Systemic Ubiquitin Release After Blunt Trauma and Burns: Association With Injury Severity, Posttraumatic Complications, and Survival

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**Background:** Recent data suggest that ubiquitin (Ub) is systemically released after trauma, has pleiotropic effects on host defense mechanisms, and that Ub administration reduces fluid shifts into tissues during inflammation. Ub release after burns (B) has not been studied and its association with injury severity and outcome after blunt trauma (T) is unknown. Thus, we evaluated Ubs association with injury severity and outcomes after B and T.

**Methods:** Injury severity was assessed with the Injury Severity Score (ISS) in T and burn size (% total body surface area, %TBSA) in B. A total of 129 T (ISS: 26 ± 13) and 55 B (46% ± 18% TBSA) were observed for sepsis/multiple organ failure (MOF) and survival. In B, sequential organ failure assessment scores were documented daily. Fifty volunteers served as controls (C) Ub serum levels were measured on day 0 (admission), 1, 3, 5, and 7 by enzyme-linked immunosorbent assay. Data were analyzed using bivariate or partial correlation analyses, t test, and analysis of variance with Tukey post-hoc test for multiple comparisons (two-tailed p < 0.05).

**Results:** Ub was significantly elevated in patients. Peak levels (ng/mL) were detectable on day 0 (C: 118 ± 76; T: 359 ± 205; B: 573 ± 331) and increased with increased ISS, %TBSA, and presence of inhalation injury. In T, Ub normalized by day 3, but remained elevated in B. In B, Ub correlated significantly negative with sequential organ failure assessment scores (r: −0.143; p = 0.0147), sepsis/MOF development (r: −0.363; p = 0.001), and survival (r: −0.231; p = 0.009). Compared with B who recovered uneventfully, Ub levels were significantly lower on days 1 to 7 and on days 5/7 in B who developed sepsis/MOF or died, respectively.

**Conclusion:** Ub concentrations reflect the extent of tissue damage. Along with Ubs previously described anti-inflammatory properties, this study suggests that its systemic release is protective, that burn patients who develop sepsis/MOF have a relative Ub deficiency and that Ub could play an important role during the physiologic response to burn injury.

**Key Words:** Fluid shifts, Capillary leak, Organ failure, Tissue damage, Immune modulation.

UBiquitin (Ub) is a small, heat stable, and highly conserved protein in all eukaryotic cells. It fulfills its major biologic role through covalent ligation (ubiquitylation or ubiquitination) to intracellular proteins, and thus labels the target protein for degradation via the proteasome or reversibly regulates the targeted protein’s function. Today, ubiquitylation is regarded as the second most common post-translational protein modification after phosphorylation. Although the discovery of Ub introduced this protein as a molecule with extracellular actions, little attention has since been paid to its possible role outside the cell. Ub is a natural constituent of extracellular fluids, such as serum, plasma, or cerebrospinal fluid, and increased extracellular concentrations have been described in some diseases. However, no pathophysiologic role was attributed to extracellular Ub in these studies.

Recently, we showed that Ub is systemically released after trauma and sepsis in patients, that it is involved in the regulation of leukocyte function and inhibits the lipopolysaccharide (LPS)-evoked tumor necrosis factor-α and interleukin-8 response of blood and isolated peripheral blood mononuclear cells in vitro. Because other investigators provided evidence that it also regulates growth and apoptosis, and possesses antimicro-

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brial activity,15–17 extracellular Ub may have pleiotropic effects on host defense mechanisms. Subsequently, we were able to confirm Ub’s immune modulatory properties in vivo and showed that it has profound anti-inflammatory effects in various models of infectious and noninfectious inflammation.18–22 Altogether, these data suggest that Ub is an endogenous anti-inflammatory mediator that could play an important role during the inflammatory response to mechanical and thermal injury. However, its association with injury severity and outcomes in blunt trauma patients is unknown and Ub release after thermal injuries has not been studied. Therefore, we conducted a prospective observational trial in blunt trauma and burned patients to evaluate whether Ub systemic concentrations are associated with injury severity, development of severe posttraumatic complications, and survival.

PATIENTS AND METHODS
Patients, Participants, and Study Protocol
The Institutional Review Boards of the Universities of Miami, Berlin, Munich, and Essen approved the protocol that we used. Informed consent was obtained from all participants. The study population comprised of 129 patients with blunt injuries and 55 patients with burns who were admitted to the emergency room of the participating hospitals, and of 50 healthy blood donors. Exclusion criteria were age under 18 years, hospital admission later than 6 hours after injury, transfer from other hospitals and severe preexisting infectious, and immunologic or cardiovascular diseases that required long-term medication. Sixty-six blunt trauma patients were recruited in Miami, 53 in Berlin, and 10 in Essen. Burned patients were recruited in Munich. Healthy blood donors were recruited in Miami (n = 20), Munich (n = 20), and Essen (n = 10). Blood donors (age: 43 ± 11 years [mean ± SD]; 68% male), had no signs of infection at least 4 weeks before the blood draw.

