RAGE and the innate immune response in infection and inflammation
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Pulmonary levels of high mobility group box (HMGB)1 during mechanical ventilation and ventilator–associated pneumonia

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Abstract

High mobility group box (HMGB)1 is a recently discovered pro-inflammatory mediator that contributes to acute lung injury. We determined HMGB1–levels in bronchoalveolar lavage fluid (BALF) of patients during mechanical ventilation and ventilator–associated pneumonia (VAP). BALF was obtained from patients who were ventilated for 5 h because of an elective surgical procedure (“short-term mechanical ventilation”; n = 40) or for several days because of respiratory failure without acute lung injury (“long–term mechanical ventilation”; n = 10) and from patients who developed unilateral VAP (n = 4). Ten healthy volunteers served as controls. HMGB1–levels were low in healthy volunteers (median 1.6 [IQR: 0.7 – 3.7] ng/ml). Although HMGB1–levels were elevated after short–term mechanical ventilation, differences were not statistically significant compared with healthy volunteers (1.7 [0.8 – 8.5] ng/ml, P = 0.493 vs. healthy volunteers; P = 0.250 vs. start of mechanical ventilation). HMGB1–levels, however, were significantly higher in “long–term” mechanical ventilation patients (11.7 [8.7 – 37.0] ng/ml, P < 0.0001 vs. healthy volunteers). With unilateral VAP, HMGB1–levels from the infected lung were 17.4 [8.5 – 23.2] ng/ml (P = 0.014 vs. healthy controls); these levels were not different from those measured in the contralateral non–infected lung (P = 0.625). Summarized, “long–term” mechanical ventilation is associated with increased HMGB1–levels, in contrast to “short–term” mechanical ventilation. In addition, HMGB1–levels during VAP are increased compared to healthy volunteers, however; they are not different from those found in patients intubated and mechanically ventilated for a similar period of time.
INTRODUCTION

Mechanical ventilation may aggragate pre–existing lung injury or even initiate pulmonary damage in patients without lung injury at the start of mechanical ventilation (a phenomenon refered to as ventilator–associated lung injury (1,2)). The mechanisms underlying ventilator–associated lung injury are beginning to be understood, despite the difficulties in distinguishing the effects of mechanical ventilation from those of underlying diseases for which mechanical ventilation was started. Similar to acute lung injury/acute respiratory distress syndrome (ARDS) and pneumonia, ventilator–associated lung injury is associated with local production of pro-inflammatory mediators. Indeed, during ventilator–induced lung injury, the experimental counterpart of ventilator–associated lung injury, cytokines are released in the pulmonary compartment (3-5). These ventilation induced pro-inflammatory changes have been confirmed in clinical studies in patients with (6-9) and without acute lung injury/ARDS at onset of mechanical ventilation (10).

High mobility group box (HMGB)1 is a recently discovered mediator of pro-inflammatory responses that contributes to acute lung injury (11-13). Local and systemic levels of HMGB1 were found increased in mice that were instilled with lipopolysaccharide (LPS) via the airways (11). In addition, acute lung injury induced by either LPS (intranasally or intratracheally), cecal ligation puncture or pancreatitis, was diminished by administration of anti–HMGB1 antibodies (11,12,14,15). Furthermore, intratracheal administration of HMGB1 has been found to induce acute lung injury (11,12). Finally, in rabbits bronchoalveolar lavage (BAL) fluid (BALF) levels of HMGB1 were 5–fold higher after 4 h of mechanical ventilation with large tidal volumes as compared to lower tidal volumes. In this ventilator–associated lung injury model administration of anti–HMGB1 antibodies attenuated lung injury (16).

The aim of this study was to investigate the local release of HMGB1 in the pulmonary compartment during mechanical ventilation and ventilator–associated pneumonia (VAP). For this, we obtained BALF from patients on “short–term” (hours) mechanical ventilation and “long–term” (days) mechanical ventilation without evidence of pre-existing lung injury and from patients who developed a VAP during the course of mechanical ventilation. Healthy volunteers served as controls.
Chapter 9

MATERIALS AND METHODS

Study design
We collected BALF samples of patients in one prospective investigation on unilateral VAP (17) and one randomized controlled trial in which patients were randomized to be mechanically ventilated with a lung–protective ventilation strategy or a conventional ventilation strategy (see below) (18). Patients were only eligible for participation in these two studies if they had no history of any lung disease, use of immunosuppressive medication, recent infections, previous thromboembolic disease and recent ventilatory support. The separate protocols were reviewed and approved by the Medical Ethics Committee of the University of Amsterdam, Amsterdam, The Netherlands. Written, informed consent from all patients/closest relatives or volunteers was obtained before inclusion.

