patients with severe sepsis [7].

We presently show that during the pathogenesis of pneumonia the PC pathway is locally suppressed. We suggest that this is the net result of increased PC consumption, cleavage of PC by neutrophil elastase, as well as inadequate PC activation due to oxidation of TM and shedding of TM from the cell surface (resulting in soluble fragments of TM) [6]. Presumably, the insufficient anticoagulant PC system contributes to the local procoagulant environment at the site of infection during pneumonia. Correction of the local PC system may be a target in the treatment of pneumonia.

Acknowledgments

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References

rhAPC Inhibits Local and Systemic Activation of Coagulation without Influencing Inflammation during *P. aeruginosa* Pneumonia in Rats

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Abstract

**Background.** It has been proposed that recombinant human activated protein C (rhAPC) exerts lung-protective effects via anticoagulant and anti-inflammatory pathways. We investigated the role of the protein C system in pneumonia caused by *Pseudomonas aeruginosa*, the organism that is predominantly involved in ventilator-associated pneumonia.

**Methods.** Five patients with unilateral ventilator-associated pneumonia were included and bilateral bronchoalveolar lavage was performed. Protein C and activated protein C were determined in lavage fluid. Sixty-two Sprague-Dawley rats were challenged with intratracheal *P. aeruginosa* (10^8 colony forming units), inducing pneumonia. Rats were randomized to treatment with normal saline, rhAPC, heparin, or recombinant tissue-type plasminogen activator. Markers of coagulation, fibrinolysis, and inflammation were measured in blood, bronchoalveolar lavage fluid, and lung tissue of infected rats.

**Results.** Patients with pneumonia demonstrated suppressed levels of protein C and activated protein C in bronchoalveolar lavage fluid obtained from the infected site compared to the contralateral uninfected site. Intravenous administration of rhAPC in rats with *P. aeruginosa* pneumonia limited bronchoalveolar generation of thrombin-antithrombin complexes, largely preserving local antithrombin activity. Yet, rhAPC did not have effects on neutrophil influx and activity, expression of pulmonary cytokines, or bacterial clearance.

**Conclusion.** In patients with ventilator-associated pneumonia the pulmonary protein C pathway is impaired at the site of infection, and local anticoagulant activity may be insufficient. Recombinant human activated protein C prevents procoagulant changes in the lung, however it does not seem to alter the pulmonary host defense against *P. aeruginosa* pneumonia.
Alveolar fibrin deposition is a hallmark of the acute respiratory distress syndrome [1] and pneumonia [2-4]. Excessive fibrin deposition within the airways results from severe inflammation and consequent activation of bronchoalveolar coagulation, which are not compensated by the anticoagulant and fibrinolytic systems [5].

In severe sepsis, which is characterized by a strong systemic inflammatory response and coagulation disturbances, a reduction in patient mortality was shown with administration of recombinant human activated protein C (rhAPC), one of the major natural anticoagulants [6]. Interestingly, in this trial, rhAPC treatment led to more rapid resolution of respiratory failure [7], and patients with community-acquired pneumonia as a primary source of infection seemed to benefit most from treatment [8]. The beneficial effects of rhAPC are not only attributed to its systemic anticoagulant properties [9,10], but also to its anticoagulant and anti-inflammatory effects in the lungs [11,12].

In the current study, crucial components of the protein C system, i.e., levels of protein C (PC), APC, and soluble thrombomodulin (sTM) were measured in bronchoalveolar lavage fluid of human patients with unilateral ventilator-associated pneumonia (VAP). Furthermore, rhAPC was administered to rats infected with Pseudomonas aeruginosa, a Gram-negative microorganism which is most frequently involved in VAP [13]. We hypothesized that the protein C pathway would become insufficient to maintain the hemostatic balance in pulmonary infection, and in addition, that rhAPC would have significant anticoagulant and anti-inflammatory effects in an in vivo model of Gram-negative bacterial pneumonia.

Material and Methods

Patient Study

Design. A bilateral bronchoalveolar lavage (BAL) was performed in 5 patients with a unilateral VAP. Ten mechanically ventilated patients without any signs of pulmonary infection served as controls. Details of the patients and BAL procedure have been published previously [4] (chapter 5). The study protocol was approved by the Medical Ethics Committee of the University of Amsterdam, and written informed consent was obtained from all subjects or their legal representatives.

Assays. APC was measured with an enzyme capture assay, using monoclonal antibody HAPC 1555 and chromogenic substrate Spectrozyme PCa (American Diagnostica, Greenwich, CT) [14]. PC activity was determined with an amidolytic assay using chromogenic substrate
S2366 (Chromogenix, Milan, Italy). sTM was measured by ELISA (Diagnostica Stago, Asnières-sur-Seine, France).

**Rat Study**

*Rats.* Male Sprague-Dawley rats, weighing approximately 250 gram, were purchased from Harlan Sprague-Dawley, The Hague, The Netherlands. The rats were allowed to acclimatize to laboratory conditions for at least 7 days (12:12 hrs day-night cycle at a constant temperature of 22 °C). The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments. All animals were handled in accordance with the guidelines prescribed by Dutch legislation and the International Guidelines on protection, care, and handling of laboratory animals.

*Experimental Protocol.* Pneumonia was induced by intratracheal instillation of $10^8$ colony forming units (CFU) of *P. aeruginosa* (PAO1, in a total volume of 250 µL bacterial suspension), which was cultured as previously described [15]. Rats were randomized to a saline control group or a treatment group. Treatment groups received rhAPC (drotrecogin alfa (activated), Eli Lilly, Indianapolis, IN), unfractionated heparin (Leo Pharma, Ballerup, Denmark), or recombinant tissue-type plasminogen activator (tPA; tenecteplase, Boehringer Ingelheim, Ingelheim, Germany). All agents were administered in intravenous bolus injections of 2 mL/kg under light sedation with a 2% isoflurane, 30 minutes before induction of pneumonia. Doses of each of the agents were based on data from previous studies: rhAPC, 500 µg/kg [16]; heparin 300 IU/kg [16]; tPA, 1.25 mg/kg [17]. Considering the plasma clearance, rats sacrificed at 16 hrs after *P. aeruginosa* inoculation, received additional injections of rhAPC, heparin, or tPA at 6 and 12 hrs; control rats were administered the same volume of normal saline (2 mL/kg). Healthy rats (n = 4) were used as uninfected controls.

At 6 and 16 hrs after inoculation, rats were sacrificed with intraperitoneal injections of ketamine 80 mg/kg (Eurovet, Bladel, The Netherlands) and medetomidine 0.5 mg/kg (Novartis, Arnhem, The Netherlands). Blood (5 mL) was collected from the inferior *vena cava* in citrated (0.109 M) vacutainer tubes. The right lung was ligated, and the left lung was lavaged three times with 2 mL ice-cold sterile saline (average total recovery 4.5 to 5 mL). The right superior lobe was fixed in 10% buffered formalin and embedded in paraffin. The remaining lung lobes were weighed and homogenized in 4 volumes of sterile saline (i.e., $4 \times$ lung weight [mg] in mL) using a tissue homogenizer (Biospec Products, Bartlesville, OK). Total cell numbers in each lavage sample were determined by a Coulter Counter (Coulter
Neutrophil counts in lavage fluids were performed on cytopsin preparations stained with Giemsa.

For bacterial quantification in lungs and blood, serial 10-fold dilutions of lung homogenates and whole blood were made in sterile isotonic saline and plated onto sheep-blood agar coated plates, incubated at 37 °C in 5% CO₂, and counted after 20 hrs. For coagulation assays, cell-free supernatants from blood and lavage fluid were used. For cytokine and chemokine measurements in lungs, cell-free supernatants were used from lung homogenates that were diluted 1:1 in lysis buffer, containing 150 mM NaCl, 15 mM Tris, 1 mM MgCl₂, 1 mM CaCl₂, 1% Triton, and pepstatin A, leupeptin, and aprotinin (all 20 ng/mL).

Assays. Thrombin-antithrombin complexes (TATc; Behring, Marburg, Germany) and fibrin degradation products (FDP; Asserachrom D-Di, Diagnostica Stago, Asnières-sur-Seine, France) were measured using ELISA. Antithrombin (AT), plasminogen activator activity (PAA), and plasminogen activator inhibitor (PAI)-1 activity were measured by an automated amidolytic assays [18-20]. Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant (CINC)-3 were measured using ELISA (R&D Systems, Abingdon, United Kingdom). Myeloperoxidase (MPO) activity was determined by measuring the H₂O₂-dependent oxidation of 3,3',5,5'-tetramethylbenzidine, and was expressed as activity per gram lung tissue [21]. BAL fluid (BALF) total protein was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Histologic Examination. Four micrometer sections were stained with hematoxylin and eosin, and analyzed by a pathologist who was blinded for group identity. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, pleuritis, and thrombus formation, as described previously [22]. Each parameter was graded on a scale of 0 to 4 (0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe). The total histopathology score was expressed as the sum of the scores for all parameters.

Statistical Analysis

In the clinical study, Wilcoxon signed-rank test was used to compare paired BALF-samples from infected and uninfected lungs in patients with a unilateral pneumonia. Differences between patients with pneumonia and controls were analyzed using Mann-Whitney U test. To detect differences between treatment groups and untreated controls in the rat study, Dunnett’s method and Dunn’s method were used, in conjunction with one-way ANOVA and Kruskal-
Wallis tests. For both the clinical and rat study, a $p$-value of less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL).

**Results**

**Low Protein C Levels in Infected Lungs of Patients with Pneumonia**

Five patients with unilateral VAP fulfilled the inclusion criteria. Basic characteristics and microbiology data of the patients were presented in a previous report [4] (chapter 5). All patients underwent two-sided BAL within 12 hrs of fulfilling the inclusion criteria, and recovered uneventfully with antibiotic therapy. Unilateral VAP patients and mechanically ventilated control patients were matched in age, ventilation duration, and mechanical ventilator settings.

In BALF obtained from the infected lungs, there were relatively more neutrophils than in BALF from uninfected lungs (neutrophil differentials: mean ± SD, 62.3 ± 6.3 versus 31.8 ± 11.4 %, $p < 0.05$). Pulmonary levels of PC and APC were consistently lower in infected lungs compared with uninfected lungs (figure 1, $p < 0.05$) and lungs of mechanically ventilated
controls \( (p < 0.05) \). Levels of sTM were significantly higher in infected lungs than in uninfected and ventilated control lungs \( (p < 0.05 \) and \( p < 0.01 \), respectively).

**Effects of APC, Heparin, and tPA on Hemostatic Parameters in Rat Pneumonia**

*P. aeruginosa* pneumonia in rats caused activation of coagulation and inhibition of fibrinolytic activity, reflected in increased TATc and lower PAA compared to uninfected control rats (figure 2 and 3). In BALF, there was marked reduction of pneumonia-induced TATc levels with rhAPC treatment at both timepoints, largely preserving AT activity, and limiting FDP

![Figure 2](image_url)

**Figure 2.** Thrombin-antithrombin complexes (TATc, A), antithrombin activity (AT, B), plasminogen activator activity (PAA, C), plasminogen activator inhibitor type 1 activity (PAI-1, D), and fibrin degradation products (FDP, E) were measured in bronchoalveolar lavage fluid from rats treated with saline, recombinant human activated protein C (APC), heparin, or recombinant tissue-type plasminogen activator (tPA) at 6 and 16 hrs after intratracheal instillation of \(^{10^9}\) colony forming units of *Pseudomonas aeruginosa* (PAO1). Controls are uninfected rats. Data represent mean ± SEM. Dunnett’s test: *\( p < .05 \), †\( p < .01 \), ‡\( p < .001 \) versus saline.
Figure 4. Histopathologic changes in *Pseudomonas aeruginosa* pneumonia. Shown are representative hematoxylin and eosin stained photomicrographs (magnification ×100) of lung tissue from rats treated with saline (A), recombinant human activated protein C (B), heparin (C), recombinant tissue-type plasminogen activator (D) at 16 hrs after intratracheal instillation of 10^8 colony forming units of *Pseudomonas aeruginosa* (PAO1). Lungs show dense inflammatory infiltrates, consisting mainly of neutrophils. Total histopathologic scores are presented in the text. No differences were encountered between the different treatment groups (Kruskal-Wallis test). [See also foldout inside back cover]
generation (all $p < 0.001$ versus saline). Heparin attenuated both TATc and PAA at 6 hrs ($p < 0.01$ and $p < 0.001$ versus saline), and led to increased PAI-1 activity at 16 hrs ($p < 0.05$ versus saline). tPA treatment led to high systemic and bronchoalveolar PAA, exceeding assay maximum (>200%), and completely abolished bronchoalveolar PAI-1 activity (below detection limit) (all $p < 0.001$ versus saline). In plasma, TATc generation was significantly attenuated with rhAPC treatment (figure 3A; $p < 0.001$ at 6 hrs, $p < 0.05$ at 16 hrs versus saline). Furthermore, heparin treated rats had increased TATc levels ($p < 0.01$ versus saline) and lower PAA ($p < 0.01$ versus saline) in plasma at 16 hrs (figure 3).

Unaltered Inflammatory Response in rhAPC, Heparin, and tPA Treatment in Rat Pneumonia

There were no differences in wet lung weight between the study groups (data not shown). There was evident increase in total cell number in the lungs during *P. aeruginosa* pneumonia, which was mostly contributed to neutrophil influx (table 1), but relative or absolute number of neutrophils in BALF was not affected by any treatment, nor was MPO activity altered. At 16 hrs after inoculation with *P. aeruginosa*, histopathology of the lungs showed dense inflammatory infiltrates consisting chiefly of neutrophils (figure 4, see also foldout inside back cover). There were no differences in lung histopathology scores between rhAPC-, heparin-, or tPA-treated rats and saline-treated rats (median [IQR]): saline, 8.0 (6.3-8.8); rhAPC, 8.0 (4.0-11.0); heparin, 7.0 (5.0-9.3); tPA, 6.0 (4.0-7.0).

There were no differences in pulmonary levels of TNF-α and IL-6, and CINC-3 during *P. aeruginosa* pneumonia (table 1).

Bacterial Clearance

Bacterial loads of *P. aeruginosa* in lungs were similar in all groups, both at 6 and 16 hrs after inoculation (figure 5). There was no bacteremia observed in any of the rats.