All patients requiring surgical intervention received standard surgical care and postoperative intensive care unit (ICU) treatment according to the standard treatment protocols of the individual hospitals. For the assessment of injury severity in blunt trauma patients, the Injury Severity Scores (ISS) were calculated based on the final patient records.\(^{23}\) In burned patients injury severity was assessed by the percentage of at least second-degree burned total body surface area (%TBSA), as documented in the patient records. All patients were prospectively observed for the development of severe sepsis, septic shock, or multiple organ failure (sepsis/MOF). Severe sepsis and septic shock were diagnosed according to the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) consensus conference criteria.\(^{24}\) MOF was diagnosed using a scoring system modified according to Goris et al.\(^{25–27}\) In brief, failure of a single organ was assumed when the following criteria were present for at least 3 consecutive days: lung, mechanical ventilation with positive end-expiratory pressure (PEEP) >10 cm H\(_2\)O and/or FIO\(_2\) >0.4; heart, dopamine >700 µg or catecholamine treatment; kidney, hemofiltration or hemodialysis; liver, bilirubin >102 µmol/L or aspartate aminotransferase (AST) >100 U/L; blood, hemorrhagic diathesis or leukocytes >60 k/µL or <2.5 k/µL; gastrointestinal, parenteral nutrition; brain, treatment for increased intracranial pressure. MOF was diagnosed when these criteria were fulfilled for two or more organs. Because MOF without positive criteria of severe sepsis or septic shock after blunt trauma and burns was expected to be rare, these posttraumatic complications were combined and documented as sepsis/MOF positive or negative. In addition, in burned patients the sepsis-related organ failure or sequential organ failure assessment (SOFA) scores\(^{28,29}\) were calculated daily and the volumes of fluids infused and urine output were documented.

Duration of ICU treatment and hospital survival were documented based on the final patient records. The epidemiologic and clinical characteristics of the blunt trauma and burned patients are shown in Table 1.

Venous blood samples (1–2 mL) were drawn immediately after admission to the emergency room (day 0) and in the morning of the subsequent days 1, 3, 5, and 7 between 7 AM and 9 AM along with the routine laboratory work-up. Because blood samples could not be obtained from each patient at each individual time point, the exact number of analyzed blood samples is provided for each time point in the Results section. Blood samples were collected in serum test tubes, which were in routine use in the participating hospitals, serum prepared according to the standard hospital procedures and stored at -70°C until further analysis. After all blood samples were collected and patient data recorded, Ub serum concentrations were determined with the investigators blinded to the patient-related data.

Ubiquitin Enzyme-Linked Immunosorbtent Assay (ELISA)
Ub serum concentrations were quantified with a competitive ELISA, in which biotinylated Ub and Ub in the test samples compete for a limited number of binding sites in the

| Table 1 Epidemiologic and Clinical Characteristics of the Patient Populations |
|-----------------------|-----------------------|
|                       | Trauma | Burns |
| N                     | 129    | 55    |
| Age                   | 45 ± 20| 46 ± 18|
| Male (%)              | 72.8   | 83.6  |
| ISS                   | 26 ± 13| N/A   |
| TBSA (%)              | 0      | 39 ± 16|
| with inhalation injury| 0      | 34.5  |
| ICU (d)               | 21 ± 29| 47 ± 83|
| Sepsis/MOF            | 20.1%  | 45.5% |
| Mortality             | 12.4%  | 23.6% |

