Burn injury induces high levels of phosphorylated insulin-like growth factor binding protein-1

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Abstract: Purpose: Burn injury is associated with early apoptotic death of T cells. Insulin-like growth factor-1 (IGF-I) is able to protect T cells from apoptosis. Association of IGF-I with its IGFBP (Binding Protein)-1 limits its bioavailability and serine phosphorylation of IGFBP-1 lowers this further because of an increased affinity for IGF-I. The level of phosphorylated IGFBP-1 has been shown to increase in pediatric burn patients. Thus we hypothesized that a longitudinal study of burn patients would demonstrate 1) increased IGFBP-1 levels, 2) increased IGFBP-1 phosphorylation and 3) decreased IGF-I levels over time. Methods: We conducted a prospective observational study in adult burn patients admitted to UNC Jaycee Burn Center. Plasma levels of insulin, insulin-like growth factor 1 (IGF-I) and insulin-like growth factor binding protein 1 (IGGBP-1) were measured on admission up to 10 days post admission. ELISA was used to measure serum levels of insulin, IGF-I and IGFBP-1. Serine phosphorylation of IGFBP-1 was measured by Western blot with and without the incubation of calf intestinal phosphatase (CIP). Significant findings: There was a significant positive correlation of increasing %TBSA burn and increasing levels of serum IGFBP-1 from admittance blood draws. Levels of IGF-I also decreased with increasing Total Body Surface Area (TBSA, p<0.05). In patients studied longitudinally (n=84) we found that IGFBP-1 levels are significantly (p<0.05) increased 1-72 hours post burn (mean±SEM serum concentration; burn=172±23 ng/mL, normal=13±3 ng/mL) and that levels of IGF-I are reduced. IGFBP-1 is serine phosphorylated in burn patients. In patients surviving past 72 hours IGF-BP1 remained phosphorylated over the study period. Conclusions: IGFBP-1 and its serine phosphorylation regulate and limit IGF-I bioavailability. Our results suggest that increases in IGFBP-1 and persistent serine phosphorylation of IGFBP-1 correlate with the severity of burn injury, and may contribute to burn-associated T cell apoptosis and subsequent immune dysfunction by reducing the bioavailability of this important cell survival factor.

Keywords: Insulin-like growth factor, insulin-like growth factor binding protein-1, trauma, burns

Introduction

The leading causes of death from serious burn injury following resuscitation are sepsis and multiple system organ failure secondary to profound immune dysfunction [1]. Burn is associated with apoptosis of lymphocytes in the thymus and in the peripheral lymphoid organs [2, 3]. The consequence of these events is suppression of the inflammatory immune response in burn patients, thereby leading to an increased susceptibility to infection. Insulin-like Growth factor-1 (IGF-I) has been shown to be immunomodulatory primarily via its ability to act as a potent stimulator of T-cell survival and function. The IGFs (IGF-I and II) are 7.5 kDa conserved secreted polypeptides with 70% homology with each other and 50% with insulin [4, 5]. They are known to have diverse effects including metabolic insulin-like effects and effects on cell growth, differentiation and survival acting in an endocrine, autocrine and paracrine manner. Specifically, in the case of T cells IGF-I has been shown to protect activated T cells from apoptosis [6, 7].

IGF Binding Protein (IGFBP)-1 is the major modulator of the bioactivity of free IGF-I. The majority of IGF-I in the circulation is associated with IGFBP-3 [4, 5]. Serine phosphorylation of IGFBP-1 increases its affinity for IGF-I [8], thereby sequestering IGF-I away from its receptor and reducing its bioactivity [9]. It has been suggested that a sustained high serum concentra-
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Figure 1. A model of the changes that occur in the insulin-like growth factor 1 (IGF-I) axis in burn patients. Phosphorylation of IGF binding protein 1 (IGFBP-1) would occur in burn patients which increases binding affinity to IGF-I. IGF-I is a well characterized T cell survival factor, therefore in the presence of elevated IGFBP-1 and reduced IGF-I levels, the immunosuppressive state of the adaptive immune system would be significantly increased.