Discussion

In the present report, we have demonstrated suppression of the protein C system at the site of infection in patients with VAP. Both PC and APC were markedly reduced in BALF obtained from infected lung sites, compared to BALF from contralateral uninfected lungs or from mechanically ventilated, non-injured lungs. It is likely that in patients with pneumonia
Figure 5. Number of bacterial colony forming units (CFU) in lungs after Pseudomonas aeruginosa pneumonia. Bacterial CFU was determined in lung homogenates from rats treated with saline, recombinant human activated protein C (APC), heparin, or recombinant tissue-type plasminogen activator (tPA) at 6 and 16 hrs after intratracheal instillation of $10^8$ CFU of P. aeruginosa (PAO1). Data represent median ± IQR. There were no differences between treatment groups and saline (Kruskal-Wallis test).

Table 1. The pulmonary inflammatory response during Pseudomonas aeruginosa pneumonia

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<th>Total BALF Cells</th>
<th>BALF Neutrophils</th>
<th>MPO (U/g)</th>
<th>Total Protein (μg/mL)</th>
<th>TNF-α (ng/mL)</th>
<th>IL-6 (ng/mL)</th>
<th>CINC-3 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls (n = 4)</strong></td>
<td>18 (15-21)</td>
<td>0</td>
<td>1.8 (1-2.8)</td>
<td>137 (71-192)</td>
<td>0.09 (0.09-0.12)</td>
<td>0.81 (0.62-0.97)</td>
<td>0.3 (0.2-0.3)</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa (t = 6 hrs)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n = 8)</td>
<td>237 (186-326)</td>
<td>218 (171-300)</td>
<td>20.2 (17.2-24.3)</td>
<td>400 (239-685)</td>
<td>2.36 (1.01-3.99)</td>
<td>4.83 (3.63-5.27)</td>
<td>1.74 (0.81-2.64)</td>
</tr>
<tr>
<td>rhAPC (n = 8)</td>
<td>169 (128-350)</td>
<td>156 (117-322)</td>
<td>20.0 (12.8-31.1)</td>
<td>453 (259-506)</td>
<td>1.85 (1.24-3.16)</td>
<td>5.58 (4.43-7.13)</td>
<td>1.88 (1.10-3.30)</td>
</tr>
<tr>
<td>Heparin (n = 8)</td>
<td>192 (109-253)</td>
<td>176 (100-233)</td>
<td>17.0 (14.6-20.9)</td>
<td>421 (313-447)</td>
<td>1.85 (1.35-2.95)</td>
<td>3.80 (3.18-5.72)</td>
<td>1.63 (1.03-2.54)</td>
</tr>
<tr>
<td>tPA (n = 8)</td>
<td>212 (190-265)</td>
<td>195 (175-239)</td>
<td>18.3 (16.0-22.2)</td>
<td>389 (234-490)</td>
<td>2.13 (1.60-3.07)</td>
<td>5.47 (4.65-7.46)</td>
<td>2.32 (1.57-3.76)</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa (t = 16 hrs)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n = 8)</td>
<td>210 (179-272)</td>
<td>179 (144-237)</td>
<td>19.2 (12.8-23.1)</td>
<td>641 (532-739)</td>
<td>0.25 (0.14-0.30)</td>
<td>0.71 (0.52-1.21)</td>
<td>2.60 (1.43-6.44)</td>
</tr>
<tr>
<td>rhAPC (n = 8)</td>
<td>162 (117-274)</td>
<td>147 (96-216)</td>
<td>21.0 (14.2-23.5)</td>
<td>604 (486-791)</td>
<td>0.31 (0.24-0.41)</td>
<td>1.07 (0.72-1.18)</td>
<td>3.99 (2.56-5.34)</td>
</tr>
<tr>
<td>Heparin (n = 6)</td>
<td>245 (219-356)</td>
<td>216 (173-300)</td>
<td>19.4 (11.0-28.2)</td>
<td>603 (462-721)</td>
<td>0.30 (0.12-0.44)</td>
<td>0.88 (0.51-1.40)</td>
<td>3.03 (1.35-2.91)</td>
</tr>
<tr>
<td>tPA (n = 8)</td>
<td>186 (151-259)</td>
<td>165 (119-222)</td>
<td>23.8 (18.9-34.0)</td>
<td>597 (513-878)</td>
<td>0.42 (0.27-0.72)</td>
<td>1.20 (0.81-2.01)</td>
<td>4.12 (2.45-17.32)</td>
</tr>
</tbody>
</table>

Total bronchoalveolar lavage fluid (BALF) cells and neutrophils are expressed as median (IQR) × 10⁴ cells per milliliter BALF at 6 and 16 hrs after inoculation with $10^8$ colony forming units of P. aeruginosa. All other data are also expressed as median (IQR). Controls are uninfected rats. BALF, bronchoalveolar lavage fluid; CINC-3, cytokine-induced neutrophil chemoattractant; IL-6, interleukin-6; MPO, myeloperoxidase activity. TNF-α, tumor necrosis factor-α. Kruskal-Wallis tests, all $p > 0.05$. 
localized impairment of the protein C system contributes to increased bronchoalveolar thrombin generation. Furthermore, in a rat model of P. aeruginosa pneumonia, we have shown that systemic infusion of rhAPC strongly inhibited bronchoalveolar activation of the coagulation system. Yet, this treatment did not influence pulmonary inflammation or bacterial clearance from the respiratory tract. Taken together, our data suggest that local suppression of the procoagulant response in pneumonia is not necessarily detrimental to the host.

APC is one of the major physiological coagulation inhibitors that exerts its anticoagulant activity by catalyzing the proteolytic inactivation of factors Va and VIIa [23]. APC thereby attenuates coagulation without directly interacting with thrombin, unlike AT, one of the other major natural anticoagulants, which forms complexes with thrombin (TATc) during coagulation activation. Moreover, APC is involved in the inactivation of PAI-1, limiting inhibition of plasminogen activator activity and thus enhancing fibrinolysis [23]. Insufficiency of the protein C system within the pulmonary compartment was previously demonstrated in healthy volunteers with endotoxin-induced lung injury (12), surgical patients who were ventilated for 5 hrs with higher tidal volumes and no positive-end expiratory pressure [24], mechanically ventilated patients developing VAP [25], and patients with acute lung injury [26]. Relative insufficiency of the protein C system may be caused by increased consumption and increased degradation of PC by neutrophil elastase [27]. Thrombomodulin (TM) is the main receptor involved in thrombin-mediated activation of PC. There are consistent reports on high levels of sTM at inflamed lung sites [12,24-26], suggesting that shedding of TM from the cell surface contributes to protein C insufficiency.

In pulmonary infection, anticoagulant therapy with rhAPC seemed particularly appealing, not only because of its anticoagulant activity but also because of its anti-inflammatory properties shown in several preclinical studies [23]: in rodents, intratracheal [28] and systemic [29,30] application of APC attenuated bleomycin-induced and endotoxin-induced lung injury, respectively. Recently, Laterre et al. demonstrated that rhAPC had mainly been effective in reducing mortality in sepsis patients who presented with severe community-acquired pneumonia [8]. Patients with nosocomial pneumonia had not benefited from rhAPC treatment [8], suggesting that there are differences in the pathogenesis of pneumonia that should account for differences in therapy effects. In our rat pneumonia model with P. aeruginosa, a Gram-negative microorganism that is predominantly involved in nosocomial infections, we could not demonstrate any alterations in host response despite clear anticoagulant effects in the lungs. Of course, based on our study, it is not valid to conclude that rhAPC is not effective in P. aeruginosa induced pneumosepsis. Of note, pathogens behave differently with regard to the
induction of pulmonary inflammation [31], and further studies should elucidate the mechanisms in which various pathogens induce changes in hemostasis.

Inflammation and coagulation play pivotal roles in host defense. As phylogenetically old responses, there is extensive crosstalk between inflammation and coagulation in mounting an adequate immune response against potentially injurious stimuli [32]. Interference with either the inflammatory or coagulation response may impede the primary host defense mechanisms. In the treatment of sepsis, anti-inflammatory strategies have failed to demonstrate improvements in patient outcome so far [33]. Similarly, systemic anticoagulant strategies may not always be beneficial to the patient. Indeed, treatment with tissue factor pathway inhibitor and AT have failed to increase survival rates in severe sepsis [34,35], while it remains unclear what exact mechanisms are involved during APC treatment. With regard to the use of rhAPC in severe pulmonary infections, Robriquet et al. have recently reported detrimental effects [36]. Administration of rhAPC in the early stage of P. aeruginosa induced lung injury in rats tended to increase lung edema with loss of the inflammatory response compartmentalization [36]. Kipnis et al. have shown similar results with AT treatment in a mouse model of P. aeruginosa induced lung injury [37]. These studies, in fact, suggest that interference with bronchoalveolar coagulation can be harmful in the very first hours after microbial challenge. The data presented by drs. Robriquet, Kipnis, and colleagues [36,37], however, are not consistent with our data. rhAPC treatment in our model significantly inhibited TATc generation in the lungs, and no differences in inflammatory parameters were noted. Discrepancies in study results may have originated from differences in study design and severity of the model. Still, it remains to be established what effects anticoagulant therapy has in pneumonia caused by other pathogens, with Gram-positive bacteria in particular (since most experimental studies in this field have been performed with endotoxin and P. aeruginosa).

Unexpected effects were observed in the group of rats treated with heparin. Heparin was used as an anticoagulant, but increased systemic TATc levels at 16 hrs after induction of pneumonia; this may be due to the fact that heparin facilitates binding of AT to thrombin [38]. Underdosing may have been an issue, but attenuation of bronchoalveolar TATc at 6 hrs after inoculation with P. aeruginosa suggests that there was sufficient heparin to limit bronchoalveolar coagulation. There are actually conflicting data in the literature with regard to lung-protective effects of heparin: in a rat model of endotoxemia-related lung injury heparin alone did not exert any beneficial effects [39], in contrast with a similar model in sheep [40], whereas low-molecular weight heparin limited lung injury in endotoxemic swine [41] and
mice [42]. The major anticoagulant effects of heparin are via AT, and it may well be that AT consumption (as seen in our study, figure 2B) leads to impaired actions of heparin.

There are several limitations to our study. First and foremost, our experimental model does not correspond with the clinical practice, and interpretation of the data should be done carefully. The high number of bacterial CFU instilled intratracheally does not match the bacterial challenge as observed in patients with VAP, nor does it mimic the clinical emergence of VAP. Also, it is noted in patients with VAP that inadequate antibiotic therapy leads to sustained procoagulant activity within the pulmonary compartment, associated with hypoxemia [43], whereas rats did not receive antibiotics. On the other hand, it may be reassuring for clinicians that pharmacological agents used in sepsis (rhAPC), prevention of deep venous thrombosis (heparin), and thrombolysis (tPA) had no major impact on dissemination of *P. aeruginosa* in our current report. Practical limitations to our study include that we were not able to directly measure pulmonary levels of PC, APC, or sTM in rats. Furthermore, rhAPC was delivered by bolus injections, in stead of continuous intravenous infusion. Nevertheless, high levels of bronchoalveolar TATc suggested that activation of coagulation is not completely counterbalanced by the natural anticoagulant pathways in rats, while the delivered rhAPC induced significant anticoagulant effects. And indeed, for continuous infusion prolonged anesthesia and surgical procedures would have been necessary, inducing unforeseen effects on pulmonary inflammation and coagulation which we tried to control as much as possible in this experimental setting.

In conclusion, during pulmonary infection, insufficiency of the protein C system contributes to the establishment of a local procoagulant environment within the airways. Interference with the procoagulant response by rhAPC treatment does not seem to alter the inflammatory response to *P. aeruginosa* nor influence bacterial clearance.

**Acknowledgments**

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References


Antithrombin Inhibits Bronchoalveolar Activation of Coagulation and Limits Lung Injury during *S. pneumoniae* Pneumonia in Rats

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submitted for publication
Abstract

**Background.** It has been proposed that natural inhibitors of coagulation, including activated protein C (APC), antithrombin (AT), and tissue factor pathway inhibitor (TFPI), exert lung-protective effects via anticoagulant and possibly anti-inflammatory pathways. We investigated the role of these natural anticoagulants in *Streptococcus pneumoniae* pneumonia.

**Methods.** Patients with unilateral community-acquired pneumonia were included and underwent bilateral bronchoalveolar lavage. Protein C, AT, and TFPI were measured in lavage fluid. Ninety-eight rats were challenged intratracheally with *S. pneumoniae* (serotype 3, $10^6$ colony forming units), inducing pneumonia. Rats were randomized to treatment with normal saline, APC, AT, TFPI, heparin, or tissue-type plasminogen activator. Markers of coagulation, fibrinolysis, and inflammation were measured in blood, bronchoalveolar lavage fluid, and lung tissue of infected rats.

**Results.** Patients with pneumonia demonstrated compartmentalized suppressed levels of protein C, AT, and TFPI at the site of infection. Rats infected with *S. pneumoniae* had increased thrombin-antithrombin complexes in bronchoalveolar lavage fluid, with decreased levels of antithrombin activity and fibrin degradation products. Administration of APC, AT, and TFPI significantly limited these procoagulant changes. Furthermore, AT treatment resulted in less bacterial outgrowth of *S. pneumoniae* and less histopathological damage in lungs.

**Conclusion.** Impairment of the natural anticoagulant pathways may contribute to the increased coagulation activity at the site of pulmonary infection. Anticoagulant treatment attenuates pulmonary coagulopathy. AT seems to exert significant lung-protective effects in *S. pneumoniae* pneumonia in rats.
Alveolar fibrin deposition is a hallmark of acute respiratory distress syndrome (ARDS) [1-3], but has also been demonstrated in patients with pneumonia [3-5]. Excessive fibrin deposition within the airways results from severe inflammation with increased activation of coagulation, and may compromise pulmonary integrity and function [6,7].

In severe sepsis, which is characterized by a strong systemic inflammatory response and coagulation disturbances, improved survival has been shown with the infusion of recombinant human activated protein C (APC), one of the major natural inhibitors of coagulation [8]. Interestingly, in this trial APC treatment led to more rapid resolution of respiratory failure [9], while patients with community-acquired pneumonia seemed to benefit most from APC infusion [10]. The beneficial effects of APC are not only attributed to its systemic anticoagulant properties [11,12]. Indeed, it has been proposed that anticoagulant and antiinflammatory effects in the lungs contribute to better outcome [13,14]. Lung-protective effects have also been attributed to the other natural anticoagulants, i.e., antithrombin (AT) and tissue factor pathway inhibitor (TFPI). However, improvement in lung function by AT and TFPI has only been demonstrated in a relatively limited number of patients with sepsis [15,16], and were not confirmed in larger phase III clinical trials [17,18].