Data are means ± SD or percentages.
anti-Ub antibody according to that shown in Refs. 14 and 18 to 22. In brief, microtiter plates (Nalge Nunc International, Rochester, NY) were coated with anti-Ub (Sigma-Aldrich, St. Louis, MO) and incubated for 18 hours at 4°C. The plates were washed three times with 0.05% Tween 20 in phosphate buffered saline and were incubated with blocking buffer (0.5% bovine serum albumin [Sigma-Aldrich] 0.05% Tween 20, in phosphate buffered saline) for 1.5 hours. After washing three times, 50 μL/H9262L of the standards or samples were mixed with 50 μL/H9262L of biotinylated Ub (Boston Biochem, Boston, MA) and placed in the plates. Each sample was tested in eight dilutions. Dilutions for the standard curve and the test samples were prepared in blocking buffer. After incubation for 1.5 hours the plates were washed again and a peroxidase-labeled anti-biotin antibody (Amersham Biosciences, Buckinghamshire, UK) was added. After incubation for 1.5 hours the plates were washed again and 100 μL 3, 3′, 5, 5′, tetramethylbenzidine ELISA solution (Sigma-Aldrich) was added. After incubation for 20 to 40 minutes, the reaction was stopped by addition of 100 μL HCl and optical densities were measured using a micro-ELISA autoreader (μQuant, Bio-Tek Instruments Inc., Winooski, VT; test filter: 450 nm; reference filter: 540 nm). The Ub concentration in the test sample was calculated with the KC4 for windows program, version 3.02 (Bio-Tek Instruments Inc.), from a four-parameter logistic fit employing Ub as standard (0–1700 ng/mL, Sigma-Aldrich). The correlation coefficients for each standard curve were 0.98 to 1. This ELISA predominantly detects free Ub. Comparison of Ub concentrations measured by ELISA and western blot showed that concentrations determined by ELISA were 107% ± 9% (n = 4) of the concentrations for free Ub that were determined by western blot. Thus, because of some cross-reactivity with ubiquitylated proteins, the ELISA measurements overestimate the concentration of free Ub by approximately 7%. Addition of purified hemoglobin to the assay did not affect measurements. The recovery of Ub in spiked serum and plasma was 94% to 105%.

Fig. 1. Ubiquitin serum concentrations in blunt trauma and burned patients during a 7-day observation period. Time points not sharing the same letter are significantly different (analysis of variance/Tukey). Data are mean ± SEM. (A) ●. Blunt trauma patients, day 0: n = 116, day 1: n = 66; day 2: n = 63, day 5: n = 58, day 7: n = 26. □. Healthy blood donors, n = 50. Inserts, time course of Ub serum levels in patients recruited at the different study sites. (B) ■. Burned patients, day 0: n = 37; day 1: n = 46; day 2: n = 45, day 5: n = 41, day 7: n = 37. □. Healthy blood donors, n = 50.
Statistical Analysis

Data are described as mean ± SD and are presented in the graphs as mean ± SEM along with the exact number of measurements to improve the interpretation of the data. Differences in Ub serum concentrations were analyzed using Student’s t test or analysis of variance with Tukey post hoc correction to correct for multiple testing. Correlations were assessed using Spearman correlation coefficients ($r_s$) and partial correlation coefficients ($r_p$) that describe the relationship between two variables while controlling for the effects of one or more additional variables. Statistical analyses were calculated with the SPSS for windows program (SPSS Inc., Chicago, IL). Differences were considered significant on a two-tailed $p < 0.05$ level.

RESULTS

The time course of Ub serum concentrations in trauma and burned patients is shown in Figure 1A and B. Ub serum levels were significantly elevated in both patient populations, as compared with healthy volunteers ($n = 50$, 118 ng/mL ± 76 ng/mL Ub). Ub serum

