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Decreased levels of perforin-positive lymphocytes are associated with posttraumatic complications in patients with major trauma

Rong Hua [a,*], Yongmei Zhang [b,*], Fuxing Chen [c], Zhonghai Zhou [c], Xi Li [d], Boming Shao [b], Shangjing Wang [a], Yujing Zhang [d], Xiaoting Lv [c]

[a] Department of Emergency Medicine, The 97th Hospital of PLA, Xuzhou 221000, China
[b] Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical College, Xuzhou 221000, China
[c] Department of Central Laboratory, The 97th Hospital of PLA, Xuzhou 221000, China
[d] Department of Trauma Central, The 97th Hospital of PLA, Xuzhou 221000, China

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ABSTRACT

Objective: Posttraumatic immune disorder can cause complications including systemic inflammatory response syndrome (SIRS) and multiple-organ dysfunction syndrome (MODS). Cytotoxic granules containing perforin and granzyme-B (GrB) are released by cytotoxic $CD8^+$ T lymphocytes, NK and γδT cells after major trauma. This prospective clinical study was designed to analyze the association between these immune components and complications after major trauma.

Methods: We retrospectively studied 48 patients aged between 16 and 65 years admitted within 90 min of major trauma (Injury Severity Score > 16) and surviving beyond 7 days, and 20 healthy controls. Blood samples were drawn on admission and after 1, 3 and 7 days. $CD8^+$ T, NK and γδT cell counts in peripheral blood and the levels of perforin and GrB in these cells were analyzed by flow cytometry. Clinical aspects of MODS and SIRS were recorded daily.

Results: $CD8^+$ T cell counts were not significantly different in patients with SIRS or uncomplicated group, but were depressed in the MODS group after trauma. However, NK cell counts in patients with MODS were significantly depressed only at day 7 after injury, and γδT cell counts were significantly depressed after trauma. Perforin levels in $CD8^+$ T, NK and γδT cells in patients with MODS were depressed after trauma. GrB levels in NK, $CD8^+$ T and γδT cells in patients with MODS were significantly depressed at 3 and 7 days post trauma.

Conclusion: Posttraumatic MODS is associated with early, sustained, and severe depression of lymphocytes.

Introduction

Major traumatic injury is the leading cause of death and morbidity in patients younger than 45 internationally [1,2], and subsequent posttraumatic multiple organ dysfunction syndrome (MODS) is the primary cause of morbidity [2]. Posttraumatic MODS remains a frequent concern despite enormous technical progress in organ-supportive therapy [2].

A multitude of immunological alterations are involved in the development of MODS after major trauma [3,4]. Cytotoxic $CD8^+$ T lymphocytes (CTLs), NK and γδT cells exert their biological activity through two distinct mechanisms [5–7]: synthesis and release of soluble mediators such as cytokines, and release of cytotoxic granules inducing apoptosis in neighbouring cells [8,9]. The density of granules and their content in cytotoxic mediators such as perforin and granzymes in CTLs, NK cells, and γδT cells might indicate the potential activity of these cells [10], and overall status of host cellular immunity [11].

The main role of perforin is to permeabilise target membranes to allow entry of granzymes such as granzyme-B (GrB) [12,13]. GrB then triggers an apoptotic caspase cascade [14,15]. Downregulation of perforin lymphocyte subsets following trauma is associated with unfavourable outcomes [16], and GrB-deficient individuals are more susceptible to LPS-induced toxic shock [5]. However, the relationship between the content of cytotoxic granules and the incidence of complications after major trauma remains to be determined.

In this study, we measured the perforin and GrB content of CTLs, NK cells and γδT cells in the peripheral blood of severe trauma

* Corresponding authors. Tel.: +86 0516 83349121; fax: +86 21 64085875.
E-mail address: HuarongSCI@163.com (R. Hua).

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patients at different time points after the onset of trauma in order to test the hypothesis that decreased cytotoxic granule loading was associated with complications after major trauma.

Patients and methods

Subjects

We retrospectively studied the medical records of 48 adult patients with major trauma (diagnosed with Injury Severity Score [ISS] $\geq$ 16 [17]) who reached the emergency department within 90 min of traumatic event. Patients were admitted to the Emergency Department and the Department of Trauma Center in the 97th Military Hospital between January 2009 and January 2013. The present study is a supplementary analysis using the samples and data of another study. The study was approved by the Ethics Committee of the 97th Military Hospital, and informed consent was originally obtained from all patients or the patients' legal representative, as well as their permission to use data and samples for further analyses. Patients with penetrating injuries, isolated brain jury, immunological or chronic inflammatory disease, viral or intracellular parasitic infections, cancer, and those who died within 7 days post trauma were excluded.