We sought to determine the role of the natural anticoagulant systems in the host defense against bacterial pneumonia. First, we measured levels of protein C, AT, and TFPI in bronchoalveolar (BAL) fluid (BALF) from patients with community-acquired pneumonia. Since the anticoagulant pathways seemed to be impaired in infected lungs, we hypothesized that restoration of the anticoagulant systems would have lung-protective effects by hampering both coagulation activation and inflammation. This was investigated using recombinant human APC, plasma-derived AT, and recombinant human TFPI in a *Streptococcus pneumoniae* pneumonia model in rats.

**Material and Methods**

**Patient Study**

*Design.* BAL was performed in 4 patients with a unilateral community-acquired pneumonia. Nine healthy volunteers served as controls. Details of the patients and BAL procedure have been published previously [4]. The study protocol was approved by the Medical Ethics Committee of the University of Amsterdam, and written informed consent was obtained from all patients and volunteers, or their legal representatives.
Assays. PC activity was determined with an amidolytic assay using chromogenic substrate S2366 (Chromogenix, Milan, Italy). Antithrombin was measured by an automated amidolytic technique [19]. TFPI activity was measured on the Behring Coagulation System (Behring, Marburg, Germany) [20].

Rat Study

Rats. All experiments were approved by the institutional Animal Care and Use Committee of the Academic Medical Center. Male Sprague-Dawley rats, weighing 250-300 gram, were used (Harlan Sprague Dawley, The Hague, The Netherlands).

Experimental Protocol. In 98 rats, pneumonia was induced by intratracheal instillation of $5 \times 10^6$ colony forming units (CFU) of *S. pneumoniae* (serotype 3, ATCC 6303, in a total volume of 250 µL bacterial suspension), which was cultured as described previously [21]. Rats were randomized on a daily basis to a saline control group or a treatment group. Treatment groups received APC (drotrecogin alfa (activated), Eli Lilly, Indianapolis, IN), AT (plasma-derived antithrombin III, Baxter, Vienna, Austria), TFPI (tifacogin, Chiron, Emeryville, CA), unfractionated heparin (Leo Pharma, Ballerup, Denmark), or recombinant tissue-type plasminogen activator (tPA; tenecteplase, Boehringer Ingelheim, Ingelheim, Germany). All agents were administered in bolus injections of 2 mL/kg under light sedation with 2% isoflurane, 30 minutes before induction of pneumonia, and every 6 hrs after inoculation. Control rats were administered the same volume of normal saline (2 mL/kg). Total doses of each of the agents were determined using data from previous studies and pilot studies with endotoxin-induced lung injury (Chapter 4): APC, 500 µg/kg [22]; AT, 250 U/kg [22,23]; TFPI, 1.5 mg/kg [16,18,24]; heparin 300 IU/kg [22]; tPA, 1.25 mg/kg [25]. Healthy rats (n = 4) were used as uninfected controls.

At 24 and 48 hours after inoculation, rats were sacrificed with intraperitoneal injections of ketamine 80 mg/kg (Nimatek, Eurovet, Bladel, The Netherlands) and medetomidine 0.5 mg/kg (Domitor, Novartis, Arnhem, The Netherlands). Blood (5 mL) was collected from the inferior vena cava in citrated (0.109 M) vacutainer tubes. The left lung was lavaged three times with 2 mL ice-cold sterile saline (average total recovery 4.5 to 5 mL). The right superior lobe was fixed in 10% buffered formalin and embedded in paraffin. The remaining lung lobes were weighed and homogenized in 4 volumes of sterile saline (i.e., $4 \times$ lung weight [mg] in mL) using a tissue homogenizer (Biospec Products, Bartlesville, OK). Total cell numbers in lavage
samples were determined by a Coulter Counter (Coulter Electronics, Hialeah, FL). Neutrophil counts in lavage fluids were performed on Giemsa-stained cytospin preparations.

For bacterial quantification in lungs and blood, serial 10-fold dilutions of lung homogenates, bronchoalveolar lavage fluids, and whole blood were made in sterile isotonic saline and plated onto sheep-blood agar plates. After a 16 hrs incubation at 37 °C in 5% CO₂, number of CFU was counted. For coagulation assays, cell-free supernatants from blood and lavage fluid were used. For cytokine and chemokine measurements in lungs, cell-free supernatants were used from lung homogenates that were diluted 1:1 in lysis buffer, containing 150 mM NaCl, 15 mM Tris, 1 mM MgCl₂, 1 mM CaCl₂, 1% Triton, and pepstatin A, leupeptin, and aprotinin (all 20 ng/mL).

Histologic Examination. Four micrometer sections were stained with hematoxylin and eosin, and analyzed by a pathologist who was blinded for group identity. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, pleuritis, and thrombus formation, as described previously [26]. Each parameter was graded on a scale of 0 to 4 (0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe). The total histopathology score was expressed as the sum of the scores for all parameters.

Assays. Thrombin-antithrombin complexes (TATc; Behring, Marburg, Germany) and fibrin degradation products (FDP; Asserachrom D-Di, Diagnostica Stago, Asnières-sur-Seine, France) were measured using ELISA. Antithrombin (AT), plasminogen activator activity (PAA), and plasminogen activator inhibitor (PAI)-1 activity were measured by automated amidolytic assays [27-29]. Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant (CINC)-3 were measured using ELISA (R&D Systems, Abingdon, United Kingdom). Myeloperoxidase (MPO) activity was determined by measuring the H₂O₂-dependent oxidation of 3,3'5,5' tetramethylbendsidine [21]. Total protein in BALF was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Statistical Analysis

For the patient study, Wilcoxon signed-rank test was used to compare paired BALF-samples from infected and uninfected lungs in patients with a unilateral pneumonia. Differences between patients with pneumonia and controls were analyzed using Mann-Whitney U test. To detect differences between treatment groups and untreated controls in the rat study, Dunnett’s method and Dunn’s method were used, in conjunction with one-way ANOVA and Kruskal-
Wallis tests. For both the clinical and rat study, a $p$-value of less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL) and Prism 4.0 (GraphPad software, San Diego, CA).

**Results**

**PC, AT, and TFPI in Pneumonia Patients**

Patient characteristics and microbiology data of patients with unilateral pneumonia were presented in a previous report [5], and are summarized in table 1. Bronchoalveolar levels of PC, AT, and TFPI were consistently lower in infected lungs compared with opposite uninfected lungs, but differences were not statistically significant ($p = 0.067$; figure 1). Levels of PC, AT, and TFPI were significantly lower in infected lungs than in lungs from healthy volunteers.

**Effects of Anticoagulants on Hemostatic Parameters**

In rats infected with *S. pneumoniae*, a marked increase in bronchoalveolar TATc was observed. Pneumonia-induced TATc levels were attenuated with APC, AT, and TFPI.

![Figure 1](image.png)

**Figure 1.** Levels of protein C (A), activated protein C (APC, B), and soluble thrombomodulin (sTM, C) in bronchoalveolar lavage fluids from patients with unilateral ventilator-associated pneumonia (VAP) and mechanically ventilated control patients without lung injury. The infected site was localized by chest radiography. Wilcoxon matched-samples test: *$p < 0.05$ versus uninfected site. Mann-Whitney U test: † $p < 0.01$ versus healthy controls.*
Table 1. Clinical and microbiological data of patients with a community-acquired pneumonia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Site of infiltrate</th>
<th>BALF-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>42</td>
<td>Right middle lobe</td>
<td>Streptococcus pneumoniae</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>47</td>
<td>Right middle lobe</td>
<td>...&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>40</td>
<td>Right upper lobe</td>
<td>Streptococcus pneumoniae</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>36</td>
<td>Right middle and lower lobe</td>
<td>...&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

BALF, bronchoalveolar lavage fluid.

<sup>a</sup>site of infiltrate was determined by chest radiography; <sup>b</sup>no micro-organisms cultured or isolated.

Figure 2. Thrombin-antithrombin complexes (TATc, A), antithrombin activity (AT, B), plasminogen activator activity (PAA, C), plasminogen activator inhibitor type 1 activity (PAI-1, D), and fibrin degradation products (FDP, E) were measured in bronchoalveolar lavage fluid from rats treated with saline, recombinant human activated protein C (APC), plasma-derived antithrombin (AT), recombinant human tissue factor pathway inhibitor (TFPI), heparin, or recombinant tissue-type plasminogen activator (tPA) at 24 and 48 hrs after intratracheal instillation of 10^6 colony forming units of *Streptococcus pneumoniae*. Controls are uninfected.
treatment at both timepoints (figure 2A), with consequent limitation of the generation of FDP (figure 2E). TFPI was less capable of maintaining AT activity during pneumonia than APC and AT (figure 2B). Although none of the interventions had an effect on PAI-1 activity (figure 2D), bronchoalveolar PAA was differentially altered (figure 2C). tPA was able to increase PAA, increasing fibrin degradation and abolishing PAI-1 activity (figure 2C-D).

In plasma, levels of TATc were higher and PAA lower in rats with *S. pneumoniae* pneumonia, compared to uninfected control rats. APC and AT treatment attenuated systemic TATc generation, and was associated with less suppression of PAA (figure 3).

**Inflammatory Response in Rats with *S. pneumoniae* Pneumonia**

There was evident increase in total cell number in the lungs during *S. pneumoniae* pneumonia, mostly contributed to neutrophil influx. As shown in table 2, AT treatment led to less BALF cells and neutrophils. MPO activity was not affected by any treatment. Although lung levels of TNF-α, IL-6, and CINC-3 were highly variable, these mediators were consistently lower in lungs of AT treated rats, notably at 48 hrs (table 2).

![Figure 4](image.jpg)

**Figure 4.** Histopathologic changes in *Streptococcus pneumoniae* pneumonia. Shown are representative hematoxylin and eosin stained photomicrographs (magnification × 100) of lung tissue from rats treated with saline (A), recombinant human activated protein C (B), plasma-derived antithrombin (C), recombinant human tissue factor pathway inhibitor (D), heparin (E), recombinant tissue-type plasminogen activator (F) at 24 hrs after intratracheal instillation of 10⁶ colony forming units of *S. pneumoniae*. [See also foldout inside back cover]
Figure 3. Thrombin-antithrombin complexes (TATc, A) and plasminogen activator activity (PAA, B) were measured in plasma from rats treated with saline, recombinant human APC, plasma-derived AT, recombinant human TFPI, heparin, or recombinant tPA at 24 and 48 hrs after intratracheal instillation of $10^6$ colony forming units of Streptococcus pneumoniae. Controls are uninfected rats. Data represent mean ± SEM. Dunnett’s test: *p < 0.05, ‡p < 0.001 versus saline.

Table 2. The pulmonary inflammatory response during Streptococcus pneumoniae pneumonia

<table>
<thead>
<tr>
<th></th>
<th>Total BALF Cells</th>
<th>BALF Neutrophils</th>
<th>MPO (U/g)</th>
<th>BALF Protein (μg/mL)</th>
<th>TNF-α (ng/mL)</th>
<th>IL-6 (ng/mL)</th>
<th>CINC-3 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Saline (n = 11)</td>
<td>176</td>
<td>134</td>
<td>13.9</td>
<td>601</td>
<td>0.64</td>
<td>0.90</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>(110-307)</td>
<td>(70-245)</td>
<td>(6.8-25.1)</td>
<td>(418-665)</td>
<td>(0.21-1.93)</td>
<td>(0.80-0.97)</td>
<td>(5.2-44.3)</td>
</tr>
<tr>
<td>APC (n = 8)</td>
<td>136</td>
<td>112</td>
<td>19.9</td>
<td>434</td>
<td>0.72</td>
<td>0.72</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>(87-204)</td>
<td>(57-185)</td>
<td>(9.3-38.0)</td>
<td>(301-739)</td>
<td>(0.14-1.59)</td>
<td>(0.68-1.16)</td>
<td>(3.3-11.4)</td>
</tr>
<tr>
<td>AT (n = 7)</td>
<td>85</td>
<td>68</td>
<td>11.3</td>
<td>486</td>
<td>0.13*</td>
<td>0.72</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>(47-193)</td>
<td>(29-141)</td>
<td>(7.2-14.9)</td>
<td>(305-600)</td>
<td>(0.012-0.16)</td>
<td>(0.67-0.79)</td>
<td>(4.6-8.0)</td>
</tr>
<tr>
<td>TFPI (n = 6)</td>
<td>172</td>
<td>138</td>
<td>11.5</td>
<td>548</td>
<td>0.22</td>
<td>0.83</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>(121-200)</td>
<td>(87-152)</td>
<td>(9.4-14.7)</td>
<td>(420-784)</td>
<td>(0.16-0.33)</td>
<td>(0.63-0.91)</td>
<td>(4.8-9.3)</td>
</tr>
<tr>
<td>Heparin (n = 7)</td>
<td>260</td>
<td>192</td>
<td>17.9</td>
<td>811</td>
<td>1.04</td>
<td>0.86</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>(97-373)</td>
<td>(74-298)</td>
<td>(11.7-27.0)</td>
<td>(501-1079)</td>
<td>(0.42-3.24)</td>
<td>(0.80-1.25)</td>
<td>(8.3-46.8)</td>
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<tr>
<td>tPA (n = 7)</td>
<td>177</td>
<td>147</td>
<td>19.5</td>
<td>545</td>
<td>0.90</td>
<td>0.82</td>
<td>16.2</td>
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<tr>
<td></td>
<td>(62-250)</td>
<td>(43-198)</td>
<td>(12.8-25.2)</td>
<td>(400-888)</td>
<td>(0.16-1.88)</td>
<td>(0.79-1.08)</td>
<td>(7.7-31.1)</td>
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</table>

Streptococcus pneumoniae (t = 24 hrs)