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**Fig. 2.** Association of ubiquitin serum concentrations at admission with injury severity and duration of ICU treatment. Data are means ± SEM. Groups not sharing the same letter are significantly different (analysis of variance/Tukey). (A) Ubiquitin serum concentrations in blunt trauma patients that were grouped according to their ISS. Healthy blood donors, $n = 50$ (open bars). Blunt trauma patients with ISS in the given ranges (ISS 0: $n = 5$; ISS 1–9: $n = 9$; ISS 10–19: $n = 17$; ISS 20–29: $n = 49$; ISS 30–39: $n = 23$; ISS 40+: $n = 13$) (grey bars). (B) Ubiquitin serum concentrations in burned patients that were grouped according to their %TBSA and according to the presence (yes) or absence (no) of inhalation injury. Healthy blood donors, $n = 50$ (open bars). Burned patients with %TBSA in the given ranges (%TBSA < 25: $n = 9$; %TBSA 25–35: $n = 10$, %TBSA 36–45: $n = 11$, %TBSA 46+: $n = 7$) (grey bars). Inhalation injury: yes: $n = 10$; no: $n = 27$. *$p < 0.05$; t test. (C) Ubiquitin serum concentrations in blunt trauma and burned patients that were grouped according to their duration of ICU treatment. Blunt trauma patients (ICU days 0: $n = 23$, ICU days 1–7: $n = 21$, ICU days 8–21: $n = 30$, ICU days 22+: $n = 32$) (left). groups not sharing the same letter are significantly different (analysis of variance/Tukey). Burned patients (ICU days ≤ 30: $n = 30$, ICU days > 30: $n = 17$) (right). *$p < 0.05$; t test.
Ubiquitin serum concentrations after blunt trauma by gender, age, sepsis/MOF, and survival. Data are means ± SEM. (A) Female (day 0: n = 31, day 1: n = 19; day 2: n = 18, day 5: n = 17, day 7: n = 8) (open bars). Male (day 0: n = 85, day 1: n = 47; day 2: n = 45, day 5: n = 41, day 7: n = 18) (grey bars). (B) Less than 30 years (day 0: n = 31, day 1: n = 20; day 2: n = 21, day 5: n = 18, day 7: n = 7) (open bars). Thirty to 50 years (day 0: n = 40, day 1: n = 21; day 2: n = 19, day 5: n = 18, day 7: n = 6) (light grey bars). More than 50 years (day 0: n = 45, day 1: n = 22; day 2: n = 21, day 5: n = 20, day 7: n = 10) (dark grey bars). (C) No sepsis/MOF (ISS: 24±13 [mean ± SD]; day 0: n = 91, day 1: n = 48; day 2: n = 45, day 5: n = 42, day 7: n = 15) (striped bars). No sepsis/MOF - ISS matched (ISS: 29 ± 10 [mean ± SD]; day 0: n = 69, day 1: n = 41; day 2: n = 39, day 5: n = 38, day 7: n = 13) (white bars). Sepsis/MOF (ISS: 33 ± 11 [mean ± SD]; day 0: n = 25, day 1: n = 12; day 2: n = 13, day 5: n = 13, day 7: n = 8) (grey bars). (D) Survived (ISS: 24 ± 12 [mean ± SD]; day 0: n = 101, day 1: n = 58; day 2: n = 54, day 5: n = 50, day 7: n = 22) (striped bars). Survived - ISS matched (ISS: 30 ± 8 [mean ± SD]; day 0: n = 71, day 1: n = 46; day 2: n = 43, day 5: n = 41, day 7: n = 16) (white bars). Died (ISS: 33 ± 11 [mean ± SD]; day 0: n = 15, day 1: n = 8; day 2: n = 9, day 5: n = 8, day 7: n = 8) (grey bars).
Table 2: Correlation of Ubiquitin Serum Concentrations During the 7-d Observation Period With Epidemiologic and Clinical Patient Characteristics After Burns

<table>
<thead>
<tr>
<th></th>
<th>Age (n=116)</th>
<th>Female (n=59)</th>
<th>TBSA (%) (n=116)</th>
<th>FB (n=116)</th>
<th>SOFA (n=116)</th>
<th>Sepsis/MOF (n=116)</th>
<th>Survival (n=116)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ub</td>
<td>0.11 (0.126)</td>
<td>0.153 (0.033)*</td>
<td>0.065 (0.461)</td>
<td>-0.143 (0.047)*</td>
<td>-0.155 (0.031)*</td>
<td>-0.051 (0.497)</td>
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<tr>
<td>Age</td>
<td>0.182 (0.225)</td>
<td>-0.154 (0.308)</td>
<td>-0.002 (0.979)</td>
<td>0.245 (0.001)*</td>
<td>0.156 (0.302)</td>
<td>0.469 (0.01)*</td>
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</tr>
<tr>
<td>Female</td>
<td>-0.119 (0.43)</td>
<td>-0.048 (0.577)</td>
<td>-0.105 (0.118)</td>
<td>-0.258 (0.08)</td>
<td>0.086 (0.572)</td>
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</tr>
<tr>
<td>TBSA (%)</td>
<td>0.300 (0.001)*</td>
<td>0.312 (0.001)*</td>
<td>0.393 (0.007)*</td>
<td>0.375 (0.01)*</td>
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<tr>
<td>FB</td>
<td>0.067 (0.438)</td>
<td>0.046 (0.597)</td>
<td>0.229 (0.007)*</td>
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<tr>
<td>SOFA</td>
<td>0.55 (0.001)*</td>
<td>0.432 (0.001)*</td>
<td>0.459 (0.001)*</td>
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<td>Sepsis/MOF</td>
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<td>Survival</td>
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Table 3: Partial Correlation Analyses of Ubiquitin Serum Concentrations, Clinical and Epidemiologic Patient Characteristics With Outcomes After Burns

<table>
<thead>
<tr>
<th></th>
<th>Sepsis/MOF (n=23)</th>
<th>Survival (n=116)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ub</td>
<td>-0.363 (0.001)</td>
<td>-0.231 (0.009)</td>
</tr>
<tr>
<td>TBSA (%)</td>
<td>0.246 (0.005)</td>
<td>0.448 (0.001)</td>
</tr>
<tr>
<td>FB</td>
<td>0.003 (0.970)</td>
<td>0.112 (0.211)</td>
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<tr>
<td>Age</td>
<td>0.233 (0.009)</td>
<td>0.547 (0.001)</td>
</tr>
<tr>
<td>Female</td>
<td>-0.282 (0.001)</td>
<td>-0.016 (0.855)</td>
</tr>
<tr>
<td>SOFA</td>
<td>0.319 (0.001)</td>
<td>0.285 (0.001)</td>
</tr>
</tbody>
</table>

