High mobility group box-1 expression correlates with poor outcome in lung injury patients

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ABSTRACT

Chest trauma is frequently followed by pulmonary contusion and sepsis. High mobility group box-1 (HMGB-1) is a late mediator of severe sepsis that has been associated with mortality under experimental conditions. We studied HMGB-1 mRNA expression in patients with lung injury and its relationship with the severity of trauma and survival.

A total of 24 consecutive patients with chest trauma referring to the Intensive Care Unit of Messina University Hospital, were enrolled. Lung trauma was established on the basis of chest X-ray and computed tomography. Injury Severity Score (ISS), Revised Trauma Score (RTS) and Glasgow Coma Scale (GCS) were also assessed. Accordingly to these results 6 patients were considered as controls because of no penetrating trauma and low ISS.

Blood and broncho-alveolar lavage fluid (BALF) from chest trauma patients were withdrawn at admission and 24 h after the beginning of the standard therapeutic protocol.

HMGB-1 mRNA increased significantly in blood ($r = 0.84$) and BALF ($r = 0.87$) from patients with trauma and pulmonary contusion and positively correlated with the severity of trauma (based on ISS and RTS) and the final outcome. HMGB-1 protein levels were also elevated in BALF macrophages from severe trauma patients compared to control subjects, furthermore TNF-$\alpha$ and its receptor TNFR-1 mRNA levels were also markedly increased in patients with a poor outcome respect to other subjects.

Our study suggests that HMGB-1 may be an early indicator of poor clinical outcome in patients with chest trauma.

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1. Introduction

Pulmonary injuries resulting from blunt chest trauma remain a common clinical entity for critical care. Pulmonary contusion is a common lesion occurring in patients sustaining severe blunt chest trauma. Alveolar haemorrhage and parenchyma destruction are maximal during the first 24 h after injury and then usually resolve within 7 days. The diagnosis of traumatic lung injury is usually made clinically with confirmation by chest computed tomography. Early detection and intervention toward minimizing injury progression provides the greatest chance for survival. Complications include pneumonia and adult respiratory distress syndrome, which may occur in up to one-half of all cases [1].

After trauma, an immediate inflammatory response occurs as a consequence of an efficient host defence system; it requires the intervention of several cytokines and one of these, HMGB-1, seems the most involved in determining survival. HMGB-1 has been known as a small DNA-binding protein, however, evidences suggest that HMGB-1 has pro-inflammatory cytokine-like properties [2]. HMGB-1 was demonstrated to be a potent inducer of TNF-$\alpha$ production in cultured macrophages [3], furthermore the up-regulation of...
the cell death receptor TNF-1 has been postulated for regulating post-traumatic changes in intracellular matrix integrity and in turn to enhance the apoptotic cascade [4].

HMGB-1 release from cells may occur as a passive process as cells undergo necrotic death. However, active mobilization of HMGB-1 out of the cell nucleus and into the extracellular environment also appears to occur in response to stressors [5,6]. Recently, elevated levels of HMGB-1 have been observed in the serum of adult human trauma patients compared with healthy volunteer [6]. Since HMGB-1 is a late mediator in sepsis, but it can also be released early after a traumatic insult, we studied its expression as mRNA at two different intervals (at hospital admission and the day after) and in two different sites (the lung as the site of injury and in whole blood) in order to evaluate its role in the clinical course of patients with blunt chest trauma.

