TRAUMA CAUSES EARLY RELEASE OF SOLUBLE RECEPTORS FOR TUMOR NECROSIS FACTOR

Larissa R. Tan, MD,* Kenneth Waxman, MD,* Gianna Scannell, MD,* Gene Ioli,* and Gale A. Granger, PhDb,c

The importance of tumor necrosis factor (TNF) in the pathophysiology of trauma and hemorrhagic shock is not known. In addition, TNF bioactivity may be modulated by soluble forms of the 55-kd and 75-kd membrane receptors (TNFR). This study was undertaken to determine circulating levels of TNF and TNFR after trauma. Nine severely injured male patients were studied. The mean age was 30 ± 10 years (range, 15-45). The mean Injury Severity Score (ISS) was 31.3 ± 17.6 (range, 10-59), and the mean Revised Trauma Score (RTS), 5.7 ± 2.2 (range, 0.7-7.8). Serum was obtained immediately upon arrival at our trauma center, within 1 hour of injury. The TNF and TNFR levels in the serum were measured using ELISA techniques. After trauma, 55-kd and 75-kd TNFR levels were significantly elevated above those of controls (6.99 ± 4.57 ng/ml and 5.42 ± 1.88 ng/ml, respectively, p < 0.01); TNF levels were not increased. Patient serum containing TNFR inhibited in vitro TNF cytotoxicity and correlated with 55-kd TNFR levels (p < 0.05). We conclude that TNF is a strong releasing factor for TNFR; the presence of TNFR may be indirect evidence that TNF is present after trauma, despite low measured levels. Both TNF and TNFR may be more important in trauma and hemorrhagic shock than previously thought.

THE ROLE OF TUMOR NECROSIS FACTOR (TNF) in the pathophysiology of trauma and hemorrhagic shock remains unclear. Several animal studies have demonstrated systemic release of TNF after hemorrhagic shock. However, previous human studies of both elective surgery and trauma have not demonstrated significant circulating levels of TNF.

Although the numerous functions of TNF have been extensively studied, there is a relative paucity of information regarding the regulation of TNF. In recent years, two soluble receptors for TNF (TNFR) have been identified. These soluble receptors have been found in the serum and urine of patients with chronic renal failure as well as in the serum and ascites of cancer patients. These TNFR are shed or secreted from the extracellular segments of the 55-kd and 75-kd TNF membrane receptors and may be important in the regulation of TNF activity. The TNFR are capable of binding TNF and preventing binding to cellular membrane receptors, thus decreasing the biologic effects of the cytokines. In vitro the TNFR have been found to inhibit the lytic activity of TNF on murine L929 and WEHI 164 cells. In addition, TNFR inhibits in vivo necrosis of cutaneous Meth A tumors, and decreases mortality in animal models of septic shock.

The purpose of this study was to determine if TNF and TNFR were released early after injury. Serum was obtained from patients within 2 hours of traumatic injury and levels of TNF and its soluble receptors determined. Patient sera were also tested for the ability to suppress in vitro TNF cytotoxicity.

MATERIALS AND METHODS

Patient Population

This study was approved by the Human Subjects Research Committee of the University of California, Irvine. Nine severely injured male patients were studied (Table 1). The mean age was 30 ± 10 years (range, 14-45). Five were injured in automobile or motorcycle crashes; most had multiple injuries, although one patient had an isolated head injury. Two had gunshot injuries, one a 60% total body surface area burn, and one fell from a ten-story building. Severity of injury was quantitated with the Injury Severity Score (ISS) and the Revised Trauma Score (RTS). The TRISS score, an estimated probability of survival, was determined using weighted values of the RTS, ISS, and patient age. The mean ISS was 31.3 ± 17.6 (range, 10-59), the mean RTS was 5.7 ± 2.2 (range, 0.7-7.8), and the mean probability of survival was 0.69 ± 0.36 (range, 0.07-1.00). Five patients died. Serum samples from eight noninjured subjects (four men and four women) with mean age 34 ± 9 years served as controls. All patients except the burned patient arrived

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Presented at the Fifty-second Annual Session of the American Association for the Surgery of Trauma, September 17-19, 1992, Louisville, Kentucky.