<table>
<thead>
<tr>
<th></th>
<th>Total BALF Cells</th>
<th>BALF Neutrophils</th>
<th>MPO (U/g)</th>
<th>BALF Protein (μg/mL)</th>
<th>TNF-α (ng/mL)</th>
<th>IL-6 (ng/mL)</th>
<th>CINC-3 (ng/mL)</th>
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</thead>
<tbody>
<tr>
<td>Saline (n = 11)</td>
<td>168</td>
<td>72</td>
<td>9.6</td>
<td>1333</td>
<td>0.19</td>
<td>0.87</td>
<td>2.8</td>
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<tr>
<td></td>
<td>(91-257)</td>
<td>(41-141)</td>
<td>(6.1-15.0)</td>
<td>(427-2779)</td>
<td>(0.14-1.06)</td>
<td>(0.71-1.18)</td>
<td>(1.7-15.5)</td>
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<tr>
<td>APC (n = 11)</td>
<td>100</td>
<td>27</td>
<td>9.4</td>
<td>638</td>
<td>0.15</td>
<td>0.76</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>(55-206)</td>
<td>(13-69)</td>
<td>(5.7-16.0)</td>
<td>(485-756)</td>
<td>(0.14-0.29)</td>
<td>(0.59-1.05)</td>
<td>(1.8-2.7)</td>
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<tr>
<td>AT (n = 7)</td>
<td>29</td>
<td>8</td>
<td>6.8</td>
<td>364</td>
<td><strong>0.10</strong></td>
<td><strong>0.51</strong></td>
<td><strong>0.3</strong></td>
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<tr>
<td></td>
<td>(26-59)</td>
<td>(7-29)</td>
<td>(8-2.9)</td>
<td>(262-470)</td>
<td>(0.10-0.11)</td>
<td>(0.41-0.59)</td>
<td>(0.3-0.4)</td>
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<tr>
<td>TFPI (n = 7)</td>
<td>87</td>
<td>62</td>
<td>7.9</td>
<td>1241</td>
<td>0.15</td>
<td>0.72</td>
<td>2.1</td>
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<tr>
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<td>(66-172)</td>
<td>(52-107)</td>
<td>(7-1.98)</td>
<td>(920-3634)</td>
<td>(0.14-0.19)</td>
<td>(0.66-1.06)</td>
<td>(1.7-3.5)</td>
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<tr>
<td>Heparin (n = 7)</td>
<td>156</td>
<td>68</td>
<td>5.1</td>
<td>1255</td>
<td>0.20</td>
<td>0.90</td>
<td>5.0</td>
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<tr>
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<td>(110-283)</td>
<td>(38-105)</td>
<td>(4.7-9.3)</td>
<td>(1126-1972)</td>
<td>(0.13-0.22)</td>
<td>(0.82-1.26)</td>
<td>(1.6-6.8)</td>
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<tr>
<td>tPA (n = 9)</td>
<td>98</td>
<td>76</td>
<td>9.7</td>
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<td>4.1</td>
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<td>(47-179)</td>
<td>(23-142)</td>
<td>(6.7-15.8)</td>
<td>(405-2022)</td>
<td>(0.16-1.36)</td>
<td>(7.31-2.85)</td>
<td>(2.8-44.6)</td>
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</table>

Total bronchoalveolar lavage fluid (BALF) cells and neutrophils are expressed as median (IQR) × 10⁴ cells per milliliter BALF at 24 and 48 hrs after inoculation with $10^6$ colony forming units of S. pneumoniae. All other data are also expressed as median (IQR). Controls are uninfected rats. APC, activated protein C; AT, antithrombin; BALF, bronchoalveolar lavage fluid; CINC-3, cytokine-induced neutrophil chemoattractant; IL-6, interleukin-6; MPO, myeloperoxidase activity; TFPI, tissue factor pathway inhibitor; TNF-α, tumor necrosis factor-α; tPA, tissue-type plasminogen activator. Dunn’s test: *p < 0.05, ‡p < 0.01 versus saline at the same timepoint.
Histopathology of the Lungs and Bacterial Outgrowth

At 24 hrs after induction of *S. pneumoniae* pneumonia, pulmonary histopathology showed dense inflammatory infiltrates, consisting predominantly of neutrophils, localized in the interstitium, alveolar space, and bronchial lumina (figure 4, see also foldout back cover).

Furthermore, endothelialitis, pleuritis and edema were present to a variable extent. Total histopathology scores are shown in figure 5. AT treatment resulted in significantly lower total scores compared to saline.

**Figure 5.** Total lung histopathologic scores at 24 hrs after *Streptococcus pneumoniae* infection were determined by an independent pathologist who was blinded for group identity. Total histopathologic scores are presented as median with interquartile range. Dunn’s test: *p < 0.05 versus saline.

**Figure 6.** Cultured *Streptococcus pneumoniae* colony forming units (CFU) were determined in lung homogenates (*A*) and bronchoalveolar lavage fluid (BALF, *B*) from rats treated with saline, recombinant human activated protein C (APC), plasma-derived antithrombin (AT), recombinant human tissue factor pathway inhibitor (TFPI), heparin, or recombinant tissue-type plasminogen activator (tPA) at 24 and 48 hrs after intratracheal instillation of $10^6$ CFU of *S. pneumoniae*. Bars represent mean ± SEM. Data are presented as the number of bacteremic rats per group (%).
From both lung and BALF from rats treated with AT, there were less *S. pneumoniae* CFU cultured (figure 6). There was no bacteremia observed in any of the rats at 24 hrs. At 48 hrs after *S. pneumoniae* inoculation, 6 of 11 (55 %) rats treated with saline had bacteremia. The proportion of bacteremic rats was only significantly different with the heparin-treated group in which all rats (7 of 7, 100%) developed bacteremia (figure 6).

**Discussion**

We demonstrate in patients with pneumonia that the natural anticoagulants PC, AT, and TFPI are suppressed in the bronchoalveolar compartment. This localized impairment of the physiological anticoagulants may contribute to enhanced bronchoalveolar thrombin generation in patients with pneumonia. Moreover, we used a rat model of *S. pneumoniae* pneumonia to further elucidate the potential role of anticoagulant therapy in bacterial pneumonia. Intravenous treatment with APC, AT, and TFPI limited bronchoalveolar coagulation. However, only AT treatment resulted in significant lung-protective effects, i.e., less bacterial outgrowth and less histopathologic lung injury.

Antithrombin is one of the major physiological inhibitors of coagulation, capable of inactivating thrombin and factors Xa, IXa, and VIIa bound to tissue factor [30]. Severe inflammatory processes result in increased consumption of AT, which has been demonstrated in patients with sepsis [31]. In a small group of sepsis patients, AT treatment seemed to improve lung function [15]. In a larger phase III trial with sepsis patients, AT treatment reduced the prevalence of new pulmonary dysfunction, but patient outcome was unchanged [17]. In addition, outcome of preexistent respiratory failure was unfortunately not assessed or not reported [17]. In trauma patients, AT did not influence the rate of respiratory failure, duration of mechanical ventilation, or duration of ARDS [32]. Mechanisms by which AT exerts its protective effects may be via binding with heparin-like glycosaminoglycan receptors [33,34] and increased prostacyclin-mediated inhibition of cytokines, decreased NF-κB activation [35], and inhibition of leukocyte activation and migration [36-39]. Also, AT could compete with bacterial toxins for binding on endothelial cell proteoglycans [40], thereby limiting the inflammatory response upon bacterial challenge [41]. Here we show that in *S. pneumoniae* challenged rats, bacterial outgrowth is strongly inhibited with AT treatment, limiting neutrophil influx and inflammatory reactions. It remains unclear whether these effects are associated with prostacyclin formation or interference with bacterial toxins (e.g.,
pneumolysin), but at least it is most likely that there are processes involved that are independent of anticoagulant activity.

APC is also a natural inhibitor of coagulation, and exerts its anticoagulant activity by catalyzing the proteolytic inactivation of factors Va and VIIa, and profibrinolytic activity by inactivating PAI-1 [42]. It thereby attenuates coagulation and promotes fibrinolysis. In pulmonary infection, anticoagulant therapy with APC seemed particularly appealing, because of anticoagulant and antiinflammatory effects in the pulmonary compartment [13,14]. Several reports have already addressed the beneficial effects of anticoagulation in experimental lung injury models: in rodents, intratracheal [43] and systemic [44,45] application of APC has been shown to attenuate bleomycin-induced and endotoxin-induced lung injury, respectively. Our current data confirm that APC is a potent inhibitor of bronchoalveolar coagulation activation in the presence of *S. pneumoniae* pneumonia. The profibrinolytic and antiinflammatory effects of APC could not be demonstrated in this study. Most importantly, we found that the inflammatory host response against *S. pneumoniae* was not significantly altered.

TFPI is the third natural anticoagulant which is of importance in regulating activation of coagulation. The main anticoagulant actions are via binding and inactivation of the tissue factor-factor VIIa complex and factor Xa. There is ongoing debate on the antiinflammatory properties of TFPI, which were primarily shown in experimental animal studies [46,47]. No survival benefit was demonstrated in sepsis patients treated with TFPI [18]. However, TFPI improved lung dysfunction in patients with severe sepsis and ARDS [16] while patients with severe community-acquired pneumonia did seem to benefit from treatment [48]. In our pneumococcal pneumonia model, TFPI was a less potent inhibitor of coagulation than APC and AT, although in previous experiments APC, AT, and TFPI were equally effective in inhibiting endotoxin-induced coagulation using the same dosages (chapter 4).

Heparin is a broadly used anticoagulant that acts by binding antithrombin, but had hardly any anticoagulant effects in our study. This may have been due to a dosing problem, but it is also possible that increased consumption of AT (as shown in figure 2B) has led to impaired activity of heparin. Somewhat surprising was that all rats treated with heparin developed bacteremia, suggesting that heparin interferes with microbial containment or facilitates dissemination. This may be related to the fact that heparin neutralizes antiinflammatory effects of AT [39], but the exact mechanism remains unresolved since inflammatory mediators were not altered by heparin treatment. Certainly, this result changes the perspective on heparin after several reports describing lung-protective effects in endotoxemia-related lung injury in sheep [49], swine [50] and mice [51].
Facilitation of bacteremia by heparin underlines that coagulation and inflammation are pivotal host defense mechanisms, and interference with these pathways should be performed with great care. In the recent past, antiinflammatory strategies have failed to improve outcome of patients with sepsis [52]. Similarly, anticoagulant treatment with AT and TFPI both failed to increase survival rates in severe sepsis [17,18], while there is ongoing debate on the efficacy of APC. Robriquet et al. recently reported detrimental effects of APC-administration in the early stage of P. aeruginosa induced lung injury [53], whereas Kipnis et al. showed similar results with AT-treatment [54]. These studies, in fact, suggest that interference with bronchoalveolar coagulation can be very detrimental in the very first hours after microbial challenge. Our heparin-treated rats seem to concur the findings presented by Drs Robriquet and Kipnis [53,54], but data from our current report (with APC, AT, and TFPI) and from a previous report (using recombinant nematode anticoagulant protein c2 in murine pneumococcal pneumonia)[4] suggest that anticoagulant therapy does not necessarily lead to increased bacterial dissemination. Of note, studies by Drs Robriquet, Kipnis, and ourselves are experimental models in which both the emergence of pneumonia (overload of bacteria to healthy animals), absence of concomitant medication (e.g., antibiotics), and timing of treatment (before inoculation with bacteria) do not correspond with the clinical practice. Therefore, clinical trials are warranted to determine the effects of natural anticoagulants in patients with pneumonia without sepsis. In the light of our current study results, it is unfortunate that AT is not being investigated as a therapeutic agent in non-septic pneumonia patients, as opposed to ongoing trials with APC (NCT00112164, patients with acute lung injury; ISRCTN52566874, patients with community-acquired pneumonia) and TFPI (NCT00084071, patients with community-acquired pneumonia).

In conclusion, during pulmonary infection, insufficiency of the natural anticoagulant pathways contributes to the establishment of a local procoagulant environment within the airways. AT exerts lung-protective effects in S. pneumoniae pneumonia by inhibiting bacterial outgrowth, limiting histopathological damage, and hampering the inflammatory response. These effects are most likely independent of coagulation inhibition, and further studies are needed to unravel the underlying mechanisms.

Acknowledgments

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References


Ventilator-Associated Lung Injury
Mechanical Ventilation with Lower Tidal Volumes and PEEP Prevents Alveolar Coagulation in Patients without Lung Injury

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Esther K. Wolthuis
Paul Bresser
Marcel Levi
Tom van der Poll
Misa Dzoljic
Margreeth B. Vroom
Marcus J. Schultz

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Abstract

Background. Alveolar fibrin deposition is a hallmark of acute lung injury, resulting from activation of coagulation and inhibition of fibrinolysis. Previous studies have shown that mechanical ventilation with high tidal volumes may aggravate lung injury in patients with sepsis and acute lung injury. We sought to determine the effects of mechanical ventilation on the alveolar hemostatic balance in patients without preexistent lung injury.

Methods. Patients, scheduled for an elective surgical procedure (lasting ≥ 5 hours), were randomized to mechanical ventilation with either higher tidal volumes of 12 mL/kg ideal body weight and no positive end-expiratory pressure (PEEP) or lower tidal volumes of 6 mL/kg and 10 cm H2O PEEP. After induction of anesthesia and 5 hours later bronchoalveolar lavage fluid and blood samples were obtained, and markers of coagulation and fibrinolysis were measured.

Results. In contrast to mechanical ventilation with lower tidal volumes and PEEP (n = 21), the use of higher tidal volumes without PEEP (n = 19) caused activation of bronchoalveolar coagulation, as reflected by a marked increase in thrombin-antithrombin complexes, soluble tissue factor, and factor VIIa after 5 hours of mechanical ventilation. Mechanical ventilation with higher tidal volumes without PEEP caused an increase in soluble thrombomodulin in lavage fluids and lower levels of bronchoalveolar activated protein C in comparison to lower tidal volumes and PEEP. Bronchoalveolar fibrinolytic activity did not change by either ventilation strategy.

Conclusion. Mechanical ventilation with higher tidal volumes and no PEEP promotes procoagulant changes, which are largely prevented by the use of lower tidal volumes and PEEP.
Pulmonary inflammation is characterized by local generation of proinflammatory mediators and a procoagulant shift of the alveolar hemostatic balance, promoting fibrin depositions within the airways [1,2]. Indeed, disturbances in alveolar fibrin turnover have been demonstrated in patients with pneumonia [3-6] and acute respiratory distress syndrome (ARDS) [3,7]. Whereas fibrin formation may aid in host protection, such as the containment of infectious agents during pulmonary infection and in maintaining or repairing the endothelial-epithelial barrier, on the other hand, coagulation products such as thrombin and fibrin have significant proinflammatory properties, potentially compromising pulmonary integrity and function [1,2]. In its most extreme form bronchoalveolar fibrin formation may compromise pulmonary function, as may occur with severe ARDS.

In severe lung injury, ventilatory support is almost invariably mandatory, but it is increasingly recognized that mechanical ventilation itself may aggravate or even initiate lung injurious processes [8,9]. The so-called ventilator-associated lung injury is characterized by several pathophysiological sequelae, including local generation of inflammatory mediators, constituting a pulmonary environment which is highly proinflammatory. Another hallmark of ventilator-associated lung injury in patients with severe lung injury is the activation of bronchoalveolar coagulation [5,6]. In patients with ARDS mechanical ventilation with lower tidal volumes improves patient survival [10], most likely by limiting generation of proinflammatory mediators, both locally in the lungs and systemically [11]. It is unknown whether (mechanical ventilation induced) alterations in the alveolar hemostatic balance contribute to outcome in mechanically ventilated patients. Moreover, there is ongoing debate on whether patients without preexistent lung injury would benefit from mechanical ventilation with lower tidal volumes, since large clinical trials have only investigated patients with acute lung injury and ARDS in the intensive care unit. Recently, the pulmonary and systemic inflammatory effects of mechanical ventilation were investigated in patients during major surgery, showing little alterations in the inflammatory responses [12,13].