2. Methods

2.1. Subjects

In this study were enrolled 24 consecutive patients with traumatic chest injury. Eligibility criteria were 18–65 years of age, the presence of a chest trauma, no penetrating trauma, no history of spontaneous pneumothorax, no presence of confounding concomitant pathological conditions. Lung injury was assessed by antero-posterior chest X-ray and confirmed by computed tomography. Of all the enrolled patients 6 showed only traumatic rib fractures without penetrating trauma confirmed by absence of lung contusion or haemorrhage and were used as controls. This choice has been made in order to discriminate whether the trauma itself could represent a critical condition for the expression of HMGB-1, and it has been made at the time of admission. The remaining 18 patients presenting lung contusion were further divided in two groups accordingly to the final outcome, deceased (n = 6) or discharged (n = 12). The protocol was approved by the Ethics Committee of Messina University. Informed consent was obtained from all subjects before sample collection. All pharmacological treatments have been done following the guidelines of Messina University Hospital for trauma patients. Broncho-alveolar lavage fluid (BALF) and blood were collected at the time of acceptance before any drug administration and 24 h after the beginning of the therapy. Severity of trauma was assessed at admittance by using the Revised Trauma Score (RTS), the Glasgow Coma Scale (GCS) and the Injury Severity Score (ISS) as previously described [7,8].

2.2. Broncho-alveolar lavage

All patients underwent broncho-alveolar lavage that has been performed by the Unit of Thoracic Surgery. The bronchoscope (Olympus, USA) was wedged into the segmental bronchus of the injured lobe and 20 ml × 7, a total 140 ml of sterile saline solution (0.9% NaCl) was infused. Fluid was gently aspirated immediately after the infusion has been completed and was collected into a sterile container. BAL cell differential counts were performed by counting at least 300 cells in cytocentrifuge preparations stained with eosin–haematoxylin.

2.3. HMGB-1, TNF-α and TNFR-1 mRNA quantification

HMGB-1, TNF-α and TNFR-1 mRNA were quantified as previously described [9]. Briefly total RNA was extracted from BALF and whole blood, after reverse transcription cDNA was stored at −80 °C and at the end of the enrollment was used to quantify the amount of HMGB-1, TNF-α and TNFR-1 cDNA by Real-Time Polimerase Chain Reaction method (Real-Time RT-PCR), as well as β-actin cDNA as endogenous control. The results for the target genes were expressed as an n-fold difference relative to the endogenous control gene (relative expression levels).

2.4. Isolation of cytoplasmatic proteins and western blot analysis for HMGB-1

After BALF macrophages isolation by two layer density gradient centrifugation (Histopaque, Sigma, Milan, Italy), proteins were homogenized in 1 ml lysis buffer (25 mM Tris/HCl, pH 7.4, 1.0 mM EGTA, 1.0 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, aprotinin, leupeptin, pepstatin A (10 μg/ml each) and Na3VO4 100 mM). The homogenate was subjected to centrifugation at 15,000 × g for 15 min. The supernatant containing cytoplasmic protein was collected and stored at −80 °C. The concentration of total proteins was determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). Protein samples (10 μg) were denatured in reducing buffer (62 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue) and separated by electrophoresis on a SDS (12%) polyacrylamide gel. The separated proteins were transferred on to a nitrocellulose membrane using the transfer buffer (39 mM glycine, 48 mM Tris pH 8.3, 20% methanol) at 100 mV for 1 h. The membranes were stained with Ponceau’S (0.005% in 1% acetic acid) to confirm equal amounts of protein and blocked with 5% non-fat dry milk in TBS–0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS–0.1% Tween, and incubated with a primary for the phosphorylated form of HMGB-1 (Abcam, UK) overnight at 4 °C. After being washed three times for 10 min each in TBS–0.1% Tween, the membranes were incubated with a specific peroxidase-conjugated secondary antibody (Pierce, UK) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacture’s protocol (Amersham, UK). The protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil, Celbio, Milan, Italy). The results from each experimental group were expressed as relative integrated intensity compared with control measured with the same batch. Equal loading of protein was assessed on stripped blots by immunodetection of β-actin with a rabbit monoclonal antibody (Cell Signaling, USA) and a peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, UK). All antibodies were purified by protein A and peptide affinity chromatography.

2.5. Statistical analysis

Normal distribution of data was verified by Kolmogorov Smirnov test in each group before the analysis. The results were expressed as mean ± SD. Data were analyzed by two-side ANOVA and analysis of variance (Sigma Stat, Jandel). Linear regression analysis for the goodness-of-fit was used to determine r values for HMGB-1 levels. The level for statistical significance was set at p < 0.05.