Ubiquitin Release After Blunt Trauma and Burns

ng/mL). Similarly, blunt trauma patients who did not require ICU treatment (ISS: 13 ± 11) had significantly lower Ub serum levels at admission (289 ng/mL ± 141 ng/mL) than patients with more than 7 days of ICU treatment (ISS: 30 ± 11; 420 ng/mL ± 238 ng/mL) (Fig. 2C).

When burned patients were grouped according to their %TBSA, Ub serum levels on day 0 increased from 321 ng/mL ± 91 ng/mL with <25% TBSA (n = 9) to 465 ng/mL ± 175 ng/mL (n = 10), 697 ng/mL ± 412 ng/mL (n = 11) and 713 ng/mL ± 356 ng/mL (n = 7) with 25% to 35% TBSA, 35% to 46% TBSA, and more than 46% TBSA, respectively (Fig. 2B). Furthermore, the presence of inhalation injury was associated with significantly higher Ub concentrations (Fig. 2B; no inhalational injury [n = 27]: 506 ng/mL ± 342 ng/mL; inhalational injury [n = 10]: 782 ng/mL ± 254 ng/mL; p = 0.029). Surprisingly, the initial Ub serum levels were significantly lower in burned patients who required ICU treatment for more than 30 days (36% ± 10% TBSA; 501 ng/mL ± 193 ng/mL) than in patients with a shorter duration of ICU treatment (43% ± 14% TBSA; 779 ng/mL ± 774 ng/mL; p = 0.03) (Fig. 2C).

Ub concentrations showed no sex or age-related differences and no differences between blunt trauma patients with and without development of sepsis/MOF or blunt trauma survivors and nonsurvivors at any time point (Fig. 3).

Because we did not detect time-related changes of Ub serum levels after burns, the correlations between Ub concentrations, %TBSA, fluid balance (fluids infused minus urine output), age, sex, SOFA scores, and outcomes were analyzed for the 7 days observation period (Table 2). In a bivariate correlation analysis, Ub levels correlated significantly positive with %TBSA and negative with SOFA scores and sepsis/MOF development. As expected, %TBSA, age, fluid balance, and SOFA scores also correlated with outcomes. Thus, we used partial correlation analyses to adjust Ub correlation with outcome for possible confounding factors (Table 3). When correlations with sepsis/MOF development and survival were corrected for the effects of all other variables, Ub concentrations, as well as age and %TBSA correlated significantly with sepsis/MOF development and survival. In contrast, fluid balances during the entire obser-
revision period did not show independent correlations with these outcomes.

Ub serum levels showed no differences when burned patients were grouped by age and sex (Fig. 4A and B). As indicated by the correlation analyses, burned patients with development of sepsis/MOF had a significantly higher %TBSA than patients without development of this complication (sepsis/MOF \( n = 25 \) 48\% ± 15\%TBSA; no sepsis/MOF \( n = 30 \) 32\% ± 13\% TBSA; \( p < 0.001 \)) and their average Ub serum concentration during the 7 days observation period was significantly lower (sepsis/MOF: 459 ng/mL ± 274 ng/mL; no sepsis/MOF: 591 ng/mL ± 421 ng/mL; \( p < 0.05 \)). This difference was even more pronounced when burned patients with sepsis/MOF development were compared with patients without this complication who were matched for %TBSA (n = 12, 42\% ± 9\%TBSA; \( p > 0.05 \) vs. burned patients with sepsis/MOF development; 904 ng/mL ± 438 ng/mL, \( p < 0.001 \) vs. burned patients with sepsis/MOF development). Similarly, burned patients who died (n = 13; 54\% ± 15\% TBSA) had significantly lower average Ub serum concentrations (474 ng/mL ± 300 ng/mL) than survivors with similar injury severity (n = 14, 46\% ± 11\%TBSA, \( p > 0.05 \) vs. nonsurvivors; 838 ng/mL ± 392 ng/mL, \( p < 0.001 \) vs. nonsurvivors).