Table 1. Patient characteristics. Data on the total body surface area (TBSA) burn, presence of inhalational injury and patient age on admission were collected from patient charts. A total of 84 patients were included in the study. These patients were arranged into 4 groups in respect to burn size. Mean age and TBSA are shown ± Standard Deviation from the mean.

<table>
<thead>
<tr>
<th>Burn Injury TBSA (%)</th>
<th>Number of patients</th>
<th>Mean age (years)</th>
<th>Mean TBSA (%)</th>
<th>Inhalation injury (% of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-15</td>
<td>37</td>
<td>36±18.2</td>
<td>7±4.3</td>
<td>0</td>
</tr>
<tr>
<td>16-30</td>
<td>10</td>
<td>32±26.3</td>
<td>21±4.6</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>31-50</td>
<td>19</td>
<td>44±14.0</td>
<td>41±6.1</td>
<td>8 (42%)</td>
</tr>
<tr>
<td>51-95</td>
<td>18</td>
<td>37±20.2</td>
<td>68±12.3</td>
<td>15 (83%)</td>
</tr>
</tbody>
</table>

Given the potential role of IGF-I as a T cell survival factor this decrease in IGF-I may contribute to the loss of T cell function. Clinical studies in which IGF-I (associated with its principal binding protein IGFBP-3 which prolongs its half-life and reduces associated side effects, notably hypoglycemia) was administered to burn patients showed improvement in outcome [15, 18]. An alteration in the cytokine profile of patients following IGF-I/IGFBP-3 administration was observed suggesting that the positive effects of IGF-I were mediated at least in part through its ability to modify the immune response [16, 19].

We therefore hypothesized that the decrease in IGF and increase in IGFBP-1 levels after burn is associated with IGFBP-1 phosphorylation (Figure 1). To test this, we obtained peripheral blood from burn patients admitted to the Jaycee Burn Center within the first 24 hours after injury and daily thereafter up to 10 days post burn. We used ELISA to measure the serum levels of IGF-I and IGFBP-1. We also characterized the phosphorylation status of IGFBP-1 within each sample by calf intestinal phosphatase (CIP) treatment followed by Western blot. We observed 1) increased IGFBP-1 levels, 2) decreased IGF-I levels over time and 3) increased and persistent IGFBP-1 phosphorylation.

Materials and methods

Patients

With the approval of the University of North Carolina Healthcare Internal Review Board (IRB), samples were collected from excess serum originally obtained for clinical use. The Internal Review board approved the waiver of consent as the study did not incur additional risks and patient specimens were systemati- cally de-identified. All patients admitted to the Jaycee Burn Center were eligible to be included in the study. Patients enrolled in the study had peripheral blood samples collected within 24 hours of admission and were followed up to 10 days post admission.

Data on the total body surface area (TBSA) burn, presence of inhalational injury and patient age on admission were collected from patient...
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charts. A total of 84 patients were included in the study. These patients were arranged into 4 groups in respect to burn size. This included a group of burn patients with TBSA burn injury of 1-15%, 16-30%, 31-50% and 51-95% (Table 1).

**Insulin/insulin-like growth factor/insulin-like growth factor binding protein axis measurements**

Excess serum samples were obtained from burn patients within 24 hours of injury and daily thereafter up to 10 days. Insulin, insulin-like growth factor I (IGF-I) and insulin-like growth factor binding protein 1 (IGFBP-1) concentrations were measured by enzyme-linked immunosorbent assays (ELISA, Diagnostic Systems Laboratories, Webster, TX, USA). Serum analyzed included burn patient specimens and normal controls. Normal controls were obtained from healthy volunteers.