3. Results

Patients with severe blunt chest trauma accompanied by pulmonary contusion, confirmed by chest computed tomography (CT), showed alveolar haemorrhage and parenchyma destruction. All subjects (n = 24), aged 18–63 years, were administered with antibiotic to avoid bacterial infections, additionally subjects with severe lung injury (n = 10) received also glucocorticoid therapy (Table 1). Two days after admittance, 6 out of 24 patients with chest trauma and a low RST and GCS (4.54 ± 1.128 and 4.8 ± 2.1 respectively; p < 0.001 vs controls; Table 2), which constitute a high risk status.
died because of respiratory complications, more specifically critical respiratory failure occurred caused by pneumonia in 2 patients, haemothorax in 1 and onset of pneumothorax in the other 3. However in this 6 subjects the Injury Severity Score (ISS) was not significantly different from controls or discharged patients ($p = 0.06$ vs discharged and $p = 0.28$ vs controls).

Total BAL cell counts, evaluated in all chest trauma subjects, increased the day after trauma (total cell count day 0 = $140 \pm 43 \times 10^3$ and day 1 = $145 \pm 45 \times 10^3$; $p < 0.05$). In the group of subjects with lung contusion ($n = 18$) the presence of alveolar macrophages increased on day 1 (day 0 = $75 \pm 8\%$ and $88 \pm 9\%$; $p < 0.05$), while the percentage of neutrophils did not change (day 0 = $21 \pm 3\%$ and $19 \pm 4\%$). In subjects of the control group subjected to BAL fluid aspiration ($n = 6$) total cell count, as well as cellular percentages did not vary between day 0 and day 1 (results not shown).

Control subjects ($n = 6$) underwent another CT the day after admittance to exclude any possible late lung involvement. In these subjects, HMGB-1 expression in BAL fluid and blood collected at admittance, was very low ($0.1 \pm 0.03$ and $0.5 \pm 0.15$ n-fold).

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**Table 1**

Clinical features of the study subjects.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age</th>
<th>Pulmonary contusion</th>
<th>Outcome</th>
<th>Therapy</th>
<th>Additional injuries</th>
<th>Previous conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls ($n = 6$)</td>
<td>$40 \pm 20$ years</td>
<td>Not present at chest X-ray or computed tomography</td>
<td>Discharged ($n = 6$)</td>
<td>Antibiotic ($n = 4$)</td>
<td>Not present ($n = 6$)</td>
<td>Diabetes ($n = 1$)</td>
</tr>
<tr>
<td>Chest trauma ($n = 12$)</td>
<td>$42 \pm 21$ years</td>
<td>Present at chest X-ray or computed tomography</td>
<td>Discharged ($n = 12$)</td>
<td>Antibiotic ($n = 5$); antibiotic + glucocorticoids ($n = 7$)</td>
<td>Head trauma ($n = 3$); pneumothorax ($n = 6$); abdominal trauma ($n = 2$)</td>
<td>Diabetes ($n = 3$); cardiovascular diseases ($n = 2$); arthritis ($n = 1$)</td>
</tr>
<tr>
<td>Chest trauma ($n = 6$)</td>
<td>$38 \pm 20$ years</td>
<td>Present at chest X-ray or computed tomography</td>
<td>Deceased ($n = 6$)</td>
<td>Antibiotic ($n = 3$); antibiotic + glucocorticoids ($n = 3$)</td>
<td>Head trauma + haemothorax ($n = 1$)</td>
<td>Diabetes ($n = 1$); arthritis ($n = 1$)</td>
</tr>
</tbody>
</table>