The Ub levels on the individual days of the observation period in burned patients with and without sepsis/MOF development and in survivors and nonsurvivors with burns are shown in Figures 5 and 6, respectively. Patients who developed sepsis/MOF had significantly lower Ub serum levels on days 1 to 7 than patients with a comparable burn size who recovered uneventfully (Fig. 5A). The total volumes of fluids infused (Fig. 5B), urine output (Fig. 5C), and hematocrit values (Fig. 5D) were similar between these groups. The comparison of survivors and nonsurvivors with burns showed the same tendency and group differences reached a level of statistical significance on days 5 and 7 after injury (Fig. 6A–D).

The SOFA scores during the observation period averaged 6.5 ± 2.5 in burned patients with development of sepsis/MOF and 2.5 ± 1.4 in burned patients who recovered uneventfully (\( p < 0.001 \)). Average SOFA scores were 3.3 ± 2 and 7 ± 2.8 in burned survivors and nonsurvivors, respectively (\( p < 0.001 \)). As shown in Figure 7A, Ub serum levels in the entire population of burned patients were significantly higher on days with SOFA scores of 0 to 1 than on days with SOFA scores of 2 or higher. After exclusion of patients with inhalation injury, Ub concentrations were also significantly higher on days with SOFA scores of 2 to 6 than on days with SOFA scores of more than 7 (Fig. 7B).

**DISCUSSION**

This is the first description of Ub serum concentrations after burn injuries and the initial association of Ub serum levels with injury severity and outcomes after blunt trauma and burns in patients. There are several new findings from the present study: first, Ub release increases with increased injury...
severity after mechanical and thermal injury. Second, high amounts of Ub are systemically released in burned patients. Third, in contrast to blunt trauma patients, Ub is not cleared from the systemic circulation within the first week in burned patients. Fourth, Ub serum levels in burned patients who develop sepsis/MOF and in those who die after burns are lower than in patients with comparable injury severity who recover uneventfully or survive. Fifth, low Ub concentrations are associated with a higher degree of organ dysfunction or failure after burns.

The Ub serum concentrations and their time-related changes that were detected by ELISA in the present study confirm our initial measurements in a small number of blunt trauma patients, that were detected previously using a competitive binding immunoassay. The finding that Ub serum levels at admission correlate with injury severity in both trauma populations indicates passive release from damaged tissues as its major cellular source. Because we showed recently that the lung is among the organs that contain the highest concentrations of free intracellular Ub per gram of tissue, the increased Ub serum concentrations in burned patients with additional inhalation injury further support this assumption.

In line with cell damage as major cellular source of Ub, we did not detect any age or sex-related differences after blunt trauma or burns. In contrast to the ISS that was used to assess injury severity in blunt trauma patients, %TBSA is a measure of the overall size of injured tissue. Thus, the stronger correlation between Ub levels and %TBSA than between Ub levels and ISS further indicates Ub as a marker of cell damage. Because the serum half-life of extracellular Ub is approximately 1 hour, the finding that it is not cleared from the systemic circulation after burns suggests ongoing cell damage for at least 1 week.

Fig. 5. Ubiquitin serum concentrations and fluid balance in burned patients with and without sepsis/MOF development. Data are means ± SEM. (A) Ubiquitin serum concentrations. No sepsis/MOF (day 0: n = 20, day 1: n = 23; day 2: n = 23, day 5: n = 21, day 7: n = 19) (stripped bars). No sepsis/MOF – %TBSA matched (day 0: n = 6, day 1: n = 11; day 2: n = 11, day 5: n = 10, day 7: n = 10) (white bars). Sepsis/MOF (day 0: n = 17, day 1: n = 23; day 2: n = 22, day 5: n = 20, day 7: n = 18) (grey bars). *p < 0.05 (analysis of variance/Tukey). (B–D) Same patients as in (A). No sepsis/MOF (open circles). No sepsis/MOF – %TBSA matched (open squares). Sepsis/MOF (grey circles). (B) Fluids infused (mL/24 h). (C) Urine output (mL/24 h). (D) Hematocrit (%).
Because it has been shown that Ub is also stored together with catecholamines in secretory granules of chromaffin cells in the adrenal medulla and secreted with catecholamines into the circulation upon chromaffin cell stimulation, its release during the stress response could also contribute to its systemic concentrations. However, patients who were admitted to the emergency room after an accident but did not suffer from any injuries (ISS 0) did not show a significant increase in Ub serum concentrations, as compared with healthy volunteers. Thus, the contribution of Ub that is actively secreted during the stress response to its overall concentration in the systemic circulation after blunt trauma and burns is probably small.

Although Ub serum levels were not associated with sepsis/MOF development or survival after blunt trauma, significantly lower Ub concentrations were detectable in burned patients with poor outcomes and Ub concentrations after burns correlated significantly negative with SOFA scores. This association with poor clinical outcome and organ dysfunction or failure explains the negative correlation of its concentrations with the duration of ICU treatment.