The aim of the current study was to characterize the effects of mechanical ventilation on the alveolar hemostatic balance. A randomized controlled trial was performed comparing two mechanical ventilation strategies in patients without preexistent lung injury who were scheduled for a major surgical procedure.
Material and Methods

Patients

The study protocol was approved by the Medical Ethics Committee of the University of Amsterdam, and informed consent was obtained from all patients. Adult patients were eligible if scheduled for a surgical procedure of \( \geq 5 \) hrs, and all involved physicians (surgeon, anesthesiologist, pulmonologist) consented with the study procedures, assuring safety of the patient. Exclusion criteria included a history of any lung disease, use of immunosuppressive medication, recent infections, previous thromboembolic disease, recent admission to the intensive care unit for ventilatory support, and participation in another clinical trial.

Study Protocol

All patients received routine anesthesia according to protocol, including intravenous propofol (2-3 mg/kg, thereafter 6-12 mg/kg/hr), fentanyl (2-3 μg/kg, thereafter as required), and rocuronium (as required); and epidural bupivacaine (0.125%) / fentanyl (2.5 μg/mL). The ventilatory protocol consisted of volume-controlled mechanical ventilation, at an inspired oxygen fraction of 0.40, inspiratory to expiratory ratio of 1:2, and a respiratory rate adjusted to normocapnia. Randomization was performed by drawing a presealed envelope; patients were randomized to mechanical ventilation with either tidal volumes of 12 mL/kg (ideal body weight, calculated according to the formula as described before) \([10]\) and no positive end-expiratory pressure (PEEP) or 6 mL/kg and 10 cm H\(_2\)O PEEP. Anesthesiologists were allowed to change the ventilation protocol at any time point upon surgeon’s request, or if there was any concern on patient’s safety. If the surgical procedure exceeded 5 hrs, anesthesiologists were allowed to change the ventilation strategy after the second sampling (blood and bronchoalveolar lavage). Patients were followed until hospital discharge or death.

Specimen Processing

Bronchoscopy and bronchoalveolar lavage were performed twice on all patients: the first just after initiation of mechanical ventilation in either the right middle lobe or lingula, the second performed in the contralateral lung 5 hrs thereafter, either perioperatively or directly postoperatively. Lavage fluid was obtained as previously describe \([4,6,14]\). In short, the bronchoalveolar lavage was performed by an experienced pulmonologist in a standardized fashion according to the guidelines of the American Thoracic Society, using a flexible fiberoptic videobronchoscope. Seven successive 20 mL aliquots of prewarmed normal saline
were instilled and aspirated immediately with low suction (general recovery 10-15 mL). For coagulation assays, sodium citrate and benzamidin were added to the lavage fluids to a final concentration of 10 mM and 20 mM, respectively. Citrated (0.109 M) blood samples were drawn prior to both lavages, and hourly blood gas analyses were performed. Cell free supernatants were stored at −80 °C until analysis.

Assays for Coagulation and Fibrinolysis

Thrombin-antithrombin complex (TATc), soluble tissue factor, factor VIIa, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1), plasminogen activator activity, soluble thrombomodulin and activated protein C (APC) concentrations were measured as described before [15-17].

Statistical Analysis

The required sample size was calculated from data from our previous investigations on pulmonary hemostasis [5,6]. To detect differences in bronchoalveolar TATc concentrations in
the study groups at a two-sided significance level of 5 percent with a power of 80 percent, the number of patients to be studied in each group was at least 19. Baseline characteristics of the randomized patient groups were compared with Student’s t test or Mann-Whitney U test, where appropriate. For categorical data \( \chi^2 \) test was used. Differences within groups were analyzed with a Wilcoxon signed-rank test for paired samples comparing \( t = 5 \) versus \( t = 0 \) hrs, Mann-Whitney U test was used to compare the changes over time between the two randomization groups. All results are expressed as mean ± SD. A \( p \)-value of less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL).

### Table 1. Baseline characteristics of patients

<table>
<thead>
<tr>
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<th>LV(T) / PEEP (n = 21)</th>
<th>HV(T) / ZEEP (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, years</td>
<td>62 ± 9.8</td>
<td>61 ± 9.5</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>14 (67)</td>
<td>14 (74)</td>
</tr>
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<td>ASA, median (range)</td>
<td>2 (1-4)</td>
<td>2 (1-3)</td>
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<tr>
<td>Height, mean ± SD, cm</td>
<td>176 ± 8.7</td>
<td>174 ± 10.0</td>
</tr>
<tr>
<td>Weight, mean ± SD, kg</td>
<td>79 ± 14.4</td>
<td>76 ± 13.7</td>
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<tr>
<td>IBW, mean ± SD, kg</td>
<td>70 ± 9.5</td>
<td>69 ± 10.6</td>
</tr>
<tr>
<td>Tobacco use, n (%)</td>
<td>9 (43)</td>
<td>6 (32)</td>
</tr>
<tr>
<td>Surgical procedure</td>
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<td>5 Whipple’s Procedure(^a)</td>
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<td>5 Lap. Radical Prostatectomy</td>
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</tr>
<tr>
<td>6 Hemihepatectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Retroperitoneal Tumor Resection</td>
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<td></td>
</tr>
<tr>
<td>2 Total Pancreatectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Open Prostatectomy(^b)</td>
<td></td>
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</tbody>
</table>

ASA, American Society of Anesthesiologists (physical status classification); IBW, ideal body weight; HV\(T\) / ZEEP, higher tidal volumes / zero positive end-expiratory pressure; LV\(T\) / PEEP, lower tidal volumes / positive end-expiratory pressure. 
\(^a\) Whipple’s procedure is a pancreaticoduodenectomy. 
\(^b\) The open prostatectomy was performed after an initial laparoscopic approach.

### Table 2. Perioperative parameters

<table>
<thead>
<tr>
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<th>LV(T) / PEEP (n = 21)</th>
<th>HV(T) / ZEEP (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV duration, mean ± SD, min</td>
<td>304 ± 35</td>
<td>308 ± 52</td>
</tr>
<tr>
<td>Blood loss, median (IQR), mL</td>
<td>1550 (800-2325)</td>
<td>1000 (463-1675)</td>
</tr>
<tr>
<td>Transfused red cells, median (IQR), units</td>
<td>0 (0-1.5)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>Transfused plasma, median (IQR), units</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Colloids, median (IQR), L</td>
<td>0.5 (0.5-1.5)</td>
<td>0.5 (0.5-1.5)</td>
</tr>
<tr>
<td>Crystalloids, median (IQR), L</td>
<td>4.5 (2.75-5.75)</td>
<td>4.0 (2.5-5.5)</td>
</tr>
<tr>
<td>Lowest Hb, mean ± SD, mmol/L(^a)</td>
<td>6.0 ± 1.2</td>
<td>6.2 ± 1.0</td>
</tr>
<tr>
<td>Highest SBP, mean ± SD, mmHg</td>
<td>122 ± 17</td>
<td>135 ± 21*</td>
</tr>
<tr>
<td>Lowest SBP, mean ± SD, mmHg</td>
<td>82 ± 9.6</td>
<td>87 ± 14.9</td>
</tr>
</tbody>
</table>

MV, mechanical ventilation; IQR, interquartile range; Hb, hemoglobin; SBP, systolic blood pressure. 
\(^a\) p < 0.05 versus LV\(T\) / PEEP; 
\(^{a}\) Hemoglobin, 1 mmol/L = 1.61 g/dL.
Results

Patients

Seventy-four consecutive patients who were scheduled for an elective surgical procedure of 5 hrs or more were screened in the period December 2003 through March 2005 (figure 1). Twenty-eight patients were excluded, leaving 46 patients for randomization. Five patients were randomized but excluded from final analysis, because the initial surgical procedure was converted by the surgeon into another shorter operation (< 3 hrs), and only one bronchoalveolar lavage was performed. One patient was randomized but no lavages were performed upon the surgeon’s request after induction of anesthesia. In total 40 patients completed the study protocol. There were no major differences between both randomization groups with regard to baseline characteristics (table 1).

There were no adverse events related to the bronchoalveolar lavages. One surgeon complained about hepatic congestion, and demanded PEEP levels to be reduced (patient ventilated with lower tidal volumes and PEEP). Aside from the mechanical ventilator settings

Figure 2. Perioperative parameters. Tidal volumes (VT), respiratory rate (Respir. Rate), maximal pressures (Pmax) in patients ventilated with lower tidal volumes and positive end-expiratory pressure (open symbols, n = 21) and patients ventilated with higher tidal volumes and no positive end-expiratory pressure (closed symbols, n = 19). MV, mechanical ventilation. Data are mean ± SD. There are no significant differences in arterial blood gas analyses at any timepoint.
Figure 3. Thrombin-antithrombin complexes (TATc, A), soluble tissue factor (sTF, B), and factor VIIa (FVIIa, C) in bronchoalveolar lavage fluid recovered at baseline (t = 0) and after 5 hrs (t = 5) from patients mechanically ventilated with 6 mL/kg and 10 cm H₂O PEEP (LV₃/PEEP, n = 21) or with 12 mL/kg and zero PEEP (HV₃/ZEEP, n = 19). Data are mean ± SD. Wilcoxon signed-rank test: *p < 0.001, †p < 0.01 versus t = 0. Mann-Whitney U test: ‡p < 0.001 between groups.

Figure 4. Plasminogen activator activity (PAA, A), plasminogen activator inhibitor type-1 (PAI-1, B), and tissue-type plasminogen activator (tPA, C) in bronchoalveolar lavage fluid recovered at baseline (t = 0) and after 5 hrs (t = 5) from patients mechanically ventilated with 6 mL/kg and 10 cm H₂O PEEP (LV₃/PEEP, n = 21) or with 12 mL/kg and zero PEEP (HV₃/ZEEP, n = 19). Data are mean ± SD. Wilcoxon signed-rank test: *p < 0.05, †p < 0.001, ‡p < 0.001 versus t = 0. Mann-Whitney U test: §p < 0.05 between groups.

Figure 5. Activated protein C (APC, A) and soluble thrombomodulin (sTM, B) in bronchoalveolar lavage fluid recovered at baseline (t = 0) and after 5 hrs (t = 5) from patients mechanically ventilated with 6 mL/kg and 10 cm H₂O PEEP (LV₃/PEEP, n = 21) or with 12 mL/kg and zero PEEP (HV₃/ZEEP, n = 19). Data represent are mean ± SD. Wilcoxon signed-rank test: *p < 0.05. Mann-Whitney U test: ‡p < 0.05.
(tidal volume, PEEP, and respiratory rate), there were no significant differences in perioperative hemodynamic parameters (table 2, figure 2). In particular, peak pressures were not different between the study groups during 5 hrs of mechanical ventilation.

**Bronchoalveolar Coagulation and Fibrinolysis**

Mechanical ventilation with higher tidal volumes and zero PEEP (HV/T/ZEEP) caused activation of bronchoalveolar coagulation, as reflected in a marked increase in TATc, soluble tissue factor, and factor VIIa after 5 hrs of mechanical ventilation (all \( p < 0.001 \) versus \( t = 0 \); figure 3). In patients ventilated with lower tidal volumes and 10 cm H\(_2\)O PEEP (LVT/PEEP), only soluble tissue factor was slightly elevated (\( p < 0.01 \) versus \( t = 0 \); figure 3B) and far less pronounced than in patients with HV/T/ZEEP (\( p < 0.001 \) between groups; figure 3B).

Neither mechanical ventilation strategies were associated with changes in bronchoalveolar plasminogen activator activity (both within groups and between groups), despite a slight upregulation of PAI-1 with HV/T/ZEEP (\( p < 0.05 \) versus \( t = 0 \); figure 4). tPA was increased in both groups (both \( p < 0.001 \) versus \( t = 0 \); figure 4C), slightly more in HV/T/ZEEP (\( p < 0.05 \) between groups).

There was a trend towards lower levels of bronchoalveolar APC with HV/T/ZEEP as opposed to a trend towards higher APC with LVT/PEEP (figure 5A). Between group analysis did show a difference in changes of APC levels over time (\( p < 0.05 \) between groups).

Mechanical ventilation with HV/T/ZEEP caused an increase in soluble thrombomodulin as measured in lavage fluids (\( p < 0.05 \) versus \( t = 0 \); figure 5B), which was not with LVT/PEEP.

**Systemic Hemostasis**

During surgery both systemic procoagulant and fibrinolytic activity were increased. In patients ventilated with HV/T/ZEEP, there was an increase in TATc (6.1 ± 0.76 versus 5.78 ± 1.10 ng/mL, \( p < 0.05 \)) and plasminogen activator activity (103 ± 5.9 versus 99 ± 6.5 %, \( p < 0.01 \)); in patients ventilated with LVT/PEEP, there was also an increase in TATc (5.63 ± 1.13 versus 4.86 ± 1.09 ng/mL, \( p < 0.01 \)), but only a trend towards higher plasminogen activator activity (102 ± 8.7 versus 99 ± 6.6 %). The changes over time were not different between the two mechanical ventilation strategies.
Postoperative Course

In the postoperative recovery, 28 patients had followup chest radiographs. There were no differences in postoperative arterial blood gas analyses (HV T/ZEEP versus LV T/PEEP): pO$_2$ 15.5 ± 5.6 versus 16.4 ± 7.1 kPa, pCO$_2$ 5.7 ± 0.6 versus 5.6 ± 0.7 kPa, and pH 7.36 ± 0.053 versus 7.34 ± 0.051. There were no differences in incidence of pulmonary complications (e.g., acute lung injury, pneumonia) between the two study groups; in each study group, there was one patient requiring prolonged mechanical ventilation for respiratory failure after surgery. One patient ventilated with LV T/PEEP died postoperatively of multiple organ failure after complicated hemihepatectomy. All other patients were discharged home.

Discussion

Although mechanical ventilation with lower tidal volumes is generally considered to be protective in patients with acute lung injury, there is ongoing debate on the ideal tidal volumes in patients without preexistent lung injury. We here demonstrated that mechanical ventilation has significant effects on bronchoalveolar hemostasis: although the duration of mechanical ventilation was only 5 hrs, and no differences were observed in clinical parameters during the surgical procedure or in the recovery phase, local procoagulant activity was increased in the group of patients with non-injured lungs ventilated with 12 mL/kg and without the use of PEEP. Furthermore, we showed that mechanical ventilation with lower tidal volumes and PEEP can largely prevent these procoagulant changes. Simultaneously, there is upregulation of plasminogen activation, which is not immediately reflected in increased fibrinolytic activity, perhaps – at least in patients ventilated with HV T/ZEEP – because of inhibitory effects of PAI-1. And finally, we demonstrated that mechanical ventilation with HV T/ZEEP causes generation of more soluble fragments of thrombomodulin in the bronchoalveolar spaces, potentially leading to an impaired activation of the protein C system. In summary, mechanical ventilation with HV T/ZEEP seems to promote fibrin depositions within the airways by three mechanisms: increased procoagulant activity via the extrinsic pathway, a relative insufficiency of the anticoagulant protein C system, and inhibition of fibrinolysis by PAI-1.

