Early inflammatory response in polytraumatized patients: Cytokines and heat shock proteins. A pilot study

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Abstract

Introduction: In the initial phases after polytrauma there is an hyperinflammatory state that sometimes leads to multiple organ dysfunction syndrome (MODS) and death, and that appears to be responsible for posttraumatic immunosuppression: among the trigger endogenous stimuli, heat shock proteins (HSPs) have been proposed. The objectives of this study were to analyze if some inflammatory mediators can be considered prognostic biomarkers of outcome, and the possible role of HSPA1A in posttraumatic immunosuppression.

Hypothesis: Cytokines and HSPs could be early prognostic biomarkers of inflammatory and immune response in polytrauma patients.

Materials and methods: A prospective observational descriptive pilot study was carried out, evaluating the early inflammatory and stress response of 18 polytraumatized patients (ISS > 16) on hospital admission, at 12 hours, 24 hours, and 48 hours posttrauma. Variables means were compared using non-parametric tests; qualitative and quantitative variables were analyzed using a Spearman’s correlation test.

Results: Seven patients met criteria for MODS. Statistically significant changes were recorded in leukocyte count, C-reactive-protein (CRP), IL-6, TNF-α, and IL-1β concentrations. HSPA1A levels were significantly higher immediately after the accident followed by gradual lowering. Anti-Hsp70 antibodies showed a significant reduction in all the studied time-points. MODS did not influence either plasma levels of leukocytes, fibrinogen, CRP or anti-Hsp70, but patients with MODS had higher plasma levels of IL-6 and TNF-α and a slower decrease of HSPA1A concentrations.

Discussion: The higher serum concentrations of TNF-α and IL-6 found in patients with MODS, suggests a possible role as potential early predictive markers for systemic inflammatory response and clinical complications. The higher levels of HSPA1A in patients with MODS, allows proposing HSPA1A as a useful prognostic trauma biomarker early after severe injury and to consider a “damage control surgery”. The significant reduction in the levels of anti-Hsp70 antibodies could reflect a part of posttraumatic immunosuppression and hydrocortisone treatment might be suggested.

Level of evidence: Level III: case-control study.

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1. Introduction

In the initial phases after injury, local tissue damage induces a local and systemic inflammatory response (SIRS), characterized by the production and release of a variety of “dangerous molecules” (danger-associated-molecular-patterns [DAMPs]) which lead to an early inflammatory and immune response [1]. Depending on the severity of the aggression and the immune status of the patients, the posttraumatic response sometimes leads to multiple organ failure (multiple organ dysfunction syndrome [MODS]) and death [2].

Elevated serum levels of cytokines TNF-α, IL-1β or IL-8 have been observed in patients with systemic inflammation, and interleukin 6 (IL-6) has been correlated with the Injury Severity Score (ISS), incidence of multiple organ failure (MODS), sepsis and survival prognosis [3]. IL-6 also has anti-inflammatory properties, both due to induction of release of prostaglandin E2 and by promoting release of IL-1β and TNF-α receptor antagonists [4]. This, together with the production of specific anti-inflammatory cytokines such as...
IL-10 or IL-4, causes the initial proinflammatory immune response to be followed by a compensatory anti-inflammatory response syndrome (CARS). This hyperinflammatory state appears to be responsible for posttraumatic immunosuppression; also called sterile inflammation due to the absence of bacterial infection at the time of the traumatic event, has led to it being suggest that this posttraumatic immunosuppression must be triggered by endogenous stimuli, among which heat shock proteins (HSPs) have been proposed [5].

Within the superfamily of the HSPs, the most widely studied family of proteins related to the biology of inflammation is HSPA1A (Hsp70) [6]. Extracellular HSPA1A has powerful immune properties participating in the processing and presentation of exogenous antigens [7]. HSPA1A also has anti-inflammatory properties through inhibition of the expression of proinflammatory cytokines and proinflammatory transcription factors such as nuclear factor κB [8]. Although there are multiple studies implicating immunity to Hsp70 in the promotion of chronic inflammatory conditions such as diabetes and cardiovascular disease, many others show that this autoimmune response attenuates inflammatory diseases [9].

The present study has a dual objective; on one hand to analyze whether the mediators of early inflammatory response in severely injured patients can be considered prognostic biomarkers of outcome, and on the other to study the possible role of HSPA1A in posttraumatic immunosuppression. Our hypothesis was that cytokines and HSPs could be early prognostic biomarkers of inflammatory and immune response in polytraumatized patients.