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Table 1
Patient characteristics and early TNFR levels

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (years)</th>
<th>Injuries</th>
<th>Mortality</th>
<th>ISS</th>
<th>RTS</th>
<th>Triss</th>
<th>55-kd TNFR (ng/mL)</th>
<th>75-kd TNFR (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>GSW; chest; pulmonary artery laceration</td>
<td>Died</td>
<td>16</td>
<td>5.15</td>
<td>0.95</td>
<td>8.19</td>
<td>5.86</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>50% Body surface area burns</td>
<td>Died</td>
<td>25</td>
<td>7.84</td>
<td>0.99</td>
<td>1.14</td>
<td>3.34</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>Spinal cord compression; ruptured spleen; sm. liver laceration</td>
<td>Survived</td>
<td>57</td>
<td>4.09</td>
<td>0.15</td>
<td>13.09</td>
<td>9.29</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>MCC; open pelvic fracture; lower extremity amputation</td>
<td>Died</td>
<td>26</td>
<td>0.73</td>
<td>0.07</td>
<td>5.16</td>
<td>5.45</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>GSW, abdomen; large liver laceration</td>
<td>Survived</td>
<td>10</td>
<td>7.84</td>
<td>1</td>
<td>15.69</td>
<td>7.28</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>MVC; pelvic fracture; pulmonary lacerations; large liver laceration</td>
<td>Died</td>
<td>59</td>
<td>5.68</td>
<td>0.41</td>
<td>6.26</td>
<td>4.91</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>MVC; cerebral contusion</td>
<td>Survived</td>
<td>10</td>
<td>6.61</td>
<td>0.99</td>
<td>7.79</td>
<td>5.55</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>MCC; ruptured spleen; lacerated kidney and lung</td>
<td>Died</td>
<td>41</td>
<td>7.84</td>
<td>0.96</td>
<td>2.50</td>
<td>2.65</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>MVC; epidural hematoma severe facial fractures</td>
<td>Survived</td>
<td>38</td>
<td>5.03</td>
<td>0.65</td>
<td>3.11</td>
<td>4.42</td>
</tr>
</tbody>
</table>

with systolic blood pressure less than or equal to 100 mm Hg.

Sample Collections

Blood samples were drawn from central venous catheters placed upon arrival at the UC Irvine trauma center. In all cases, the first sample was obtained within 1 hour of injury. Subsequent samples were drawn every 15–30 minutes for a total of 2 hours or until surgical intervention. Blood samples were immediately centrifuged, and the serum aspirated. Serum samples were stored at −20°C until assayed.

TNF ELISA

A multiple antibody sandwich ELISA (Genzyme, Cambridge, Mass) was utilized. A flat-bottomed microtiter plate was coated with mouse monoclonal anti-TNF antibody, then incubated overnight at 4°C. The plate was then washed with 1% Tween in phosphate-buffered saline. Standard dilutions of recombinant human TNF and serum samples were added to the plate, followed by incubation at 37°C for 4 hours. The plate was again washed, rabbit anti-human TNF polyclonal antibody added, and the plate incubated for an additional 2 hours at room temperature. After washing, biotin-conjugated goat anti-rabbit IgG was added and the plate incubated at room temperature for 50 minutes. The plate was washed and streptavidin-peroxidase incubated at room temperature for 40 minutes. After a final washing, substrate containing chromagen and peroxide was added. After about 5 minutes, the reaction was stopped with 2N sulfuric acid. Absorbance was measured at 492 nm. A standard curve was generated and the TNF levels in samples were calculated using the standard curve.