A ‘multiple-hit’ model of lung injury can be theorized whereby predisposing conditions, like injurious mechanical ventilation during surgery, may result in pulmonary inflammation (the ‘primary hit’). Then, several ‘second hits’, like transfusion of blood products [18], prolonged (injurious) mechanical ventilation [11,19], aspiration [10,20], shock or sepsis
[10,20], and ventilator-associated pneumonia [21] may all cause additional lung injury, finally resulting in full-blown ARDS with high morbidity and mortality. Although the present study was not designed to investigate clinical outcome, no differences were observed in the postoperative course between the study groups. However, the alterations in bronchoalveolar hemostasis may indicate that mechanical ventilation potentially has harmful effects, even in patients without acute lung injury.

The presently described changes in pulmonary hemostasis are very similar as previously described in patients with pneumonia or ARDS [3-6], and human volunteers with endotoxin-induced pulmonary inflammation [15,22]. Consistently, increased procoagulant activity is reported, mostly related to the extrinsic coagulation pathway. It is likely that this activation is mediated by tissue factor expression on epithelial cells and mononuclear cells in the bronchoalveolar compartment. In the case of activation of epithelial and endothelial cells, either by pathogens, excessive inflammation, or – as probably is the case during mechanical ventilation – mechanical strain, there will be disruption of the endothelial-epithelial barrier. Transudation of plasma into the bronchoalveolar compartment will subsequently initiate coagulation within the airways. We speculate that this is the mechanism leading to immediate ‘sealing’ of the damaged area, providing injury containment, and initiating other repair systems. Also, PAI-1 upregulation has been found consistently in patients with pneumonia [3-6], ARDS [3,7], and in our patients ventilated with HV/T/ZEEP. Although in the current clinical settings, this did not lead to a suppression of fibrinolytic activity, it may well be possible that prolonged mechanical ventilation could lead to even higher levels of PAI-1 and more interference with fibrinolytic activity, as we demonstrated in patients developing ventilator-associated pneumonia [5,6]. tPA antigens levels were increased by ventilation with both low and high tidal volumes. This is in contrast to in vitro studies by Dr Günther’s group in which various cell lines all showed a downregulation of tPA mRNA expression upon inflammatory stimuli [23]. The same group however reported in an in vivo model of endotoxin-induced lung injury, that tPA is indeed upregulated in both structural (alveolar type II cells, endothelial cells) and host defense cells (alveolar macrophages) in mouse lungs [24]. Also in our recent report on endotoxin-induced lung inflammation in human volunteers, increased levels of tPA were observed [15]. It is thought that this early activation of the fibrinolytic system is involved in tissue remodeling [25].

We have shown before that in patients with pneumonia protein C activation is suppressed, as well as after instillation of endotoxin in lungs of healthy volunteers [15,26]. Mechanical ventilation with HV/T/ZEEP induced shedding of sTM into the airspaces, which is generally
believed to represent epithelial or endothelial damage. On the other hand, there was a trend towards lower levels of APC which was clearly opposite of the effect seen with LV/PEEP ventilation. Recombinant human APC (rhAPC) has been shown to reduce mortality in patients with severe sepsis [27], in whom protective effects are believed to be beyond its anticoagulant properties. Despite vast data from \textit{in vitro} and animal studies, however, it is still unclear how rhAPC acts \textit{in vivo}. Systemic administration of rhAPC has significant effects in the lungs [15,28], suggesting that pulmonary effects of rhAPC may contribute to patient survival. Indeed, in the pivotal phase III study in patients with sepsis, the majority of patients had a pulmonary origin and moreover, patients with pneumonia benefited mostly from rhAPC treatment [27,29,30].

All of the described effects shift the hemostatic balance towards a procoagulant side, promoting fibrin depositions in the airways. The question remains whether this reflects an adaptive mechanism with host protective functions, or whether it is a harmful process, predisposing the lungs to secondary complications or perhaps with long-term effects on pulmonary function. Fibrin deposition may be an important mechanism to repair endothelial or epithelial damage, however exaggerated coagulation activation has been related to a number of detrimental sequelae. Coagulation products have important proinflammatory effects [31], and in addition, ongoing fibrin depositions inactivate surfactant proteins, causing alveolar instability and collapse. Importantly, various anticoagulant strategies have been shown to limit lung injury in experimental studies [32], but potential beneficial effects have to be confirmed in human patients.

To date, there have been few other reports on the effects of mechanical ventilation in patients with non-injured lungs. Gajic \textit{et al.} [33,34] identified mechanical ventilation with higher tidal volumes as a risk factor for the development of acute lung injury in patients who did not have lung disease at the onset of mechanical ventilation. However, these patients were critically ill patients in an intensive care unit, developing ARDS after 48 hrs or more. Wrigge \textit{et al.} [12] recently showed that in patients undergoing major surgery with up to 3 hrs of mechanical ventilation, the ventilation strategy did not affect pulmonary or systemic cytokine levels, suggesting that a brief period of mechanical ventilation does not affect patients without systemic inflammation. Most recently Wrigge \textit{et al.} extended the duration of mechanical ventilation to 6 hrs by selecting patients undergoing cardiac surgery [13]. Again, no systemic effects were observed, but postoperative levels of tumor necrosis factor alfa in bronchoalveolar lavage fluid were lower in patients ventilated with lower tidal volumes. However, measured cytokine levels were very low and highly variable. Therefore, we decided
to lavage patients twice, immediately after initiation of mechanical ventilation, and 5 hrs thereafter; this way, every patient would be his/her own control.

We here demonstrate for the first time that mechanical ventilation in patients with normal lungs induces a procoagulant shift in the alveolar hemostatic balance. Mechanical ventilation with lower tidal volumes and PEEP largely attenuates these changes in procoagulant activity within the airways. Clinical studies are warranted to establish the effects of prolonged mechanical ventilation (i.e., in an intensive care unit) on bronchoalveolar hemostasis, and the relationship between alveolar procoagulant activity and patient outcome.

References

Mechanical Ventilation with Lower Tidal Volumes and PEEP Limits Pulmonary Inflammation in Patients without Lung Injury

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Abstract

Background. Mechanical ventilation with higher tidal volumes aggravates lung injury in patients with established acute lung injury. We sought to determine the effects of mechanical ventilation on the pulmonary inflammatory response in patients without preexistent lung injury.

Methods. Patients scheduled for an elective surgical procedure (lasting ≥ 5 hours) were randomized to mechanical ventilation with either higher tidal volumes of 12 mL/kg predicted body weight and no positive end-expiratory pressure (PEEP) or lower tidal volumes of 6 mL/kg and 10 cm H2O PEEP. After induction of anesthesia and 5 hours thereafter, bronchoalveolar lavage fluid (BALF) and blood were investigated for polymorphonuclear cell influx, changes in levels of inflammatory markers and nucleosomes. In addition, mRNA from alveolar macrophages was analyzed for changes in expression of inflammatory and apoptotic mediators.

Results. Five hours of mechanical ventilation did not induce polymorphonuclear cell influx in the lungs, as determined in BALF. However, the use of higher tidal volumes without PEEP (n = 19) caused a slight but statistically significant increase of interleukin (IL)-8 and myeloperoxidase (MPO) in BALF, in contrast with the use of lower tidal volumes with PEEP (n = 21) which did not alter pulmonary IL-8 and MPO-levels. BALF-levels of IL-1α, IL-1β, IL-6, macrophage inflammatory protein (MIP)-1α, and MIP-1β did not increase in both groups. There was no direct relationship between protein levels of inflammatory mediators in BALF and mRNA expression in alveolar macrophages. Plasma levels of IL-6 and IL-8 increased with mechanical ventilation, but there was no difference between the groups. Nucleosome levels in BALF and plasma were increased after 5 hours of mechanical ventilation, which was attenuated by ventilation with lower tidal volumes and PEEP.

Conclusion. The use of lower tidal volumes and PEEP moderately limits local inflammation and apoptosis in mechanically ventilated patients without preexistent lung injury.
Mechanical ventilation may aggravate pulmonary inflammation which may be a factor in additional morbidity and mortality associated with nonprotective forms of mechanical ventilation [1,2]. Indeed, mechanical ventilation with lower tidal volumes has been found to improve survival of patients with acute lung injury and acute respiratory distress syndrome (ALI/ARDS) [3]. This so-called ‘ventilator-associated lung injury’ is characterized by local attraction of inflammatory cells and local production and subsequent dissemination of inflammatory mediators. Ranieri et al. demonstrated a reduction in bronchoalveolar lavage fluid (BALF) concentrations of polymorphonuclear cells (PMN) and proinflammatory mediators with a lung-protective mechanical ventilation strategy as compared with conventional mechanical ventilation in patients with ARDS [4]. In addition, lung-protective mechanical ventilation attenuated systemic levels of inflammatory mediators [3,4], which seems clinically relevant since higher systemic levels of these mediators were associated with more multi-organ failure [5]. Furthermore, it has been shown in experimental studies that lung-protective mechanical ventilation could significantly limit end-organ epithelial cell apoptosis, protecting organ function during mechanical ventilation [6,7].

Whether mechanical ventilation per se initiates pulmonary inflammation is an ongoing debate. Although earlier studies in animals demonstrated that mechanical ventilation with high tidal volumes caused pulmonary edema and injury [8] and inflammation [9,10], these studies are criticized because used tidal volumes were unphysiologically large. Then again, deleterious effects of high tidal volumes in patients without lung injury, have been suggested by retrospective studies [11-13].

The purpose of this study was to investigate the effects of mechanical ventilation on pulmonary inflammation and apoptosis. A randomized controlled trial was performed comparing two different mechanical ventilation strategies in patients without lung injury, who where scheduled for a major surgical procedure.

Material and Methods

Patients

The study protocol was approved by the Medical Ethics Committee of the University of Amsterdam, and informed consent was obtained from all patients. Adult patients were eligible if scheduled for a surgical procedure of \( \geq 5 \) hrs, and all involved physicians (surgeon, anesthesiologist, pulmonologist) consented with the study procedures, assuring safety of the
patient. Exclusion criteria included a history of any lung disease, use of immunosuppressive medication, recent infections, previous thromboembolic disease, recent ventilatory support, and/or participation in another clinical trial.

**Study Protocol**

All patients received anesthesia according to a local protocol, as described previously [14]. The ventilatory protocol consisted of volume-controlled mechanical ventilation, at an inspired oxygen fraction of 0.40, inspiratory to expiratory ratio of 1:2, and a respiratory rate adjusted to achieve normocapnia. Randomization was performed by drawing a presealed envelope; patients were randomized to mechanical ventilation with either tidal volumes of 12 mL/kg (ideal body weight, calculated according to the formula as described before) [3] and no positive end-expiratory pressure (PEEP) or 6 mL/kg and 10 cm H$_2$O PEEP. Anesthesiologists were allowed to change the ventilation protocol at any time point upon surgeon’s request, or if there was any concern on patient’s safety. If the surgical procedure exceeded 5 hrs, anesthesiologists were allowed to change the ventilation strategy after the second sampling (blood and bronchoalveolar lavage [BAL]).

**Specimen Processing**

Bronchoscopy and BAL were performed twice on all patients: the first just after initiation of mechanical ventilation in the right middle lobe or lingula, the second performed in the contralateral lung 5 hrs thereafter, either perioperatively or directly postoperatively. BALF was obtained and processed as previously described [15-17]. Arterial blood samples were drawn prior to both lavages, and hourly blood gas analyses were performed. Cell free supernatants from BALF and blood were stored at –80 °C until analysis. BALF cells were resuspended in icecold phosphate buffered saline. The resuspended cells were partially used for absolute cell counts (using a Bürker-Turk hemocytometer, Emergo, Landsmeer, The Netherlands) and Giemsa-stained cytospin preparation for differential counting. The remaining cells were dissolved in RNAlater (Ambion, Austin, TX), and stored at –80 °C until RNA isolation.

**RNA Isolation and Multiplex Ligation-Dependent Probe Amplification**

RNA was isolated using the RNeasy mini kit system (Qiagen, Valencia, CA), according to the manufacturer’s recommendations. Multiplex ligation-dependent probe amplification was
performed as described previously [18-20]. In the present study, multiplex ligation-dependent probe amplification was used to analyze mRNA expression of a set of proteins involved in inflammation [20,21].

**Assays**

Myeloperoxidase (MPO) was determined by ELISA [22]. Tumor necrosis factor (TNF-α), interleukin (IL)-1α, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, and MIP-1β were measured by ELISA (TNF-α, IL-6, IL-8, Sanquin, Amsterdam, The Netherlands; IL-1α, MIP-1α, MIP-1β, R&D Systems, Minneapolis, MN). Nucleosomes were measured by ELISA as described previously with slight modifications [23]. One unit (U) was arbitrarily set at the amount of nucleosomes released by 100 Jurkat cells. Detection limit of the assay is 0.1 U/mL.

**Statistical Analysis**

Baseline characteristics of the randomized patient groups were compared with Student’s *t* test, Mann-Whitney *U* test, or χ² test as appropriate. Differences within groups were analyzed with a Wilcoxon signed-rank test for paired samples comparing *t* = 5 versus *t* = 0 hrs. Mann-Whitney *U* test was used to compare the changes over time between the two randomization groups. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL).

**Results**

**Patients**

Patient characteristics and perioperative parameters were described previously [14] (chapter 10). There were no major differences in perioperative parameters, including number of transfusions, colloid/crystalloid infusions, and arterial blood gases at any time point during 5 hrs of mechanical ventilation. There were no differences in incidence of pulmonary complications (e.g., acute lung injury, pneumonia) between the study groups; in each study group, there was one patient requiring prolonged mechanical ventilation for respiratory failure after surgery. One patient ventilated with lower tidal volumes and PEEP died postoperatively of multiple organ failure after complicated hemihepatectomy. All other patients were discharged home.
Figure 1. Myeloperoxidase (MPO) in bronchoalveolar lavage fluid recovered at baseline (t = 0) and after 5 hrs (t = 5) from patients mechanically ventilated with 6 mL/kg and 10 cm H₂O PEEP (LV/PEEP, n = 21) or with 12 mL/kg and zero PEEP (HV/ZEEP, n = 19). Data represent mean ± SEM. Wilcoxon signed-rank test: *p < 0.01 versus t = 0.