**Measurements of serine phosphorylation of insulin-like growth factor binding protein 1**

The degree of IGFBP-1 serine phosphorylation was assessed by non-denaturing and non-reducing SDS PAGE immunoblotting of patient serum with anti-IGFBP-1 antibody before and after treatment with 50U calf intestinal phosphatase (CIP), as detailed by Sakai et al [8, 14]. PAGE was performed in the absence of SDS in order to alter protein migration on the basis of molecular charge as well as mass, thus permitting separation of phosphorylated from non-phosphorylated IGFBP-1. In addition, the pH during the resolving phase of electrophoresis was lowered to 8.3. Gels were analyzed for a decrease in IGFBP-1 migration after CIP-treatment indicating IGFBP-1 phosphorylation [8].

**Study end point**

Patient blood samples were included in the study up to 10 days post admission or patient demise.

**Murine model of burn injury and thymocyte culture**

Wild-type C57BL/6 female 18-20 gram, 6 week old mice underwent 20% TBSA contact burn procedure as we have detailed previously [2, 20]. Mice were anesthetized with isoflurane and received a full-contact burn using a copper rod, heated in boiling water, applied to the animal’s dorsum/flank for 10 seconds. Four applications produced an approximately 20% TBSA full-thickness burn. After burn injury, we resuscitated mice with an intraperitoneal injection of lactated Ringers solution (0.1 ml/g body weight) and gave buprenorphine (2 mg/kg body weight) for pain control immediately after injury and placed mice in separate cages. The mice were given 0.02 mg/cc of morphine sulfate (4 mg/kg/day) in drinking water. Sham-treated control groups of mice were also setup; these mice received all of the above treatments, with the exception of application the heated rod. The burn procedure was followed according to institutionally approved protocols performed in accordance with NIH guidelines.

We isolated thymocytes at 6 hours post burn from each mouse and placed thymocytes in culture (1x10^6 cells/ml in 2 ml of RPMI 1640 medium plus 1% FCS, antibiotics and glutamine) in the absence or presence of 100 ng/ml IGF-I. Eighteen hours after burn, apoptosis was assessed by staining with anti-CD8 (APC), anti-CD3 (FITC), Annexin V conjugated to PE and 7-Aminoactinomycin D (7AAD) in PBS containing calcium. The degree of apoptosis was assessed by four color flow cytometry, quantifying the percentage of CD3+CD3+T cells that are apoptotic (defined by 7AAD−, Annexin V staining).

**Statistical analyses**

Variables were recorded upon admission and up to 10 days afterward. Patient age and TBSA burn in each TBSA group was reported as a mean. Categorical data which included the presence of inhalation injury was reported as total numbers of patients and percentages of each TBSA group.

Insulin, IGF-I and IGFBP-1 were measured and the Pearson correlation coefficient was used to determine the coefficient of determination (r^2) for the serum concentrations compared to the extent of the burn injury as determined by measuring the TBSA. A two way ANOVA was used to assess continuous data at different time points after burn injury and after thymocyte culture with or without IGF-1. P<0.05 was considered statistically significant. All significance tests were two-tailed. Data was analyzed using the GraphPad Prism version 4 software (GraphPad Software Inc., 2003, San Diego, CA, USA).
Results

Levels of IGF-I and IGFBP-1 correlate with TBSA on admittance

Blood samples were drawn on admittance and analyzed by ELISA for A: insulin, B: IGF-I and C: IGFBP-1 (n=84). Serum was analyzed from normal controls. Normal controls were obtained from healthy volunteers (n=16, range is indicated by grey shading). \( r^2 \) indicates the coefficient of determination.