Patients
Healthy volunteers – these were all non–smoking individuals.
“Short–term” mechanical ventilation patients – these were patients who were expected to be intubated and mechanically ventilated for at least 5 h because of elective surgery. All patients received anesthesia according to a local protocol, including intravenous propofol (induction with 2 – 3 mg/kg, thereafter 6 – 12 mg/kg/h), fentanyl (induction with 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 to achieve normocapnia. Patients were randomized to a lung–protective mechanical ventilation strategy using lower tidal volumes of 6 ml/kg ideal body weight and positive end–expiratory pressure (PEEP) of 10 cm H2O or a conventional strategy with higher tidal volumes of 12 ml/kg ideal body weight and no PEEP.
“Long–term” mechanical ventilation patients – these were patients ventilated according to a strict local protocol in which optimal PEEP was defined as the lowest level of PEEP with maximum Pao2; in addition, patients were mechanically ventilated with tidal volumes < 8 ml/kg ideal body weight.
Unilateral VAP patients – these were patients admitted to the intensive care unit for ventilatory support. None of the patients had evidence of acute lung injury/ARDS at start of mechanical ventilation. Patients were eligible for this study when they fulfilled the following criteria: fever or hypothermia (temperature < 35.0 or > 37.7 ºC), leukocytosis or leucopenia (leukocyte count < 3 or > 10 x 10⁹/l), worsening of arterial oxygen tension (Pao2/FiO2–ratio) and a chest radiograph suspect for a novel unilateral infiltrate. Furthermore, the diagnosis had to be supported by the results of microbiological culture of
BALF or a clinical course consistent with VAP. For safety reasons, patients were excluded with $P_\text{O}_2 \leq 10 \text{ kPa}$ and $\text{FiO}_2 > 0.60$. Mechanical ventilation settings were similar to those in “long-term” ventilation patients.

**Lung compliance**
In mechanically ventilated subjects, lung compliance was calculated by dividing the tidal volume with end-inspiratory plateau pressure – PEEP difference.

**Bronchoalveolar lavage**
BAL was performed by experienced pulmonologists in a standardized fashion according to the guidelines of the American Thoracic Society by use of a flexible fiberoptic video-bronchoscope. Seven successive 20–ml aliquots of prewarmed 0.9% saline were instilled in a subsegment of the lung and each was aspirated immediately with low suction. In general, 10–15 ml of the instilled 20 ml was recovered. There was no difference between the recovered volumes between the different groups.

For healthy volunteers and “long-term” mechanical ventilation patients, BALF was obtained from the right middle lobe. For “short-term” ventilation patients, bronchoscopy and BAL were performed twice on all patients: the first just after initiation of ventilation in either the right middle lobe or the lingula, the second performed in the contralateral lung 5 h thereafter, either peri-operatively or directly postoperatively. For VAP patients, 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 a chest radiograph.

Healthy volunteers received local application of lidocain (in throat, near vocal cords) before introduction of the bronchoscope before the lavage. Short-term mechanical ventilation patients were under general anesthesia with propofol and fentanyl – no additive medication was given before the lavage. VAP patients were sedated with midazolam and morphine, no additive medication was initiated because of the lavage.

**Specimen processing**
BALF was kept at $4^\circ \text{C}$ until processing, which was performed within 30 min. The first aliquot was discarded; the second and third BALF recoveries from both sides were sent for microbial culture and virus isolation. The remaining BALF was centrifuged and cell free supernatants were stored at $-80^\circ \text{C}$ until HMGB1–levels were determined.