Figure 2. Changes in mRNA expression of interleukin (IL)-1β, IL-1 receptor antagonist (RA), macrophage inflammatory protein (MIP)-1β, and MIP-1α (all relative to β2-microglobulin) in alveolar macrophages at baseline (t = 0) and after 5 hrs (t = 5) from patients mechanically ventilated with 6 mL/kg and 10 cm H₂O PEEP (LV/PEEP, n = 21) or with 12 mL/kg and zero PEEP (HV/ZEEP, n = 19) (A). Changes in mRNA expression of all other inflammatory markers (B): a, nuclear factor-κB, subunit 1 (NF-κB-1); b, nuclear factor-κB, α; c, tumor necrosis factor (TNF)-α; d, IL-1α; e, IL-6; f, IL-8; g, IL-10; h, IL-15; i, IL-18; j, MYC; k, monocyte chemotactic protein (MCP)-1; l, serine protease inhibitor, clade B, member 9; m, platelet-derived growth factor, b polypeptide; n, polyadenylate-specific RNAse; o, thrombospondin-1; p, cyclin-dependent kinase inhibitor 1A; q, TNF receptor superfamily, 1A; r, BMI-1 oncogene homolog; s, macrophage migration inhibitory factor; t, phosphodiesterase 4B, cAMP specific; u, protein-tyrosine phosphatase, nonreceptor-type 1; v, protein-tyrosine phosphatase, type 4A, 2; w, glutathione S-transferase π. mRNA expression of interferon-γ, IL-4, IL-12, IL-13, TNF-β, MCP-2, and NFκB-2 are not shown because mRNA of these inflammatory markers was detected in less than 4 patients per group. Data represent median ± interquartile range. Wilcoxon signed-rank test: *p < 0.05, †p < 0.01 versus t = 0. Mann-Whitney U test: ‡p < 0.01 between groups.
Cellular Composition of BALF and Myeloperoxidase in BALF

BALF cells consisted for > 99% of macrophages. Mechanical ventilation did not influence cell content, and no differences were found between groups. MPO levels in BALF, however, were significantly higher after 5 hrs of mechanical ventilation with higher tidal volumes without PEEP (figure 1), while no increase in MPO levels were observed with the use of lower tidal volumes with PEEP.

Expression of Inflammatory mRNAs

Expression of mRNAs of IL-1β, IL-1 receptor antagonist, MIP-1α, and MIP-1β in alveolar macrophages was lower with higher tidal volume ventilation (figure 2). Changes in mRNA expression of all other inflammatory mRNAs were not significant.

Figure 3. Tumor necrosis factor (TNF)-α (A), interleukin (IL)-1α (B), IL-6 (C), IL-8 (D), macrophage inflammatory protein (MIP)-1α (E), and MIP-1β (F) in bronchoalveolar lavage fluid recovered at baseline (t = 0) and after 5 hrs (t = 5) from patients mechanically ventilated with 6 mL/kg and 10 cm H2O positive end-expiratory pressure (LV/PEEP, n = 21) or with 12 mL/kg and zero positive end-expiratory pressure (HV/ZEPP, n = 19). Data represent mean ± SEM. Wilcoxon signed-rank test: *p < 0.05 versus t = 0.
Protein Levels of Inflammatory Mediators in the BALF and Plasma

Mechanical ventilation minimally influenced cytokine and chemokine levels in BALF and plasma (figure 3). BALF levels of TNF-α and IL-8 were influenced by the way in which patients were ventilated, but the difference were minor. Plasma levels of IL-6 and IL-8 in plasma did significantly increased during the surgical procedure, but this increase in cytokine generation was similar in both groups (figure 4).

Nucleosome levels in BALF and plasma

Mechanical ventilation with higher tidal volumes and no PEEP caused an increase in BALF nucleosomes (figure 5A). In plasma, nucleosome levels were equally increased in both groups (figure 5B).
Discussion

In the present study we demonstrate that short-term mechanical ventilation is associated with minor inflammatory changes in the pulmonary compartment and that a lung-protective strategy attenuates these changes. From this it must be concluded that mechanical ventilation is not a strong proinflammatory stimulus in non-injured lungs. It is, however, tempting to speculate that mechanical ventilation with higher tidal volumes beyond 5 hrs would indeed a local hyperinflammatory response.

The mRNA expression of a small set of inflammatory mediators were downregulated in the alveolar macrophages from patients who were ventilated with higher tidal volumes without PEEP, but this mRNA downregulation was not reflected in decreased protein levels in BALF. It should be noted that a period of 5 hrs is probably too short to detect differences in protein levels due to modified transcriptional and translational processes. We believe that most inflammatory mediators measured in BALF were stored in alveolar macrophages and lung epithelial cells and released upon stimulation [24]. The reasons for the antiinflammatory mRNA downregulation by alveolar macrophages remain uncertain, but may indicate that macrophages are adapting to counterbalance the local proinflammatory response [25,26].

Furthermore, we showed that high tidal volume ventilation induced increased BALF levels of nucleosomes, whereas the apoptotic cyclin-dependent kinase inhibitor 1A (figure 2B, p) was not upregulated at mRNA level in alveolar macrophages. The rapid increase in BALF nucleosomes (i.e., within hrs after initiation of mechanical ventilation) most likely reflects apoptosis of pneumocytes. As far as we know, this is the first study showing an association between mechanical ventilation and alveolar apoptosis in humans. In vitro experiments have shown that mechanical strain induces proapoptotic changes in human lung epithelial cells [27,28]. Furthermore, in vivo animal experiments have shown that impairment of apoptosis pathways limited pulmonary inflammation and lung injury, and also protected against multiple organ failure and death [6,7]. It has been proposed that intraalveolar apoptosis is a potentially harmful process that could be targeted in the treatment of (ventilator-associated) lung injury [29]. On the other hand, apoptosis may be a pivotal process involved in alveolar repair mechanisms. More research is needed before clinical application of antiapoptotic strategies.

Our present data are largely in agreement with several previous studies in which mechanical ventilation strategies were investigated in patients with non-injured lungs. Wrigge et al. demonstrated that mechanical ventilation for 3 hrs during major surgery did not elicit any significant inflammatory response [30,31]. Also in other studies in which mechanical ventilation during cardiopulmonary bypass surgery, only mildly increased levels of
injury caused by the different ventilation strategies, it may well be argued that alterations observed in healthy lungs are mere physiological adaptations to the artificial process of mechanical ventilation. However, we propose that lung injury is induced by a ‘multiple-hit’ model, whereby predisposing conditions, such as injurious mechanical ventilation or major surgery, may result in (mild) pulmonary inflammation. Possible second hits, such as transfusion of blood products which may cause transfusion-related ALI, prolonged (injurious) mechanical ventilation, aspiration, shock, sepsis, and pulmonary infection may all cause additional lung injury, finally resulting in full-blown ARDS with high morbidity and mortality. There is indeed clinical evidence supporting this multiple-hit hypothesis. High tidal volume ventilation was independently associated with development of ARDS in patients who did not have ARDS at the onset of mechanical ventilation in the intensive care unit [11,12]. During mechanical ventilation of pneumonectomy patients, higher perioperative tidal volumes were identified as a risk factor of postoperative respiratory failure [13]. Furthermore, postoperative patients who were ventilated with a lower tidal volume strategy had a lower risk of pulmonary infection, and duration of intubation and length of stay tended to be shorter [36]. Therefore, we would like to encourage the use of lower tidal volumes and PEEP according to the principle *primum non nocere*: ventilator-associated lung injury can and should be limited.

Of course, above mentioned studies, including ours, have investigated patients who underwent major surgery. Inflammatory effects of the surgical procedure itself could not be excluded. However, investigating the effects of mechanical ventilation in healthy humans would lack any clinical significance. We do realize that further studies are needed to elucidate the effects of prolonged mechanical ventilation. Therefore, we have initiated a multicenter, randomized controlled trial investigating mechanical ventilation with either tidal volumes of 10 mL/kg ideal body weight or 6 mL/kg in patients without ALI at the initiation of mechanical ventilation (ISRCTN82533884).

A practical limitation was that we did not have reliable methods to obtain and isolate viable lung epithelial cells from our patients, and we could not investigate them in more detail. From a scientific point of view, it would also have been interesting to have obtained lung tissue (by biopsy) for specific staining and identification of apoptotic cells. However, we have not performed these assays, because we felt that many patients would not consent to more invasive procedures peri- or postoperatively.

In conclusion, mechanical ventilation with lower tidal volumes with PEEP limits pulmonary proinflammatory and proapoptotic changes in patients with non-injured lungs.
Even during a relatively brief period of mechanical ventilation, patients will most likely benefit from lower tidal volumes and PEEP.

References


General Discussion
Dutch Summary
General Discussion
Inflammation and coagulation play pivotal roles in host defense. As phylogenetically old responses, there is extensive crosstalk between inflammation and coagulation in mounting an adequate immune response against potentially injurious stimuli. While localized inflammation and clotting clearly have host-protective functions, it is considered disadvantageous when the inflammatory response is not limited to the primary site of injury and spreads through the body. The detrimental effects of generalized clotting are best exemplified in the clinical syndrome of sepsis, characterized by a systemic inflammatory response and accompanying excessive coagulation activation. The latter response leads to consumption of clotting factors and widespread fibrin depositions, causing diffuse endothelial damage, multiple organ dysfunction, and eventually death. Various anticoagulant treatment strategies have been tested in studies over the last decade. However, despite major success in preclinical studies, most anticoagulant therapies have failed to significantly improve outcome of critically ill patients.

**Sepsis**

To date, only recombinant human activated protein C (rhAPC) has been shown to reduce patient mortality in severe sepsis. Large randomized clinical trials suggested that rhAPC should only be given to adult patients with severe sepsis. Also, early treatment (i.e., within 24 hours of appearance of first organ dysfunction) was significantly associated with lower patient mortality. In chapter 3 the clinical experience with rhAPC at our own institute was evaluated. We found that the majority of rhAPC treated patients with sepsis were indeed treated within 24 hours of appearance of first organ dysfunction, yet not being associated with better outcome than patients treated after 24 hours. Interestingly, earlier treated patients were more likely to have had a respiratory infection. It is unlikely that intensivists were fully aware of this, but there is evidence in the literature suggesting that lung-specific effects of rhAPC contribute to better outcome in sepsis patients. First, rhAPC treatment has been associated with a more rapid recovery from respiratory failure. Second, sepsis patients with community-acquired pneumonia seemed to benefit most from rhAPC treatment. And finally, several experimental studies point out that there were significant beneficial effects of APC in the bronchoalveolar compartment.

We felt that studies were warranted to investigate what mechanisms are involved in the lung-protective effects of APC, but also the other natural inhibitors of coagulation, i.e., antithrombin (AT) and tissue factor pathway inhibitor (TFPI). We decided to investigate the
pulmonary effects of the natural anticoagulants in (ventilator-associated) acute lung injury and pneumonia.

Sepsis-Induced Acute Lung Injury

In chapter 4 we compared APC, AT, and TFPI treatment in a rat model of endotoxemia. In this chapter, we demonstrated that all natural anticoagulants were equally effective in attenuating endotoxemia-related activation of coagulation within both the systemic and the bronchoalveolar compartment. Interestingly, APC, AT, and TFPI did not have significant antiinflammatory effects in the lungs. The animal model of endotoxemia is of course the simplest model for the clinical syndrome of sepsis. We subsequently performed experiments with rat models of pneumonia involving live bacteria, because we thought that lung-protection may result from modulation of host-pathogen interactions.

Pneumonia

First, we established in patients with ventilator-associated pneumonia that procoagulant changes were actually restricted to the site of infection (chapter 5). In addition, we showed that there was prolonged bronchoalveolar coagulation activity in patients with Pseudomonas aeruginosa infection, compared with Escherichia coli or Staphylococcus aureus pneumonia, indicating differences in species-specific alterations of bronchoalveolar hemostasis (chapter 6). In chapters 6-9 we extended these findings by demonstrating a compartmentalized suppression of the natural anticoagulant systems during pulmonary infection. In human pneumonia, APC, AT, and TFPI were not able to adequately counterbalance increased coagulation activity within the airways. On the contrary, pulmonary levels of APC, AT, and TFPI were suppressed in (intubated and mechanically ventilated) patients with pneumonia.

In chapters 8-9, the role of the natural anticoagulant systems were investigated in two rat pneumonia models. To counterbalance the increased coagulation activity in the lungs, APC was used in a rat model of P. aeruginosa pneumonia. It appeared that APC was effective in limiting coagulation activity in the lungs. However, P. aeruginosa induced inflammation and lung injury remained unaffected by APC treatment. In chapter 9, we described our experiments with APC, AT, and TFPI in S. pneumoniae pneumonia in rats. As expected, bronchoalveolar coagulation was significantly attenuated by all three natural anticoagulants. Additionally, AT was able to restrict bacterial outgrowth of S. pneumoniae in the lungs, also limiting pulmonary hyperinflammation. We proposed that this lung-protective effect may be related to competition of AT with bacterial toxins for binding on proteoglycans on the
endothelial cell surface. In the light of these study results, we advocated for clinical trials with AT in patients with respiratory infections, as trials with APC and TFPI are already ongoing (ISRCTN52566874 and NCT00084071, respectively). The major limitation of our studies remains that, although live bacteria were used, both the *P. aeruginosa* and *S. pneumoniae* models are simplified models for severe respiratory tract infections in humans. The clinical emergence of pneumonia, delayed diagnosis, mechanical ventilation, and concomitant medication (e.g., antibiotics) are few of many factors that are not taken into account in the animal model. Nevertheless, chapters 4-9 have broadened our view on bronchoalveolar coagulation in acute lung injury and pneumonia caused by various pathogens.

Future research should concentrate on maximizing drug efficacy and minimizing adverse reactions. Study topics include species-specific host-pathogen interactions (should anticoagulant treatment be restricted to certain bacterial infections?), selection of patients (what patients will benefit most from anticoagulant treatment?), new diagnostic tools (e.g., biological markers), timing of treatment (is it safe to administer anticoagulants in acute infections, won’t we increase the risk of bacterial dissemination? when is it too late to initiate anticoagulant treatment?), and drug interactions (e.g., antibiotics, heparin, corticosteroids).