TNF Receptor ELISA

A polyclonal sandwich ELISA for TNFR was utilized. Human recombinant 55-kd and 75-kd TNFR were obtained from Synergen (Boulder, Colo). Immunoglobulin G against the TNFR was produced by the method of Yamamoto et al.14 96-well flat-bottomed microtiter plates were coated with rabbit IgG against 55-kd or 75-kd TNFR and incubated at 4°C overnight. Plates were washed with 0.2% Tween (Bio-Rad Laboratories, Richmond, Calif) in phosphate-buffered saline. Standard preparations of 55-kd or 75-kd TNFR were diluted in phosphate-buffered saline with 1% bovine serum albumin. The TNFR standards and human serum samples were added to the plates. Plates were again incubated at 4°C overnight, then washed with Tween. Horseradish peroxidase-bound rabbit anti-TNF IgG was added, followed by incubation for 1 hour at 37°C, and washing with Tween. Substrate containing one 2,2'-azinobis diammonium salt tablet (Pierce, Rockford, Ill) and 3 µL of 30% H₂O₂ in 10 mL of 1 mol/L acetate buffer (pH 4.2) was added to the plate. The final incubation was at room temperature for 20 minutes. Absorbance at 405 nm was read with a microplate ELISA reader. A standard curve was generated and concentrations of TNFR in samples were calculated using the standard curve.

In Vitro Assay of TNFR Bioactivity

Samples of TNFR in subsets of trauma (n = 5) and control (n = 5) serum samples were tested for in vitro activity using a TNF microplate bioassay.15 Murine L-929 fibrosarcoma cells were plated at a concentration of 20,000 cells/well in RPMI-3% in microtiter plates. Cells were treated with mitomycin C and incubated for 24 hours at 37°C. After incubation, 2 mmol/L/well of NaF and serial dilutions of TNF at 50, 5, or 0.5 ng/mL were added to the plate; wells without TNF served as controls. Trauma patient and control serum samples were added, and the plates again incubated overnight at 37°C. After incubation, the medium was aspirated and the plate stained with a 2% crystal violet solution for 5 minutes at room temperature. The plate was washed with distilled water to remove nonviable cells. Absorbance of adherent, viable cells was read at 580 nm with a microplate reader; the absorbance is directly proportional to the number of viable cells. Cell viability in the presence of patient or control sera was compared to viability with TNF alone.
Statistical Analysis

One-way ANOVA followed by Newman-Keuls' multiple range tests compared 55-kd and 75-kd TNFR levels between controls and injured patients, as well as between survivors and nonsurvivors. An ANOVA was used to compare cell survival in the TNFR bioassays. Linear regression by least squares was used to determine correlation between TNFR levels and severity of injury, as well as the significance of apparent trends in TNFR levels over time.

RESULTS

Release of TNFR After Trauma

Levels of TNF were <50 pg/mL for both trauma patients and noninjured individuals (controls). However, within 1 hour of injury, both TNFRs were markedly elevated compared with controls: 55-kd 6.99 ± 4.57 ng/mL versus 0.67 ± 0.18 ng/mL, and 75-kd 5.42 ± 1.88 ng/mL versus 1.66 ± 0.33 ng/mL, p < 0.01 (Fig. 1).

Levels of TNFR did not correlate with severity of injury by ISS, RTS, or TRISS (Table 1), nor was there a difference in TNFR levels between survivors and nonsurvivors. Levels of 55-kd TNFR decreased in six patients, remained the same in two, and increased in one (Fig. 2). Overall, 55-kd TNFR levels showed a downward trend over time, decreasing in four patients, remaining about the same in four, and increasing in one. Overall, 75-kd TNFR levels did not have a predominant trend over time (Fig. 3).

In Vitro TNFR Bioactivity

Addition of patient sera increased cell viability in the presence of 50, 5, and 0.5 ng/mL TNF by 37% ± 15% (p < 0.05), 87% ± 19% (p < 0.01), and 60% ± 6% (p < 0.01), compared with cells cultured with TNF alone. Higher 55-kd TNFR levels correlated with higher cell survival in the presence of 5 ng/mL of TNF, p < 0.05, but this correlation was not significant with 50 or 0.5 ng/mL of TNF. There was no correlation between 75-kd TNFR levels and cell survival. Addition of control sera did not increase cell viability at any concentration of TNF.