**HMGB1 measurements**
Measurements of HMGB1 in BALF were performed by ELISA with the use of monoclonal antibodies to HMGB1 and with standardization to a curve of recombinant human HMGB1 as described previously (19). Briefly, polystyrene microtiter plates were
coated with monoclonal anti-calf HMGB1 antibody. Wells were incubated with bovine serum albumin, washed and the calibrator and samples were added to the wells. After washing, another anti-human HMGB1 peroxidase-conjugated monoclonal antibody (a synthetic peptide was used as immunogen) was added to each well. After another washing step, the luminescence reagent was added to the wells. The luminescence was measured using a microplate luminescence reader.

**Statistical analysis**

All data are presented as means and standard deviations or number (percentage), except for HMGB1–levels: these are presented as medians [interquartile range]. Differences in HMGB1–levels between groups were analysed using the Mann–Whitney U–test. Wilcoxon signed–rank test was used for paired BALF samples comparing t = 5 h vs. t = 0 h and comparing infected vs non–infected lungs. Since no differences were found in pulmonary HMGB1–levels between patients mechanically ventilated with lung–protective mechanical ventilation and with conventional mechanical ventilation, the data of these patients are taken together in the statistical analysis and presentation of data. Correlations were calculated using Spearman’s rho test. A P-value ≤ 0.05 was considered statistically significant.
Results

Patients
Patient characteristic are given in Table 1. Baseline characteristics and peri–operative parameters in “short–term” mechanical ventilation patients were described in detail previously (18). In short, 74 consecutive patients who were scheduled for an elective surgical procedure of at least 5 h were screened in the period December 2003 through March 2005; 48 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 h) and only one BAL 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; 21 patients were assigned to lung-protective mechanical ventilation strategy and 19 patients to conventional strategy. There were no major differences between both randomization groups with regard to baseline characteristics. Baseline characteristics and reasons for initiation of mechanical ventilation of “long–term” ventilation patients have been described before (17-20). In summary, 10 intubated and mechanically ventilated patients without any signs of pulmonary infection or acute lung injury/ARDS and 4 patients with unilateral VAP were recruited. These 4 VAP patients recovered uneventfully with antibiotic therapy.

Mechanical ventilation
Healthy volunteers were neither intubated nor mechanically ventilated. Duration of mechanical ventilation for “short–term” mechanical ventilation patients was (mean ± SD): 306 ± 44 min. Duration of mechanical ventilation in “long–term” mechanical ventilation patients and VAP patients at the moment of BALF collection was 7.6 ± 2.6 days and 9.3 ± 3.6 days, respectively. Tidal volumes and PEEP levels in the 3 mechanical ventilation groups were in accordance with the study protocol (“short–term” mechanical ventilation) or the local mechanical ventilation guideline. Neither “long–term” mechanical ventilation patients nor VAP patients developed acute lung injury during the course of mechanical ventilation.

BAL
There were no reported adverse events related to the BAL in any study group.

HMGB1–levels during MV
HMGB1–levels in healthy volunteers were 1.6 [IQR: 0.7 – 3.7] ng/ml (Fig. 1). Patients who were intubated and mechanically ventilated for a short period had increased HMGB1–levels directly after intubation and start of mechanical ventilation although differences
Table 1 - Baseline patient and healthy control characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>“Short-term” MV</th>
<th>“Long-term” MV</th>
<th>VAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 40)</td>
<td>(n = 10)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Age, mean ± SD, yr</td>
<td>62 ± 10</td>
<td>56 ± 19</td>
<td>69 ± 17</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>28 (70)</td>
<td>7 (70)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Surgical procedure</td>
<td>Whipple procedure* (13)</td>
<td>thoracic surgery (5)</td>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>- reason for MV</td>
<td>laparoscopic radical</td>
<td>surgical management of</td>
<td>and Escherichia coli (1)</td>
</tr>
<tr>
<td>- pathogens isolated in BALF</td>
<td>prostatectomy (12)</td>
<td>abdominal aortic aneurysm (1)</td>
<td>polymicrobial flora (2)</td>
</tr>
<tr>
<td>hemihepatectomy (9)</td>
<td>subarachnoid hemorrhage (2)</td>
<td>Pseudomonas aeruginosa (1)</td>
<td></td>
</tr>
<tr>
<td>retroperitoneal tumor resection (2)</td>
<td>neurotrauma (2)</td>
<td></td>
<td></td>
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<tr>
<td>total pancreatectomy (2)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>colon conduit (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>open prostatectomy† (1)</td>
<td></td>
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</tbody>
</table>

* Whipple procedure is a pancreaticoduodenectomy.
† the open prostatectomy was performed after an initial laparoscopic approach.