IGFBP-1 is serine phosphorylated after burn injury

We hypothesized that not only the levels of IGF-I and IGFBP-1 would be altered after burn injury, but that phosphorylation of IGFBP-1 would also occur in burn patients. Immunoblotting with anti-IGFBP-1 revealed that the level of IGFBP-1 in serum from a pool of normal serum is almost undetectable whereas IGFBP-1 can be seen in the sera from burn patients (Figure 4). The quantity of IGFBP-1 was increased with higher burn area (representative bands in Figure 4 from 84, 43 and 15% TBSA patients). This
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**Figure 3.** Longitudinal levels of IGF-I are significantly decreased and IGFBP-1 levels are significantly increased after burn injury compared to normal serum and is dependent on burn TBSA. Blood samples were drawn daily (d1 = day 1, d2 = day 2, d3 = day 3, day 4-10 represent a sample taken within the day 4-10 period) after admittance and analyzed by ELISA for IGF-I and IGFBP-1. Serum was analyzed from normal controls. Patients were stratified according to their burn TBSA (A: 1-15% (n=37), B: 16-30% (n=10), C: 31-50% (n=19) or D: 51-95% (n=18) and levels of IGF-I and IGFBP-1 were compared to normal serum levels. Statistical significance is indicated by *, p<0.05; **, p<0.005 by two way ANOVA.

increase in IGFBP-1 is consistent with ELISA measurements. We ran phosphorylation analyses on all the samples. When this serum was treated with CIP there was a marked shift in the molecular mass of the IGFBP-1 indicating that the IGFBP-1 in the sera from the burn patients was heavily serine phosphorylated. We consistently observed 100% phosphorylation of IGFBP-1 in burn patients and remained fully phosphorylated (Figure 5) until the end of the study.

**IGF-I can act to prevent thymocyte apoptosis caused by burn injury**

In order to demonstrate that IGF-I can be beneficial in reducing burn injury-induced apoptosis of immune T cells, we utilized a mouse model of burn injury. Wildtype female C57/BL6 mice received either a 20% TBSA or sham treatment as we have described previously [2, 20]. We typically observe induction of thymic apoptosis *in vivo* between 12 and 24 hours after burn [2]. As can be seen in **Figure 6**, apoptosis of thymocytes from burn mice occurs even after the cells are isolated from the mouse and placed in culture. This “programmed” death is reversed by addition of IGF-I to the thymocyte culture. Addition of IGF-I returned levels of apoptosis to that of thymocytes from sham mice. This suggests that elevated levels IGF-I is able to rescue T cells from burn-mediated apoptosis.

**Discussion**

In this study, we evaluated the relationship between burn injury and the IGF-I/IGFBP-1 axis.
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Using unidentified burn patients of all ages, we discovered a direct correlation with the TBSA burn injury and IGFBP-1 at the time of admission, but an inverse relationship with IGF-I and the TBSA burn injury. We observed that these relationships continued at 10 days after injury, our endpoint. Size of the burn injury appears to play an influential role in the homeostasis of the IGF-I/IGFBP-1 axis. While these data concur with earlier studies, we demonstrate here that IGFBP-1 in burn patients is heavily phosphorylated. Increased IGFBP-1 phosphorylation increases its affinity for IGF-1, likely reducing its bioavailability. These data suggest that the increase in IGFBP-1 phosphorylation coincides with the reduction of serum IGF-I seen in burn patients and therefore reduces the survival benefit of IGF-1.

Elevated levels of IGF-I provide a survival benefit in critically ill patients, and improve survival in sepsis [10, 11, 21, 22]. IGF-I is also significantly decreased in non-survivors of multi-organ failure [22-24]. The mechanism for this is thought to be that IGF-I improves survival of immune effector cells such as T cells [7, 25, 26] and other multiple immune cell types [27, 28]. IGF-I activates specific signaling pathways including Akt and Jun N-terminal kinases which in turn promotes T cell survival from Fas-induced cell death [7]. We have presented data here demonstrating in vitro IGF-1 can protect against prior in vivo burn-induced

Figure 4. IGFBP-1 is serine phosphorylated after burn injury. Serum from normal (NS) or two burn patients (84% or 43% burn TBSA), with (+CIP) or without CIP exposure, was immunoblotted with anti-IGFBP-1 antibody. IGFBP-1 and molecular mass-shifted phosphorylated-IGFBP-1 are represented by the labels.