2. Patients and methods

2.1. Patients

Eighteen patients who were victims of multiple trauma of mechanical origin (ISS > 16) were included in a prospective observational pilot study with analytical components. Eighteen healthy volunteers grouped by similar age and sex comprised the control group. Patients with ISS < 16, malignant tumors, or chronic lung, liver or kidney disease were excluded. The ISS is defined as the sum of the squares of the single highest injury score in each of the three most severely injured body regions [10]. Additionally, the Abbreviated Injury Scale (AIS) [11], an anatomic classification that values from 1 to 6 the degree of injury of body systems (head and neck, chest, abdomen, extremities abd/or pelvic girdle, and general), and New Injury Severity Score (NISS) [12] based on the sum of the squares of the three most severe injuries regardless of the body region, were calculated. A manifest MODS was considered when the score was > 12 points on two consecutive days according to the Marshall scale based on the degree (from 0 to 4) of dysfunction of six organ systems [13]. The study was approved by the Clinical Research Ethics Committee of our institution. All patients or their direct relatives signed consent prior to inclusion in the study.

2.2. Blood samples and serum determinations

Samples of venous blood were taken for routine tests and quantification of fibrinogen, C-reactive-protein (CRP), cytokines (TNF-α, IL-6, IL-18), HSPA1A and anti-Hsp70 antibodies. Data collection was performed on hospital admission (T0), at 12 hours (T1), 24 hours (T2), and 48 hours posttrauma (T3). A single blood sample was drawn from control subjects after a 12 hour fast. Blood was centrifuged at 3500 rpm, 15 minutes at 4 °C, and serum samples were frozen at −80 °C until assayed.

CRP and cytokyns were quantified using commercial ELISA kits according to the manufacturer’s instructions (DRG Instruments GMBH, Marburg, Germany; Diacclone Research, France).

HSPA1A were quantified in diluted serum 1:5 using the Hsp70 ELISA kit (EKS-715, Assay-Designs-Stressgen, AnnArbor, MI, USA). The working range (linearity) for HSPA1A resulted in 0.34–6.25 ng/mL, and sensitivity was 0.30 ng/mL. The inter-assay and intra-assay precisions were < 10%.

Titters of anti-Hsp70 antibodies in serum samples (diluted 1:1000) were measured using the EKS-750 ELISA Kits, Assay-Designs-Stressgen. The working range resulted 31.25–1000 µg/mL and sensitivity was 6.79 µg/mL. The inter-assay and intra-assay precision were < 10%.

2.3. Statistical analysis

To examine the distribution of data a Kolmorogov-Smirnoff test was applied. As data were normally distributed variable means were compared using non-parametric tests, Mann-Whitney’s U test for two independent samples, and Kruskal-Wallis test for three independent samples and an ANOVA with Bonferroni correction for normally distributed data. All probability values are derived from 2-tailed analyses and the statistical significance level selected was P < 0.05. SPSS 18.0 software for Windows was used throughout.

3. Results

3.1. Demographic characteristics

Eighteen polytraumatized patients (10 men and 8 women) with a median age of 43.50 years (range 18–77), and 18 control subjects (11 men and 7 women) with a median age of 30 years (range 18–53) were included in the study. There were no statistically significant demographic differences between both groups. Only one of the patients died 48 hours after admission due to irreversible brain injuries.

3.2. Pattern and severity of injury

All patients met the defining criteria for polytrauma. All had an AIS greater than two in at least two ISS body regions and an ISS > 16. The traumatic mechanisms were: road traffic accident (n = 8, 44.4%), pedestrians struck by motor vehicles (n = 6, 33.3%) and accidental falls (n = 4, 22.2%). Overall, ISS was 26.83 ± 9.43, NISS was 33.72 ± 10.55, and AIS was 14.61 ± 4.60. Seven patients met criteria for MODS detailed data are shown in Table 1.

3.3. Serum and plasma parameters

Leukocyte count was significantly higher on arrival to the emergency department compared to the control group, and remained so in the subsequent hours until it was similar to 48 hours from admission (Mann-Whitney test, P < 0.05). There was a high consumption of fibrinogen in the first hours after polytrauma, which gradually recovered until concentrations significantly higher than in the control group, at all the times analyzed. This progression over time in

<table>
<thead>
<tr>
<th>Variable</th>
<th>MODS</th>
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<tr>
<td>Patients</td>
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<tr>
<td>Age (years)</td>
<td>49.57 ± 18.22</td>
<td>37.8 ± 14.03</td>
<td>42.00 ± 16.48</td>
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<td>10/8</td>
</tr>
<tr>
<td>AIS</td>
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<td>11.91 ± 3.14</td>
<td>14.61 ± 4.60</td>
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<tr>
<td>ISS</td>
<td>36.29 ± 7.67</td>
<td>20.82 ± 3.69</td>
<td>26.83 ± 9.43</td>
</tr>
<tr>
<td>NISS</td>
<td>42.14 ± 0.37</td>
<td>28.36 ± 6.62</td>
<td>33.72 ± 10.56</td>
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MODS: multiple organ dysfunction syndrome; m: man; w: woman; AIS: Abbreviated Injury Scale; ISS: Injury Severity Score; NISS: New Injury Severity Score. Data are expressed as mean ± SD.
fibrinogen concentration of, is also significant \((P<0.05\), one-way ANOVA with Bonferroni correction). Polytraumatized patients had CRP concentrations significantly higher than the control group at all study times (Mann-Whitney test, \(P<0.001\)). This concentration rose in the hours following the accident reaching maximum values at 24 and 48 hours (Table 2).