Treatments and monitoring

After the initial resuscitation and primary operative treatment in accordance with the standard of care, patients were admitted to the surgical intensive care unit. Central venous pressure, arterial blood pressure, haemodynamic parameters, body temperature, hepatic function and renal function were monitored. Haemoglobin, hematocrit, absolute leukocytes counts, potassium, sodium, glucose levels, acid–base parameters, blood gases in arterial were also monitored. Standard demographic, laboratory, and clinical data were extracted from a prospectively collected database. Clinical events were recorded for 7 days. Based on previous studies regarding the kinetic of the immune response in trauma patients [18,19], 20-ml peripheral blood samples were collected on admission, within 4 h after trauma onset (day 0) and on the following 1st, 3rd and 7th days.

Grouping

Patients with a Marshall score $\geq$ 4 [20] were diagnosed with MODS (MODS group, MG). Systemic inflammatory response syndrome (SIRS) (SIRS group, SG) was diagnosed in patients fulfilling at least two of the following criteria: temperature <36 °C or >38 °C; heart rate >90/min; tachypnea (>20 breaths/min) and/or $PaCO_2$ <32 mmHg and/or mechanical ventilation; and leukocytosis (>12,000/μL) or leukopenia (<4000/μL) and/or juvenile neutrophil granulocytes $\geq$10% [21]. Patients with a Marshall score <4 and no complications were defined as "without SIRS criteria" (uncomplicated group, UG). Blood samples were collected from 20 healthy volunteers (15 males, 5 females) who underwent routine examination at the hospital as control subjects (control group, CG). Controls' written informed consent was obtained before sample collection.

Flow cytometry detection of CTLs, NK cells and γδT cell counts, perforin and GrB in lymphocytes

The lymphocyte fraction in peripheral blood (CTLs, NK cells and γδT) and the lymphocyte fraction staining positive for perforin and GrB were measured by flow cytometry. Monoclonal antibodies directed towards human antigens were purchased from BD Biosciences (ShangHai, China): CD3-PerCP (clone SK7), CD8-APC (clone SK1), CD107a-FITC (clone H4A3), CD56-APC (clone NCAM16.2), γδTCR-APC (clone B1), GrB-PE (clone GB11), and perforin-FITC (clone δG9). IgG isotypes were used as staining controls for each antibody isotype/fluorochrome combination. For the staining of cell-surface antigens, 50 μL of well-mixed, anticoagulated whole blood was incubated with fluorochrome-conjugated monoclonal antibodies for 15 min in the dark at room temperature.

For cell-surface immunophenotypic analysis, red blood cells (RBC) were lysed with BD FACS Lysing Solution (BD Biosciences, Shanghai, China) and removed. Intracellular staining was performed with ADG FIX&PERM kits (AN DER GRUB Bio Research GmbH, Kaumberg, Austria), according to the manufacturer's instructions after cell-surface staining. Data were acquired on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using the CellQuest Pro software (BD Biosciences, San Jose, CA, USA). Gating strategy: first, lymphocytes were identified based on their characteristic properties shown in the forward scatter (FSC) and sideward scatter (SSC). Then, a representative gating was set for $CD3^+CD8^+$ (for CTLs) or $CD3^-CD56^+$ (for NK cells) or $\gamma\delta TCR^+$ (for γδT cells) cells from lymphocytes. Finally, the expression of perforin, GrB and CD107a were analyzed.

Statistical analysis

All values are expressed as means ± SD. Differences in quantitative variables were analyzed by the Mann–Whitney *U* test when comparing two groups, and by the ANOVA test when comparing multiple groups. Non-parametric data were subjected to the Chi-square test or Fisher's exact test. Pearson's rank correlation coefficient (*r*) was calculated to detect significant relationships between percentage of leukocytes subsets and values of perforin and GrB, ISS scores, APACHE II scores and volume of blood transfusion. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics of patients

Twelve patients were diagnosed with MODS, 14 with SIRS, and 22 were without complications. Detailed types of trauma are shown in Table 1.

As shown in Table 2, age and gender did not differ between the groups. The volume of crystalloid fluid administered to patients in the MODS group (MG, 1.17 ± 0.38 L) was higher than in UG (0.78 ± 0.27 L) or SG (0.88 ± 0.38 L) ($P < 0.05$). In initial resuscitation and primary operative treatment, the volume of crystalloid fluid administered to MG and SG patients (0.45 ± 0.10 L and 0.42 ± 0.12 L, respectively) were higher than in UG (0.35 ± 0.07 L, both $P < 0.05$).

The volume of packed red blood cells and fresh-frozen plasma administered to MG (1.80 ± 1.08 L and 1.57 ± 0.75 L, respectively)

Table 1
Types of trauma (*n*).

	MG (*n* = 12)	SG (*n* = 14)	UG (*n* = 22)
Head, chest, extremities	4	3	3
Head, chest, abdomen	3	3	4
Chest, abdomen, extremities	2	3	5
Chest, abdomen, pelvis	2	3	4
Abdomen, extremities, pelvis	1	1	4
Abdomen, pelvis, spine	0	1	2

MG, MODS group; SG, SIRS group; UG, uncomplicated group.

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