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**Table 2**

Severity of trauma and outcome of the study subjects.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Systolic blood pressure</th>
<th>Respiratory rate</th>
<th>Glasgow coma scale</th>
<th>Revised Trauma Score</th>
<th>Injury Severity Score (ISS)</th>
<th>Final Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls ($n = 6$)</td>
<td>$130.5 \pm 11.3$</td>
<td>$14.32 \pm 2.4$</td>
<td>$15 \pm 0$</td>
<td>$7.841 \pm 0$</td>
<td>$38.1 \pm 19.2$</td>
<td>Discharged ($n = 6$)</td>
</tr>
<tr>
<td>Chest trauma ($n = 12$)</td>
<td>$119 \pm 19.8$</td>
<td>$15.46 \pm 2.60$</td>
<td>$11.30 \pm 3.32$</td>
<td>$6.935 \pm 1.066$</td>
<td>$30.6 \pm 14.9$</td>
<td>Discharged ($n = 12$)</td>
</tr>
<tr>
<td>Chest trauma ($n = 6$)</td>
<td>$114.6 \pm 35.9$</td>
<td>$19 \pm 4.14$</td>
<td>$4.8 \pm 2.1^*,$</td>
<td>$4.541 \pm 1.128^*$</td>
<td>$47.6 \pm 20.5$</td>
<td>Deceased ($n = 6$)</td>
</tr>
</tbody>
</table>

* $p < 0.001$ vs control subjects.  
§ $p < 0.005$ vs discharged subjects with chest trauma.  
* $p < 0.005$ vs control subjects.

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![Fig. 1. HMGB-1 mRNA levels evaluated by Real-Time PCR in blood (A) and BALF (B) samples collected from either controls ($n = 6$), discharged ($n = 12$) or deceased ($n = 6$) subjects with chest trauma at day 0 (hospital admittance) and day 1 (24h after the beginning of therapy). TNF-α (C) and TNFR-1 (D) mRNA levels in BALF samples collected from either controls ($n = 6$) or discharged ($n = 12$) or deceased ($n = 6$) subjects with chest trauma at day 0 (hospital admittance) and day 1 (24h after the beginning of therapy). *$p < 0.001$ vs discharged and vs control subjects.](image-url)
vs β-actin) and did not differ at day 1. Conversely a marked increase in HMGB-1 mRNA (Fig. 1A and B) has been noted in subjects (n = 18) with pulmonary contusion (blood = 3.76 ± 1.71 and BALF = 3.78 ± 1.89 n-fold vs β-actin). In blood this value increased 24 h after the admittance despite the therapy (4.27 ± 1.32 n-fold vs β-actin), while slightly decreased in BALF (3.05 ± 1.06 n-fold vs β-actin). Considering the subgroup of deceased patients (n = 6), is of particular interest, a marked and progressive increase in HMGB-1 mRNA in both BALF (day 0 = 5.56 ± 0.76 and day 1 = 6.01 ± 1.05 n-fold vs β-actin) and blood (day 0 = 5.4 ± 1.8 and day 1 = 6.51 ± 0.89 n-fold vs β-actin), compared to those who were discharged (day 0: BALF = 1.56 ± 0.69 and blood = 3.15 ± 1.34 n-fold vs β-actin) that showed also a reduction in mRNA expression 24 h after injury (day 1: BALF = 1.06 ± 0.15 and blood = 2.25 ± 0.94 n-fold vs β-actin).

HMGB-1 protein evaluated in BALF macrophages revealed an augmented expression (p < 0.01) of this inflammatory cytokine in deceased subjects at day 1 (6.1 ± 1.3 integrated intensity) compared to the other groups (Fig. 2), while at day 0 no significant changes have been observed (data not shown). A markedly increased expression of TNF-α and its death receptor TNFR-1 was also observed in BALF from subjects with severe lung trauma (Fig. 1C and D; r = 0.83 and r = 0.86 respectively), suggesting a direct involvement of HMGB-1 in stimulating TNF-α.