Figure 5. IGFBP-1 remains serine phosphorylated for at least 5 days after burn injury. Serum from normal (NS) and longitudinal samples from two burn patients (62% or 32% burn TBSA), with (+CIP) or without CIP exposure, was immunoblotted with anti-IGFBP-1 antibody. IGFBP-1 and molecular mass-shifted phosphorylated-IGFBP-1 are represented by the labels.
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Figure 6. IGF-I can act to prevent thymocyte apoptosis caused by burn injury. Wild-type female C57/BL6 mice received either a 20% TBSA or sham treatment (n=6/group). Thymocytes were harvested at 6 hours post burn from each mouse and placed thymocytes in culture in the absence or presence of 100 ng/ml IGF-I. Eighteen hours after burn, apoptosis was assessed by staining with anti-CD8 (APC), anti-CD3 (FITC), Annexin V conjugated to PE and 7-Aminoactinomycin D (7AAD) in PBS containing calcium. The degree of apoptosis was assessed by four color flow cytometry, quantifying the percentage of CD8+CD3+T cells that are apoptotic (defined by 7AAD−,lo Annexin V+ staining). Top panel, representative flow cytometry of each group. Bottom Panel, collated groups, plotting percentage of CD8+CD3+T cells that were apoptotic, where statistical significance is indicated by **, p<0.005 by two way ANOVA.

This study provides a possible mechanism for the reduced bioavailability of IGF-1 after burn injury. The rapid and sustained phosphorylation of IGFBP-1 after burn injury represents an important biological finding. Further work will be required to identify ligands, and if phosphorylated IGFBP-1 is competing with IGFBP-3 for
IGF-1, this provides rationale for disrupting this interaction to provide a possible immunotherapeutic increasing IGF-1 availability. Identification of kinases that control IGFBP-1 phosphorylation is also a target of investigation. We hypothesize that phosphorylated IGFBP-1 may also become localized to the cell surface or extracellular matrix to deliver IGF-1. This may provide a novel mechanism for studies in which IGFBP-1 deficiency both by antibody treatment and in IGFBP-1 knockout mice increased Fas-induced hepatocyte apoptosis [37] where IGBP-1 has a protective anti-apoptotic effect.

There are some limitations to this study which primarily involve the complexity of the IGF/IGFBP axis and the contribution of other metabolic factors. Although the size of burn injury coincides with an increase in IGFBP-1, we cannot claim a direct relationship between these two variables as other variables such as age, depth of injury and presence of co-morbidities cannot be adjusted for in our statistical analysis. We also recognize that hormone levels fluctuate widely within a 24-hour period following injury [22]. Nevertheless we attempted to limit the effect of temporal variations by collecting samples at similar time points each day. Patient outcomes and mortality are not included in this study due to the restricted access of comprehensive patient data. Additionally we cannot include information pertaining to pre-existing conditions or gender. However, our results still support a prognostic role for phosphorylated IGFBP-1 in burn patients and a potential molecular mechanism for immune dysfunction after burn.

Burn injury greatly impacts the IGF-I/IGFBP-1 axis. We demonstrate that IGF-I and IGFBP-1 directly correlate with the severity of burn injury. IGF-I is reduced with increasing TBSA% burn injury, while IGFBP-1 steadily increases along with increasing TBSA% burn injury. IGFBP-1 is heavily phosphorylated in burn patients early after injury which likely contributes to the reduction of circulating IGF-I. Longitudinal outcome studies will be necessary in order to confirm the prognostic value of the serum admission levels of IGF-I/IGFBP-1 on the overall outcomes for burn patients.

Disclosure of conflict of interest

None.
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- Comprehensive review of the role of insulin-like growth factor-1 (IGF-1) in burn injury and critical illness.
- Discussion on the implications of IGF-1 in the context of hypermetabolism and sepsis.

References:


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