We showed previously that Ub has multiple anti-inflammatory effects in vitro and in vivo. Ub inhibited the LPS evoked tumor necrosis factor-α and interleukin-8 responses of whole blood and peripheral blood mononuclear cells in vitro and in vivo and also enhanced the anti-inflammatory Th2 tissue cytokine response during inflammation in vivo. These immunologic effects were accompanied by a significant reduction of third spacing of fluids in models of endotoxic shock, traumatic shock, and lung ischemia-reperfusion injury. Although the exact mechanism of Ub’s actions is currently not known, it was
shown previously that extracellular Ub can be taken up into intact cells, followed by covalent ligation of extracellular Ub to intracellular proteins. Because its uptake showed saturation kinetics with a $K_d$ value in the low nM range, these data strongly suggest a highly specific receptor through which Ub mediates its actions.

Burn injuries are always associated with a profound inflammatory response and significant fluid shifts into tissues during the early postburn period, which in turn contribute to the development of organ dysfunction and failure. Therefore, the findings of the present study suggest that Ub release could constitute an endogenous defense mechanism that is aimed to limit inflammation and possibly impaired microvascular permeability. Although effects of Ub on immune functions have been described at physiologic relevant concentrations, its effects on fluid shifts were detectable when doses were administered that produced supraphysiologic concentrations. This may explain the observation that Ub concentrations were not associated with fluid balances in the present study. However, because observational studies cannot address any functional aspects, further mechanistic studies are required to evaluate this hypothesis. In contrast to the homogenous population of patients with thermal injuries, the heterogeneity of the individual injuries in blunt trauma patients and the variability of the physiologic response to blunt trauma could explain the missing association of Ub levels with outcomes in this patient population.

Comparison of the fluid balance and hematocrit values between patients with and without sepsis/MOF or burned survivors and nonsurvivors did not show differences in our patient population. Thus, high volumes of resuscitation fluids that possibly could have caused a dilution effect in patients with poor outcomes cannot account for the lower Ub concentrations. Other possible explanations could be increased renal secretion or Ub consumption. Because we showed that only 10% of intravenously administered exogenous Ub can be recovered in the urine, increased consumption appears to be more likely to account for the lower Ub concentrations in burned patients with poor clinical outcome. Besides increased proteolytic degradation of Ub as one possible explanation, enhanced uptake could also explain the lower concentrations that were associated with sepsis/MOF or death after burns.

In conclusion, the findings of the present study strongly suggest that systemic Ub concentrations reflect the extent of tissue damage after blunt trauma and burns in patients. Furthermore, burn patients who develop sepsis/MOF or die seem to have a relative Ub deficiency. Although the wide range of Ub serum concentrations in injured patients shows that its levels will probably not be useful as a diagnostic or prognostic marker in the clinical arena, the results of the present study imply that Ub could play an important pathophysiologic role during the whole body response to burn injury and suggest that its systemic release is protective. Further mechanistic studies are required to confirm this hypothesis.

Because pharmacologic interventions that prevent fluid leakage into the extravascular compartment after thermal injury are not available, the results from the present study
along with Ubs ability to reduce fluid shifts during infectious and noninfectious inflammation in vivo provide a strong rationale to assess Ubs therapeutic potential also after burn injuries.

REFERENCES


DISCUSSION

Dr. Marc G. Jeschke (Galveston, Texas): He presented that ubiquitin protein expression, ubiquitylination plays an important role in our body, is a new marker for severe, for stress and stress response.

When I read through this paper and on this data my thoughts were, is it not just another marker? We have cytokines as predictors and as major players in the whole host
defense response and systemic inflammatory response discovered 20 years ago and they failed what they promised.

So my first question leading to this is philosophical. Is ubiquitin just another marker that we have, another tool rather than ubiquitin has any effect?

The next question I have to you is based on some confusion I had reading through your paper. You say ubiquitin reflects, its release reflects the extent of the damage and the stress. However, when you get burned your basic ubiquitin goes down so there seems to be a controversy in your data and particularly, when you show that inhalation injury causes an up-regulation of ubiquitin, however, that is actually good. So is inhalation injury then good for burn patients?

My next question is how did you measure ubiquitin? It’s an ELISA as you mentioned. Is it total? Is it free? Is it bound? Is there a different effect of the ubiquitin that you measure?

And where is ubiquitin produced? That maybe is also plays a role when you say inhalation injury increases ubiquitin concentration.

And then, lastly, I would like you to elucidate on how is ubiquitin released? Is it an active transportation? A passive? Is it just like enzymes when there is cell damage and then releases into the system?