VAP; ventilator-associated pneumonia, BALF; bronchoalveolar lavage fluid.
Fig. 1. Bronchoalveolar lavage fluid levels of HMGB1 in healthy volunteers ("healthy"), from patients intubated and mechanically ventilated for hours ("intubated": directly after intubation and start of mechanical ventilation (MV); "MV - hours": after 5 h of MV) and from patients intubated and mechanically ventilated for days ("MV - days"). *** P < 0.0001 vs. healthy volunteers.

Fig. 2. Bronchoalveolar lavage fluid levels of HMGB1 in patients with ventilator-associated pneumonia (VAP) from the infected and the non-infected, contralateral site and of patients without VAP but mechanically ventilated for a similar period of days ("no VAP").
were not statistically significant (4.8 [0.7 – 15.1] ng/ml, P = 0.147 vs. healthy volunteers).
In addition, HMGB1–levels did not change during 5 h of mechanical ventilation (1.7 [0.8 – 8.5] ng/ml, P = 0.493 vs. healthy volunteers, P = 0.250 vs. start of mechanical ventilation). There was no correlation between HMGB1 levels and lung compliance (data not shown). In contrast, “long–term” mechanical ventilation was associated with elevated HMGB1–levels (11.7 [8.7 – 37.0] ng/ml; P < 0.0001 vs. healthy volunteers).

**HMGB1–levels in BALF with VAP**
Although increased HMGB1–levels were found with VAP (17.4 [8.5 – 23.2] ng/ml; P = 0.014 vs. healthy volunteers), no differences existed between BALF levels from the infected and the contralateral, non–infected site (6.1 [5.8 – 13.2] ng/ml; P = 0.625 vs. infected site, Fig. 2). Moreover, HMGB1–levels in VAP patients did not differ from those in patients on “long–term” mechanical ventilation without pulmonary infection (P = 0.839).

**DISCUSSION**
The main finding of our study is that mechanical ventilation is associated with increased HMGB1–levels in BALF in patients without pre–existent lung injury. Secondly, we here report that VAP does not result in different HMGB1–levels compared to mechanical ventilation alone, while similar mechanical ventilation settings were used for a comparable period of time.

Our results extend, at least in part, previous experimental and clinical investigations in finding increased HMGB1–levels in the bronchoalveolar space during lung injury. First, HMGB1 in BALF is increased in murine models of LPS or hemorrhage induced–lung injury (11,13) and in a mouse model of ventilator–induced lung injury (16). Secondly, HMGB1 in pulmonary epithelial lining fluid is elevated in septic patients with acute lung injury/ARDS (11). One limitation of this last study is that it leaves unanswered to what extent the observed elevated HMGB1–levels in patients with acute lung injury/ARDS are induced by mechanical ventilation. We here report for the first time that mechanical ventilation itself is associated with elevated HMGB1–levels in patients without lung injury at onset of mechanical ventilation.

Little is known about the role of extracellular HMGB1 in the alveolar space in healthy and diseased lungs. Two earlier published studies showed that intratracheally or intranasally administered HMGB1 itself causes acute lung injury in mice, manifested by neutrophil accumulation, interstitial edema and hemorrhages in the lungs (11,12). Moreover, Ogawa et al showed that blocking endogenous HMGB1 in a rabbit model of ventilator–induced lung injury improved oxygenation, limited microvascular permeability and neutrophil
influx into the alveolar lumen, implicating HMGB1 as a deteriorating factor during the development of ventilator–induced lung injury and as an appropriate therapeutic target in ventilator–induced lung injury (16).