DISCUSSION

Although animal studies have found increased TNF levels after hemorrhagic shock, to our knowledge no study has found elevated systemic TNF levels after trauma or hemorrhage in humans. We were unable to detect elevated systemic levels of TNF after trauma. However, high levels of soluble receptors for TNF were measured. The source of these soluble receptors is not clear. It is known that membrane expression of the 55-kd and 75-kd TNFR varies among different cell types. For example, epithelial cells express primarily 55-kd receptors, monocytes express primarily 75-kd receptors, and neutrophils have approximately equal concentrations of 55-kd and 75-kd receptors. Thus the 55-kd TNFR in traumatized patients may come from neutrophils or from injured organs such as the liver, pancreas, and intestinal tract. The 75-kd TNFR may be released by circulating or fixed monocytes or neutrophils. The mechanism or mechanisms of release for TNFR are not fully understood. However, in vitro activation of human neutrophils and monocytic THP-1 cells can result in cleavage of TNFR from their cellular membranes. In addition, TNF has been found to initiate release of...
TNFR both in vitro and in vivo. Thus the soluble TNF receptors found after trauma could arise from neutrophil or monocyte activation with release of membrane receptors, a response of local or systemic TNF release, or a combination of both.

Overall, 55-kd TNFR levels decreased over time, but there was no predominant trend in 75-kd TNFR levels (Fig. 3). Decreasing 55-kd TNFR levels may be because of either increased excretion or decreased production. It has been postulated that TNFR may serve as a carrier molecule to facilitate clearance or degradation of TNF, although this purported role for TNFR has been debated. Decreasing TNFR levels could also be evidence for decreasing TNF release or decreased leukocyte activation. The source of this downward trend in 55-kd TNFR levels cannot be determined from the present data, and further study is necessary. Interestingly, only the burned patient exhibited increasing 55-kd levels over time. This release of 55-kd TNFR may have been stimulated by continued cellular injury or increased neutrophil activation. Alternatively, increased TNF production could also trigger release of TNFR, although we were unable to demonstrate any increase in systemic TNF.

The presence of TNFR may interfere with our ability to accurately measure TNF, causing falsely low measurements. Cytotoxicity assays for TNF may be inaccurate because of the ability of TNFR to bind to TNF and inhibit cell killing. Similarly, monoclonal ELISA may also be inaccurate in the presence of TNFR, which may bind to an epitope or epitopes detected by the antibody. In view of these facts, the type of assay used to measure TNF levels may be important; an ELISA using polyclonal antibodies is probably the most accurate. Our assay utilized both a polyclonal and a monoclonal antibody, perhaps interfering with our ability to measure TNF in the presence of TNFR. Local release of TNF, without elevation of systemic levels, remains a possibility. In addition, because the half-life of TNF is only 5 to 20 minutes, it is possible that TNF released immediately after trauma was cleared or degraded before our earliest measurements. The presence of high levels of TNF after trauma would be consistent with local or early release of TNF or both and could be indirect evidence that TNF was present after trauma, despite low measured levels.

The TNFR detected in trauma patients' sera was biologically active, significantly increasing in vitro cell survival in the presence of varying concentrations of TNF. In addition, there was a positive correlation between 55-kd TNFR levels and improved cell survival in the presence of 5 ng/mL TNF. The 55-kd TNFR levels did not correlate with cell survival in the presence of either 50 ng/mL or 0.5 ng/mL TNF, suggesting that the biologic effects of TNFR may be less effective at very high and very low concentrations of TNF. The 75-kd TNFR levels did not correlate with cell survival at any TNF concentration, perhaps indicating that 75-kd TNFR was inactive, or less effective than 55-kd TNFR in inhibiting TNF cytotoxicity. There is evidence that the 55-kd TNFR is responsible for mediating TNF cytotoxicity, while the 75-kd TNFR may mediate other TNF activities. Although the TNFR was protective for TNF cytotoxicity in vitro we were unable to demonstrate a protective role in vivo. Severity of injury was likely the determining factor for mortality in this study; it is possible that TNFR may be beneficial in less severely injured patients.