4. Discussion

Our study represents the first report suggesting the presence of increased levels of HMGB-1 in blunt chest trauma patients. A recent paper showed increased levels of this mediator in bronchoalveolar lavage fluid of patients during mechanical ventilation with or without ventilator-associated pneumonia, confirming a role for HMGB-1 in acute lung injury [10,11]. HMGB-1 is known to be a late mediator in severe sepsis and multi-organ dysfunction, both conditions that may lead to death. HMGB-1 has been also shown to increase after traumatic injury and during lung injury [11]. In this study we demonstrated an early but persistent increase in HMGB-1 mRNA expression, in subjects with chest trauma and consequent pulmonary contusion, in peripheral blood cells and in lung alveolar macrophages. Lung and the peripheral circulation are sites of major interest for the developing of a septic condition that may lead to death. Subjects with the maximum increase in HMGB-1 levels died in the first 48 h following the traumatic insult. Despite the same resuscitating interventions and the overlapping clinical conditions the subjects with higher HMGB-1 mRNA levels in both blood and BALF exhibited multiple symptoms of organ failure. Furthermore the increasing HMGB-1 expression in worsening subjects correlated with more severe RTS and GCS classifications. Additionally the augmented mRNA expression of HMGB-1 was also accompanied by an augmented HMGB-1 protein expression and an augmented TNF-α and TNFR-1 mRNA expression in alveolar macrophages from BALF. These observations suggest a role for HMGB-1 as a potential inducer of other cytokines as shown by other authors [12,13]. Another interesting observation arising from our results is that HMGB-1 is not increased in BALF nor in blood of patients with non-penetrating trauma used as controls, suggesting that this cytokine is more specific for pulmonary tissue contusion than from the trauma itself. This observation could be extremely relevant in the next future, in fact high mobility group box-1 might be used as an early prognostic indicator of chest trauma being highly sensitive for discriminating patients at risk of a poor outcome. Furthermore data available in the literature [10] demonstrate that control patients have a very low or even absent expression of HMGB-1 (as we showed), and therefore the expected mean difference between groups justifies the number of patients included in the present study.

Increased levels of HMGB-1 have been found elevated in myocardial ischemia, in cerebral ischemia subjects [14], and very recently in arthritis [15] and chronic kidney disease patients where well correlates with inflammatory markers in the synovial fluid as well as with a reduction in glomerular filtrate [16], suggesting a role in different human diseases. Future studies are needed to clarify whether HMGB-1 is also a prognostic marker of disease activity and severity as well as a predictor of outcome in pathological conditions. The role of HMGB-1 as a successful therapeutic target in experimental models of diverse infectious and inflammatory diseases has been recently shown [17]. Indeed it has been also shown in a case report that blockade of HMGB-1 in a patient with severe septic shock improved survival [18]. In addition HMGB-1 has been demonstrated to interact with the receptor for advanced glycation end-products (RAGE), a member of the immunoglobulin superfamily, which may promote lung dysfunction. RAGE has been found elevated in BALF in experimental lung injury [19] and in the pulmonary edema fluid of patients with acute lung injury [20]. The HMGB-1/RAGE axis in the lung seems to play an important role in several pathological conditions acting as a stimulus for pro-inflammatory cytokines and pro-fibrotic molecules, such as TGF-β and PDGF which in turn lead to scar formation and function impairment in response to injuries [21]. Therefore, explore this pathway could be extremely intriguing for a better understanding of the molecular crosstalk responsible for lung injury.

5. Conclusion

In conclusion our observations, although in a limited number of subjects, support for a role for HMGB-1 as an early predictor of survival in critical lung trauma patients and potentially as a mediator of inflammation after traumatic injury. Finally it is known that HMGB-1 is expressed at very low levels in healthy subjects, while increases dramatically during certain pathological conditions, this strengthen our findings which of course need to be confirmed in larger samples before a final conclusion on HMGB-1 role, as a death predictor, can be drawn.

Conflict of interest

The authors have no conflict of interest to declare, this work has been performed with Departmental funding only.
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