Besides its potential harmful role, HMGB1 has been suggested to have a physiologic role in the host defense. Zetterstrom et al suggested that HMGB1 may contribute to the local anti–bacterial barrier system in the upper airways (21). Furthermore, healthy subjects have 1,000 fold higher HMGB1–levels in their pulmonary epithelial lining fluid than in their plasma (11). These HMGB1 epithelial lining fluid concentrations of healthy subjects were comparable with those of patients during the acute phase of acute lung injury/ARDS. Remarkably, this phenomenon was not found in plasma: HMGB1 plasma levels were not or hardly detectable in healthy subjects, while patients with acute lung injury/ARDS with sepsis had clearly elevated HMGB1 plasma levels. So, whereas HMGB1 is not detectable in plasma from healthy subjects and is increased in plasma form septic patients, HMGB1 is (“abundantly”) present in the bronchoalveolar space from healthy subjects as much as in patients during their acute phase of acute lung injury/ARDS: a function of HMGB1 in the bronchoalveolar space in healthy lungs could underlie this phenomenon. Further investigations are warranted to investigate possible functions of extracellular pulmonary HMGB1 in healthy and diseased lungs.

Although HMGB1–levels were clearly elevated in our study several days after the start of MV (“long–term”), a shorter duration of mechanical ventilation (“short–term”) did not lead to statistically significant higher HMGB1–levels compared to levels from either healthy volunteers or directly after intubation. This finding is in line with a recent study on HMGB1 involvement during ventilator–induced lung injury in rabbits (16) in which ventilation during 4 h with tidal volumes of 8 ml/kg in rabbits did not result in elevated BALF levels of HMGB1 relative to control rabbits. Notwithstanding, BALF HMGB1–levels were 5–fold higher after 4 h of mechanical ventilation with large tidal volumes (i.e. 30 ml/kg) as compared to the lower lower tidal volumes (8 ml/kg) in this rabbit study. In the clinical setting tidal volumes are never as high as this; moreover, the tidal volumes in patients with acute lung injury/ARDS are advised not to exceed 6 ml/kg (22). We extended these findings to all patients in the ICU, whether suffering from acute lung injury/ARDS or not. Therefore, all individuals included in this analysis, except for those on conventional ventilation settings during “short–term” ventilation, were ventilated with “lower” tidal volumes. The results from our patient study with “short–term” mechanical ventilation together with the observation from the rabbit study suggest that, in contrast to ventilation for a longer time period (i.e. days), HMGB1 does not play a major role during ventilation for a short duration (i.e. hours) with tidal volumes of 6 – 12 ml/kg.

In our study, VAP patients had higher HMGB1 BALF levels compared to healthy
volunteers, similar to the “long–term” mechanical ventilation patients who did not develop VAP. In addition, HMGB1 in BALF from the infected site from VAP patients was not altered compared to the contralateral, non–infected site, neither compared to levels of “long–term” mechanical ventilation patients who did not develop VAP. However, it remains inconclusive from these data whether there is an additional or synergistic effect of VAP on mechanical ventilation induced HMGB1 levels, since we did not measure HMGB1 in the VAP patients before they developed VAP. In addition, a possible role of HMGB1 as an anti–bacterial factor (21) could implicate lower HMGB1 levels prior or during the development of VAP. We here did not find decreased levels in patients with already established VAP. It remains to be elucidated whether lower BALF levels of HMGB1 are associated with an increased risk of developing a VAP.

Since mechanical ventilation itself can induce lung injury including histopathogical changes and the production of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6 and IL-8, it is of interest to compare these inflammatory changes with local HMGB1 levels. The design of our studies did not allow us to obtain lung tissue for histopathological evaluation. We measured, however, levels of various cytokines, chemokines, markers of neutrophil activation and proteins involved with fibrin turnover. We found neither a correlation between HMGB1 levels in BALF, and levels of various cytokines, chemokines, markers of neutrophil activation, nor with proteins involved in fibrin turnover.

In conclusion, mechanical ventilation itself is associated with increased HMGB1–levels in the alveolar space in patients without pre–existent lung injury. Furthermore, bronchoalveolar HMGB1-concentrations during VAP were elevated compared with healthy volunteers, but not when compared with intubated and mechanically ventilated patients who did not develop VAP. Further studies are needed to investigate the role of HMGB1 during mechanical ventilation, VAP and acute lung injury/ARDS and its potention as a therapeutic target herein.
REFERENCES