Tumor necrosis factor receptors have been effective in ameliorating the effects of TNF in animal studies of septic shock: 20 µg TNFR-IgG (55 kd) administered to mice before an LD100 dose of endotoxin/LPS resulted in 100% survival at 72 hours. The TNFR-IgG continued to have a protective effect, although partial, up to 3 hours after administration of endotoxin/LPS. Although much further study needs to be done, TNFR could prove to have a beneficial role in trauma and hemorrhagic shock as well.

Acknowledgments

We thank Dr. Tetsuya Gatanaga for his helpful suggestions as well as Maki Gatanaga and Gary Kaml for their excellent technical assistance. We also wish to thank Synergen, Boulder, Colorado, for their gift of human r55-kd TNFR and r75-kd TNFR.

REFERENCES


DISCUSSION

Dr. C. Michael M. Dunham (Baltimore, Maryland): This important study by Doctor Tan suggests that tumor necrosis factor (TNF) may be released as a result of tissue injury and shock. However, this deduction is not conclusive and the issues are rather complex.

Tumor necrosis factor elicits a response from target cells through a multiplicity of complicated events. First, TNF cell membrane receptors are essential to invoke a biologic response and are under the control of numerous regulatory processes. Second, TNF-soluble receptors influence the availability of TNF to interact with its cell membrane receptors. However, this again is completely understood. Finally, the cellular response to TNF is influenced by many host receptor signal transduction regulatory factors. These probably include activation of genetic transcription, an array of protein kinases, G proteins and eicosanoids, phospholipase A2, lysosomal enzymes, reactive oxygen species, phospholipase C, and cyclic ADP. Also, other cytokines can effect TNF cell membrane receptors and the diverse intracellular signalling molecules just mentioned. It is important to realize that TNF in its interactions with other mediators may cause adaptive or destructive cellular tissue or host biologic responses.

I think until there is a better understanding of the biologic host net effect of mediators such as TNF and we can determine whether the response varies over time or from individual to individual, we need to pursue additional investigations with our molecular biology colleagues before we are ready to advance specific new therapeutic strategies.

I have a couple of questions for Doctor Tan. Could you further elaborate on the potential of the TNF receptors to interfere with the measurement of serum TNF concentration? Are there further remedies where one may be able to measure TNF that is bound to the soluble receptors, so we can get some idea whether indeed serum TNF is in reality elevated?

When you are measuring TNF soluble receptor concentration in the serum, does the analysis include receptors bound to TNF as well as those not bound to TNF? And as a follow-up to that, regarding the cytotoxicity assays, is it clear that there are free TNF receptors in the trauma patient serum available to bind TNF in the cell serum and TNF admixture, in other words, if the presence of TNF receptors not bound to TNF in the patient serum would enhance the likelihood that indeed the receptors were responsible for the enhanced cell viability?

Dr. Kenneth Waxman (Closing): Doctor Dunham, thank you for your comments. We also think there are important data. However, we do not have a complete explanation of the biologic significance of the soluble receptors.

Your first question was whether the soluble TNF receptors in the serum had the potential to interfere with the measurement of TNF concentration; we think that they do. The solution to this problem is not clear. Polyclonal ELISA assays, instead of monoclonal assays, might perhaps better measure TNF concentrations in the presence of receptors. We and others are looking at this issue.

We also do not know whether the ELISA assays measure just free TNF, free TNF bound to receptor, or both. That is unknown. This too is an active area of investigation. We also cannot answer whether the inhibition of TNF cytotoxicity in cell cultures is from bound or free TNF receptors.

I might add that there are soluble receptors not just to TNF, but to many, if not all, of the cytokines. There is active work being done with a soluble receptor for interleukin-1, and for IL-6, and an active search for IL-8 receptor. It is very possible that all of these soluble receptors are important in mediating the biologic response to trauma.