3.4. Cytokines

3.4.1. IL-1ß

The serum concentrations of IL-1ß in the patient group at the times analyzed were significantly higher than those of the control group, without changes between them (Fig. 1).

3.4.2. TNF-α

TNF-α values were practically undetectable in the disease-free population. In contrast, serum concentrations of TNF-α were detected in all study patients. Changes over time in the TNF-α concentration did not show significant differences.

3.4.3. IL-6

Serum levels of IL-6 were undetectable in healthy volunteers. Patients had a mean IL-6 concentration of 111.20 ± 67.69 pg/mL on admission. These figures remained persistently elevated at all study times, without statistically significant differences between them.

3.5. Heat shock proteins

3.5.1. HSPA1A

In this study, circulating HSPA1A values followed the fit to normality of the Kolmogorov-Smirnov test. Extracellular HSPA1A levels were detectable in all cases, reaching peak concentrations immediately after the accident and gradually decreasing in the following hours. In addition, they were always significantly higher than baseline values in the control group (Mann-Whitney test, \(P<0.001\)). The decrease in the concentration of HSPA1A at 24 and 48 hours after the accident was significant compared to the values at admission and at 12 hours post-injury (one-way ANOVA with Bonferroni correction) (Fig. 2).

3.5.2. Anti-Hsp70 antibodies

Anti-Hsp70 concentrations fit a normal distribution, with a mean of 589.91 µg/mL in the control group and 199.28 µg/mL in polytraumatized patients on admission. This significant reduction in anti-Hsp70 levels was maintained at all study times, without differences between them (Fig. 3).

3.6. Influence of MODS on analytical determinations

During the observed period, MODS did not influence levels of leukocytes, fibrinogen or IL-1ß (data not shown).

Patients with MODS had higher levels of IL-6 and TNF-α than patients without MODS. Differences were statistically significant 48 hours after the accident.

Extracellular HSPA1A levels were elevated at admission in patients with and without MODS, but decreased more slowly in patients with MODS. Patients with MODS had higher levels with a trend to statistical significance at 24 hours \((P<0.01)\) and clearly significant differences at 48 hours \((P<0.05)\) (Fig. 4).

The presence of MODS did not affect anti-Hsp70 concentrations.
4. Discussion

We investigated the concentrations of common parameters (leukocytes, fibrinogen, CRP), various cytokines (IL-6, TNF-α and IL-1β) and HSPA1A and anti-Hsp70 antibodies in 18 patients suffering from major trauma in the early systemic inflammatory response.

All patients, with or without MODS, had significant leukocytosis in the first 24 hours after admission as part of the SIRS [11]. Blood samples drawn on hospital admission showed a significant decrease in fibrinogen levels. In the initial phase, proinflammatory cytokines activate the coagulation cascade, which leads to an increase of fibrinogen cleavage. In following hours, deep disorder of the coagulation process determines that unusual high levels of fibrinogen are detected in plasma [14], which may explain our results. The local and systemic inflammatory response induces synthesis of acute phase proteins in the liver, including CRP. The increase in the concentration of CRP is apparent 8 hours after the trauma and usually reaches peak values at 48 hours [15], which agrees with our findings. The absence of significant changes in levels of leukocytes, fibrinogen or CRP in the presence or not of MODS is only explainable in the context of a SIRS, irrespective of other possible associated complications.

The degree of systemic response was examined by the assessment of serum IL-6, TNF-α and IL-1β levels. In this study, high IL-6, TNF-α and IL-1β concentrations were observed immediately after the injury occurred, which remained steadily elevated over the first 48 hours posttrauma.

The level of involvement of the different cytokines in the clinical course of polytraumatized patients is quite disparate, partly because of the different half-life and the time of its peak production. IL-1β has a circulating half-life of six minutes, which makes it detection after trauma more unlikely. In the current study, it was undetectable in the control group but could be detected in polytraumatized subjects, without variations in the times studied and without discriminating between patients with or without MODS. This agrees with other researchers who concluded that IL-1β is not a useful parameter for predicting organ dysfunction [16].

TNF-α acts synergistically with IL-1β. It has a plasma half-life of 14–18 minutes, peaks in 1–2 hours and may have significantly decreased by 4–6 hours. The high serum concentrations of TNF-α found in the patients were significantly higher at 48 hours post-trauma among those who developed MODS. This corresponds to the findings reported by other groups but the possible use of TNF-α as potential marker of injury severity or outcome is controversial [16].