And yet again leading to the point, what is the significance? Do we want to increase ubiquitin levels? Do we want it decreased? Do we want it affected or do we want to give it or what is the clinical role then of ubiquitin? Again, thank you very much for discussing this paper.

**Dr. Basil A. Pruitt, Jr.** (San Antonio, Texas): Interesting paper. You imply that it does something to capillary permeability. How did you assess that? In terms of lung weight? Wet and dry weight lung changes or total body fluids? We’d like to hear how you rationalized that.

**Dr. Timothy Billiar** (Pittsburgh, Pennsylvania): Along the lines, some of the mechanistic questions, has the cell surface receptor for ubiquitin been identified?

**Dr. M. Majetschak** (Miami, Florida): Thank you very much for your comments and questions. With regard to Dr. Jeschke’s questions, we showed that extracellular ubiquitin has several anti-inflammatory effects in-vitro and in-vivo. Ubiquitin inhibits LPS evoked TNFα and IL-8 production of leukocytes and a very recent study showed that it enhances the Th2 cytokine response in the lung during inflammation. Studies from other investigators showed that it also possess anti-microbial actions. More importantly, we showed in several independent animal models that it has beneficial and clinically relevant effects during infectious and non-infectious inflammation. Considering these actions along with its highly conserved protein structure and the fact that the anti-inflammatory cytokine response evolved relatively late during evolution, its actions could represent a highly conserved anti-inflammatory pathway in the innate immune system that is aimed to control the evolutionary highly conserved pro-inflammatory response. The wide range of its serum concentrations in trauma patients shows that the use of systemic ubiquitin levels as a diagnostic or prognostic marker in the clinical arena is probably not feasible. Nevertheless, the results of the present study along with its anti-inflammatory and clinical relevant effects in-vitro and in-vivo suggest that the release of endogenous ubiquitin is protective and imply that burn patients who develop sepsis or multiple organ failure have a ubiquitin deficiency. The next question was in regard to its association with injury severity and inhalation injury. As presented before, we detected that ubiquitin serum levels increased with increases in ISS, burn size and also with the presence of inhalation injury. Thus, there is no inconsistency of the results.

The next question was in regard to the ELISA measurements. The ELISA is home-made and not commercially available. This assay detects predominantly free ubiquitin. We know that it has some cross-reactivity with ubiquitin protein conjugates which are also present in serum and that the measurements overestimate the free ubiquitin concentrations by approximately 5–10%.

In regard to ubiquitins cellular origin and mechanism of release, our previous and the present study strongly suggest passive release from damaged cells and tissues. We showed, for example, that approximately 30% of ubiquitin that is released into CSF after traumatic brain injuries can be attributed to its release from lysed erythrocytes and that the remaining amounts are probably released from the damaged brain tissue. On the other hand, Kieffer et al. showed in 2003 that ubiquitin is stored together with catecholamines in secretory chromaffin granules and released into the circulation upon stimulation of chromaffin cells. Two other reports suggested that it may also be secreted by leptomeningeal cells and hairy cell leukemia cells. However, our finding that patients who were admitted to the emergency room but did not suffer any injuries did not have increased ubiquitin levels suggest that the amounts of ubiquitin that are released together with catecholamines from the adrenal medulla during the stress response is probably very small, as compared to its release due to cell damage. Finally, Dr. Jeschke asked about a possible clinical role for ubiquitin. Since we showed in multiple independent and clinically relevant animals studies that ubiquitin treatment has profound therapeutic effects after trauma and the present study clearly indicates a ubiquitin deficiency in burn patients who develop complications or die, evaluation of its therapeutic efficacy also after burn injuries appears well justified.

With regard to Dr. Pruitt’s question, we showed that ubiquitin treatment consistently reduced systemic fluid requirements to maintain hemodynamics after traumatic and endotoxic shock while hematocrit values were identical in the control and treatment groups. This was accompanied by reduced brain edema formation, improved lung mechanics and oxygenation with ubiquitin treatment. Besides this indirect evidence for effects of ubiquitin on fluid
shifts, we also showed directly that it significantly reduced wet-weight dry-weight ratios of the lung after ischemia-reperfusion injury.

The last question by Dr. Billiar was with regard to a ubiquitin receptor. We recently provided initial evidence for a receptor for ubiquitin on monocytes that can be up-regulated during inflammation. We showed that ubiquitin is taken up into the cell and then rapidly conjugated to intracellular proteins, thus being introduced into the endogenous ubiquitin proteasome system of the target cell. This uptake showed saturation kinetics and a Kd value in the very low nanomolar range, which strongly suggests a highly specific receptor mediated uptake process. However, this putative receptor has not been identified yet.

Once again, I’d like to thank all discussants for their comments and questions.