**Mechanical Ventilation**

Mechanical ventilation is one of the cornerstones in the intensive care therapy, but mechanical ventilation itself was shown to aggravate or even initiate lung injury. Although patients with preexistent lung injury clearly benefit from ‘lung-protective’ mechanical ventilation, using lower tidal volumes and positive end-expiratory pressure (PEEP), there was ongoing debate on the use of lower tidal volumes in ventilation of patients with non-injured lungs. Moreover, it was unclear whether mechanical ventilation as such would induce procoagulant changes in the lungs. In chapters 10 and 11 we sought to determine the effects of mechanical ventilation on pulmonary coagulation and inflammation in patients without evident lung injury. We studied patients who underwent major abdominal surgery to investigate the effects of mechanical ventilation on healthy lungs, and demonstrated increased coagulation activity and higher levels of inflammatory mediators in the lungs with the use of higher tidal volumes without PEEP, after 5 hours of mechanical ventilation. Ventilation with lower tidal volumes and PEEP seemed to attenuate these procoagulant and proinflammatory alterations.

We postulated that even patients without preexistent lung injury should be ventilated with lower tidal volumes and PEEP. Of course, this needs to be confirmed in a larger clinical trial.
involving critically ill patients who need mechanical ventilation for a prolonged period of time (ISRCTN82533884).

**Conclusions**

We have demonstrated that there is marked activation of bronchoalveolar coagulation in critically ill patients with pneumonia and during mechanical ventilation, not being compensated by increased natural anticoagulant or fibrinolytic activity. Bronchoalveolar coagulation was significantly limited by treatment with APC, AT, and TFPI in our experimental models, while AT even had significant lung-protective effects in a rat model of *S. pneumoniae* pneumonia. The potential role of APC, AT, and TFPI in the treatment of pneumonia and ventilator-associated lung injury remains to be established in future experimental and clinical studies.
Nederlandse Samenvatting voor Niet-Ingewijden
Het menselijk lichaam wordt constant bedreigd door schadelijke bacteriën, virussen en andere ziektemakers, alleen al door te ademen, eten en drinken. Dankzij het afweersysteem verkeert de mens doorgaans in gezondheid.

Twee van de belangrijkste afweermechanismen in ons lichaam zijn ontsteking en stolling (inflammation and coagulation). Als een bacterie ons lichaam binnendringt via de luchtwegen, wordt het afweersysteem onmiddellijk geactiveerd om de bacterie in de kiem te smoren. Gespecialiseerde witte bloedcellen (leukocyten) werken samen om de bacterie op te ruimen: sommige leukocyten zijn in staat de bacterie op te eten (fagocyteren) en daarna af te breken, andere leukocyten tasten direct de integriteit van de bacterie aan (door ‘gaten’ te schieten in de cellwand van de bacterie). Een andere categorie leukocyten maakt antilichamen die de herkening van bacteriën door fagocyterende leukocyten makkelijker maken. Tegelijkertijd met deze afweermechanismen wordt het stollingsproces geactiveerd zodat er rondom het ontstekingsproces bloedstolling plaatsvindt. In het algemeen wordt gedacht dat deze stollingsactivatie twee functies heeft. Enerzijds voorkomt het gevormde stolsel (bestaande uit bloedplaatjes in een netwerk van stollingsewijten, waaronder fibrine) dat de binnengedrongen bacterie zich verspreidt via de bloedbaan. Anderzijds vormt het stolsel de basis voor weefselherstel; onder het stolsel kunnen beschadigde delen van het orgaan (bijvoorbeeld de bloedvatwand) weer worden hersteld.

Het moge duidelijk zijn dat na het bestrijden van schadelijke micro-organismen de ontstekings- en stollingsreacties weer dienen te worden gedeactiveerd. Geactiveerde leukocyten keren terug naar hun oorspronkelijke staat (rust) en het gevormde stolsel wordt geleidelijk weer afgebroken (fibrinolysis). Echter, bij ernstige infecties lijkt er sprake van doorgeschoten, ongecontroleerde ontstekings- en stollingsreacties die zich niet beperken tot het gebied van de oorspronkelijke schade, maar zich verspreiden over het hele lichaam. Dit proces noemt men systemic inflammatory response syndrome (SIRS). In geval van SIRS met orgaanschade tot gevolg spreekt men van sepsis, in de volksmond ook wel ‘bloedvergiftiging’ genoemd. Sepsis is een ernstig syndroom, vooral als het afweersysteem uitgeput raakt door deze ongecontroleerde ontsteking en op den duur niet meer in staat is te reageren op andere infectieuze micro-organismen. Hierdoor ontstaan zogenaamde secundaire infecties bij patiënten die al ernstig ziek zijn. Niet verwonderlijk zijn ook de schadelijke effecten van gegeneraliseerde stolling: immers, door het hele lichaam hopen zich bloedstolsels op die leiden tot disfunctie en afsterven van orgaansystemen. Ook kan het stollingsproces uitgeput raken, waardoor een tekort ontstaat aan stollingsewijten en het risico op ernstige bloedingen...
toeneemt. Al met al leiden deze ontsporingen van de afweersystemen tot het falen van vitale organenfuncties en daarmee niet zelden tot de dood.

Normaal gesproken staat het stollingssysteem onder strikte regulatie van drie natuurlijke systemen: geactiveerde proteïne C (activated protein C, APC), antitrombine (AT) en tissue factor pathway inhibitor (TFPI). Alhoewel het werkingmechanisme verschilt, is de belangrijkste overeenkomstige functie van de APC, AT en TFPI het geven van tegenwicht aan de stollingsactivatie, ten einde excessieve stollingsprocessen te voorkomen (anticoagulation). Echter, in ernstig zieke patiënten bleken de concentraties van APC, AT en TFPI dusdanig verlaagd dat er nauwelijks sprake kon zijn van tegenwicht. Logischerwijs werd onderzocht of toedienen van APC, AT en TFPI het natuurlijk evenwicht zou herstellen en zou leiden tot gunstige effecten. Bij patiënten met een ernstige sepsis bleek inderdaad dat het toedienen van APC een significante verbetering tot stand bracht in de overleving. Dit helaas in tegenstelling tot behandeling met AT en TFPI, die meer bijwerkingen had dan positieve effecten. Daarom is tot op heden maar één middel geregistreerd voor de behandeling van ernstige sepsis: drotrecogin alfa (geactiveerd), een afgeleide van het humane APC. Er is echter nog veel discussie over APC behandeling. De hoge kosten van het medicijn, alsmede het verhoogde risico op bloedingen zorgen ervoor dat artsen zeer terughoudend zijn in het voorschrijven van APC. Wat bijdraagt aan de sceptische houding van artsen tegenover APC is de onduidelijkheid over het exacte mechanisme waarop APC positieve effecten teweeg brengt bij ernstige sepsis. Wij hebben de toepassing van APC geëvalueerd op de afdeling Intensive Care Volwassenen van het Academisch Medisch Centrum (hoofdstuk 3). Hieruit bleek dat relatief vroeg behandelde patiënten vaker een ernstige luchtweginfectie (pneumonie, in de volksmond ‘longontsteking’) hadden dan later behandelde patiënten. Dit was opvallend omdat uit experimenteel onderzoek belangrijke aanwijzingen naar voren waren gekomen dat APC de conditie van zieke patiënten waarschijnlijk doet verbeteren, mede door beschermde effecten in de longen. APC zou met name de stollingsactivatie in de luchtwegen kunnen voorkomen, alsmede de activatie van afweercellen in de longen. Maar soortgelijke effecten waren eerder ook beschreven voor AT en TFPI.

Het onderzoek dat wordt beschreven in deze dissertatie, richt zich op longspecifieke effecten van de natuurlijke remmers van bloedstolling (APC, AT en TFPI). Om te beginnen zijn wij op zoek gegaan naar eventuele verschillen in longbeschermende eigenschappen van APC, AT en TFPI in een eenvoudig experimenteel diermodel voor sepsis (hoofdstuk 4). Uit dit onderzoek
blijkt dat alledrie de medicijnen even effectief zijn in het voorkomen van sepsis gerelateerde stollingsactivatie in het bloed en in de longen. Daarnaast lijken APC, AT en TFPI nauwelijks invloed te hebben op de lokale ontstekingsprocessen. Onbeantwoord blijft dus de vraag wat de belangrijke verschillen zijn tussen APC, AT en TFPI.

Om de rol van natuurlijke remmers van de bloedstolling verder te onderzoeken, hebben wij ons gericht op ernstige pneumonieën. Een ieder heeft wel eens te maken gehad met luchtweginfecties, maar ernstige pneumonieën kunnen leiden tot ziekenhuisopnamen met soms de noodzaak tot (tijdelijke) mechanische beademing (ook wel ‘kunstmatige’ beademing genoemd). In de geneeskunde maakt men verschil tussen pneumonieën die buiten het ziekenhuis zijn opgelopen (community-acquired pneumonia) en die zich manifesteren na ziekenhuisopname, veelal secundair aan een ander ziekteproces (nosocomial pneumonia). Dit onderscheid is belangrijk omdat er grote verschillen zijn in ontstaanswijze, betrokken infectieuze verwekkers en daarmee ook de meest adequate antibiotische behandeling. Zo is de pneumokok (Streptococcus pneumoniae) de bekendste veroorzaker van een community-acquired pneumonie, terwijl bij nosocomiale pneumonieën Pseudomonas aeruginosa een van de belangrijkste verwekkers blijkt. Interessant genoeg vinden bij ernstige luchtweginfecties vergelijkbare processen plaats als in het bloed van patiënten met een sepsis, maar die leiden dan tot lokale ontsteking en stollingsactivatie, dat wil zeggen: lokaal in de luchtwegen. Dit proces is onder andere beschreven in hoofdstuk 5: een pneumonie die ontstaat tijdens mechanische beademing (ventilator-associated pneumonia) veroorzaakt stollingsactivatie lokaal in de longen, potentieel leidend tot stolselvorming in de luchtwegen. De mate waarin het lokale stollingsseizoen verandert, lijkt afhankelijk van het micro-organisme dat bij de pneumonie betrokken is (hoofdstuk 6), met andere woorden: sommige bacteriën veroorzaken ernstigere stollingsstoornissen dan andere. Voorts hebben wij aangetoond dat de pneumonie-gerateerde lokale stollingsactivatie niet wordt gecompenseerd door een toename in APC, AT en TFPI (hoofdstukken 7-9). Integendeel, concentraties van APC, AT en TFPI nemen sterk af in ontstoken longen.

Wij hebben getracht met verschillende middelen de stollingsactivatie in de longen te remmen en hebben de effecten daarvan bestudeerd. Hiertoe is gebruik gemaakt van twee experimentele pneumoniemodellen in ratten, met de bacteriën Pseudomonas aeruginosa (hoofdstuk 8) en Streptococcus pneumoniae (hoofdstuk 9). In de desbetreffende hoofdstukken beschrijven wij wederom dat APC, AT en TFPI ongeveer net zo goed de stollingsactivatie in de longen weten te remmen, maar ook dat AT belangrijke
longbeschermende effecten heeft tijdens *Streptococcus pneumoniae* pneumonie. Toediening van AT remt de groei van pneumokokken in de longen en gaat gepaard met aanzienlijk minder longontsteking. Hoe deze longbeschermende effecten tot stand komen, is nog niet helemaal duidelijk, maar dit krijgt de aandacht in vervolgonderzoeken van onze onderzoeksgroep.

Tot slot hebben wij onderzocht hoe mechanische beademing stolling en ontsteking in de longen beïnvloedt (hoofdstukken 10-11). Mechanische beademing vormt de hoeksteen van de intensive care behandeling, maar in de laatste jaren is gebleken dat bepaalde beademingsstrategieën longschade kunnen verergeren of zelfs induceren. Patiënten met ernstige longschade worden tegenwoordig beademd met kleine teugen, met het idee dat dit de longen beschermt omdat je beschadigde longdelen niet ‘kapot’ of ‘overblaast’ met te grote teugen. Bij patiënten met gezonde longen is er aanhoudende discussie over de beste beademingsstrategie. Wij hebben een onderzoek uitgevoerd in patiënten met gezonde longen die een langdurige operatie ondergingen. Deze patiënten werden beademd met kleine of grote teugen. De belangrijkste conclusie uit het onderzoek was dat door het gebruik van kleine teugen minder stollingsactivatie werden veroorzaakt, alsmede dat ontstekingsprocessen minder werden geactiveerd. Met deze resultaten hebben wij gepleit voor het gebruik van mechanische beademing met kleinere teugen, zelfs bij patiënten zonder evident longschade.

Samenvattend, hebben wij laten zien dat er sprake is van stollingsactivatie in de longen bij ernstig zieke patiënten met een pneumonie en tijdens mechanische beademing. Alhoewel de klinische betekenis van deze longspecifieke veranderingen open blijft staan voor discussie, behoeft het geen lang betoog dat té veel stolselvorming in de longen schadelijk kan zijn voor de patiënt. APC, AT en TFPI blijken in diermodellen uitstekende behandelingen vormen om stollingsactivatie in de longen tegen te gaan. AT lijkt zelfs longbeschermende effecten te hebben in een ernstig longontstekingsmodel met pneumokokken. De rol die APC, AT en TFPI moeten krijgen in de behandeling van humane pneumonie en beademingsgerelateerde longschade is afhankelijk van noodzakelijke, aanvullende experimenten en reeds lopende, klinische studies.
Appendices

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Acknowledgments

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Journals


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**Abstracts**


**Other**


Curriculum Vitae

Goda Choi was born April 16, 1979 in Seoul, The Republic of Korea. He expatriated in 1980 to Amsterdam, The Netherlands, only returning to Korea for occasional visits. After elementary, middle, and highschool in Amsterdam (De Praatpaal and Vossiusgymnasium), he attended medical school at the University of Amsterdam, The Netherlands (1996-2003). Among other activities, he was editor-in-chief of the faculty magazine *Emphasis*, member of the Students Council, and board member of *Particolarte* (students’ arts association).

In 2003-2006, he performed research at the Laboratory of Experimental Intensive Care and Anesthesiology and Center for Experimental and Molecular Medicine, supervised by Marcus J. Schultz. Also, he worked at the Departments of Intensive Care Medicine and Internal Medicine as a site-investigator for studies involving recombinant human activated protein C, recombinant human C1-inhibitor, and C1-esterase-inhibitor-N (primary investigator: Marcel Levi). He served as board member for the Graduate School Science Education program. In 2006 he started his residency in Internal Medicine (chairman: Peter Speelman) at the Academic Medical Center, Amsterdam, The Netherlands.

Outside working hours, he enjoys listening to music, watching sports (all sports except those involving horses or horse power), and playing music and golf. His future dreams include participating in the Summer Olympics, making his own music record, and writing a novel or children’s book. He regrets not having the talents and time to pursue all his dreams.