IL-6 is less transient than IL-1β or TNF-α and therefore more readily measurable. Very high levels of IL-6 have been shown from the first hour after trauma [17], which is in agreement with the data from this study. Moreover, we found that IL-6 level was significantly higher in patients with MODS, which coincide with the results of other authors and allows to propose IL-6 as a marker for predicting both the development of SIRS, injury severity and clinical complications [17,18].

Stress response is a conserved and universal host defense mechanism to protect cell and tissues against any aggressive agent. The main event associated with stress response is the increase of the expression of a class of proteins called stress proteins and particularly heat shock proteins (HSPs), so denominated because they were originally described when induced by the stimulus of hyperthermia. Extracellular HSPs are released from destroyed tissue and damaged cells and can exert an immune-stimulatory effect and activate the host inflammatory response [19]. This effect corresponds to the first hit phenomenon of the two hit theory and takes place in the immediate aftermath after trauma [20]. In the present study, HSPA1A (Hsp70) serum concentrations up to 10 times higher after injury were observed versus control subjects, levels that remain elevated until 48 hours after the accident following a time kinetics concordant with that previously described [21]. The high extracellular concentration of HSPA1A was even greater in patients with MODS. Although the present study did not perform follow-up of survival after hospital discharge and all patients except one survived during admission, the greater elevation of extracellular HSPA1A in patients with MODS corroborates the proinflammatory role of this protein in situations of severe trauma reported by other authors, who also correlated it with survival and morbidity [22] and even proposed it as a prime example of DAMPS [23]. Therefore, it can be suspected that the release of HSPA1 into serum could provoke the organism with a way to monitor the extent of damage to organs and tissue after trauma and that because its plasma levels
are associated with MODS raises the possibility that HSPA1A could serve as a useful prognostic trauma biomarker early after severe injury. Patients with HSPA1A sera concentrations up to 10 times higher than the normal, would be candidates for submission to a “damage control surgery” (DCS) as a treatment strategy of tem-
porarization which increases radically the survival in these most seriously injured patients [24].

Multiple injuries can lead to profound changes in the innate and adaptive immune response that result in a state of immuno-
suppression, although the mechanisms involved remain unknown. Flohé et al. [5,25] propose that HSPs released from injured tissues could contribute to the posttraumatic immunosuppression due to their effects on the innate immune reaction and their capacity to inhibit TNF-α secretion in mononuclear cells, and therefore pro-
pose HSPs as possible immunosuppressive mediators related to trauma. In our investigation, in parallel to the increase in the con-
centration of HSPA1A in serum of polytraumatized patients, we observed a significant reduction in the levels of anti-Hsp70 anti-
body. This is in agreement with the findings of a previous study by the group [26] and could reflect a part of posttraumatic immuno-
suppression at the humoral level, both in patients with and without MODS. Trauma is a major risk factor for hospital-acquired pneumo-
nia and the high rates of hospital-acquired pneumonia currently reported have already been described in patients experiencing severe trauma. Corticosteroid treatment with a stress dose of hydrocortisone for 7 days has shown to reduce the rate of acquired pneumonia together with a decreased requirement for mechanical ventilation and length of ICU stay in trauma patients [27]. Patients with very low anti-Hsp70 circulating antibodies early after injury would be subsidiary of hydrocortisone therapy to reduce death and morbidity and restore an adequate immune response to infection.

The present study has some limitations. Trauma patients are inherently heterogeneous and the response of trauma mediators shows a large interindividual variability as it has been stated by several authors [20]. Moreover, the present study was designed as an observational study to assess the association of several inflam-
mation markers with physiological variables an injury severity. Therefore, our data donor permit conclusions on the molecular mechanism regulating the systemic release of cytokines or HSPS or on the possible biological role of the decrease of anti-Hsp70 antibodies in immunosuppression and the infection and septic complications. On the other hand, the sample size is small and this study must be regarded as a providing pilot data for a future larger study.

In conclusion, we tested the hypotheses that TNF-α and IL-6 can be proposed as potential early predictive markers for systemic inflammatory response and that IL1-β is an inefficient param-
eter and, hence, in our opinion, does not warrant inclusion in future studies. Despite its limitations the present study showed that circulating HSPA1A concentrations could serve as a useful prognostic biomarker early after severe injury and that monitoring of anti-Hsp70 in the first hours after injury would be an easy to use and beneficial predictive tool of the possible progression the patient towards immunosuppression. These patients should be considered subsidiary of the “damage control surgery” (DCS) and “damage control resuscitation” (DSR), and even of a stress dose of hydrocortisone in order to reduce the occurrence of hospital-
acquired pneumonia.

Disclosure of interest

The authors declare that they have no conflicts of interest concern-
ing this article.

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