Injury, Int. J. Care Injured 48S5 (2017) S1–S7

Contents lists available at ScienceDirect

Injury

journal homepage: www.elsevier.com/locate/Injury

Frequency and perforin expression of different lymphocyte subpopulations in patients with lower limb fracture and thoracic injury

Nikola Grzalja[a], Tedi Cicvaric[a], Danijel Knezevic[b], Janja Kuharic[b], Alan Sustic[b], Bore Bakota[c], Simona Komen[a], Vlatka Sotosek Tokmadzic[b,*]

[a]Department of Surgery, Medical Faculty, University of Rijeka, Brace Branchetta 20, 51 000 Rijeka, Croatia
[b]Department of Anaesthesiology, Reanimatology and Intensive Care, Medical Faculty, University of Rijeka, Brace Branchetta 20, 51 000 Rijeka, Croatia
[c]Trauma and Orthopaedic Department, Brighton and Sussex University Hospital NHS Trust, Eastern Road, Brighton BN2 5BE, UK

KEYWORDS

lower limb fracture
thoracic injury
perforin
T lymphocytes
NK cells
NK T cells

ABSTRACT

Introduction: Trauma with multiple injuries is associated with a high risk of complications, which may be related to excessive stimulation of inflammatory and anti-inflammatory responses. Although the effects of polytrauma on the immune response have been well established at the cellular and molecular levels, there is little information about the changes in the cytolytic potential of immunocompetent cells, including expression of cytotoxic molecules such as perforin. Therefore, the objective of the present study was to analyse and compare differences in the frequency and perforin expression of leukocyte subpopulations in the peripheral blood of patients with lower limb fracture, thoracic injury, and simultaneous lower limb fracture and thoracic injury.
Patients and Methods: Forty-five patients with trauma injury (15 patients with lower limb injury, 15 patients with thoracic injury, and 15 patients with simultaneous lower limb and thoracic injury) were included in the study. Peripheral blood of 15 sex- and age-matched healthy volunteers served as the control group. Peripheral blood samples were taken from all subjects included in the study and peripheral blood mononuclear cells were isolated by gradient centrifugation. The frequency of T lymphocytes, natural killer (NK) and NK T cells, and their subsets, as well as their perforin expression levels were simultaneously detected and analysed by flow cytometry.
Results: There was a statistically significant decrease in the frequency of T lymphocytes, NK and NK T cells as well as perforin expression in the patients with simultaneous lower limb and thoracic injury compared with the other two groups, with a predominantly marked decrease in NK and NK T cells.
Conclusion: The decrease in the frequency and cytotoxic potential of peripheral blood lymphocytes is related to the severity of trauma injury, which can explain the underlying mechanism contributing to complication occurrence.

Introduction

Trauma is a leading cause of death in industrialised countries for the population under 45 years of age [1,2]. Patients with multiple body injuries account for 3% of the total number of injured patients, and have a relatively high mortality rate of 16–22% [3]. The direct and indirect annual financial costs of medical treatment for patients with multiple injuries are huge and amount to hundreds of billions of Euros [1,4]. Among all patients with multiple injuries, 86% have limb injuries, 69% have head injuries, and 62% have chest injuries. The effects of combined injuries are not comparable with those endured after an isolated injury [1–5].

Patients with multiple body injuries usually die at the scene from a serious brain injury or massive bleeding as a result of injury to the heart or great vessels. In contrast, the hospital death rate of patients with multiple injuries is frequently associated with the development of intensive systemic inflammatory response syndrome (SIRS) and subsequent systematic changes, including damage to the structure and functions of the physiological barriers and reduction in the defence capabilities, eventually leading to multiple organ dysfunction syndrome (MODS) and multiple organ failure [1,6,7].

The inflammatory response improves the potential for body healing and is required for tissue repair and is ubiquitous in all mammals. In patients with isolated, uncomplicated injuries, the inflammatory response is temporary, predictable and well-balanced between proinflammatory and anti-inflammatory factors, whereas in patients with

* Corresponding author: Department of Anaesthesiology, Reanimatology and Intensive Care, Medical Faculty, University of Rijeka, B. Branchetta 20/1, 51 000 Rijeka, Croatia, Phone: +385-51-407-400, Fax: +385-51-218-407.
E-mail: vlatkast@medri.uniri.hr (V. Sotosek Tokmadzic)

0020-1383

multiple injuries, the inflammatory response is markedly enhanced and can lead to the development of SIRS [7,8]. With the development of MODS, the mortality rate of patients with multiple body injuries increases to 50–80% [9–12]. When there is an excessive inflammatory response, an anti-inflammatory response is activated in an attempt to maintain body balance. However, such an exaggerated inflammatory response can lead to the development of compensatory anti-inflammatory response syndrome (CARS) or mixed antagonistic response syndrome (MARS). In the latter case, pro-inflammatory and anti-inflammatory responses counterbalance, and when accompanied by a state of immune paralysis, the organism is not able to react appropriately to a new threat (e.g. infection) due to changes in the mechanism of cellular immunity [2,9,13]. The resulting infection can cause serious complications, such as sepsis and septic shock with subsequent multiple organ failure [14]. The response to injury varies according to the type and severity of injury, as well as with respect to individual characteristics such as age, sex, and genetic features. These variables have made it challenging to study the impact of trauma on the immune system [15]. Numerous studies have established the basic factors contributing to the occurrence of SIRS, CAR and MARS after trauma.

The main objective of these previous studies was to gain a better understanding of the cellular and molecular mechanisms responsible for the development of post-traumatic syndromes [16,17]. Research related to the impact of trauma on the innate and acquired immune systems has shown that trauma stimulates the inflammatory and anti-inflammatory responses. It is generally believed that these changes lead to alterations in the phenotype and function of the acquired immune system, which in turn disrupt the homeostasis of the immune system to expose traumatised patients to a greater risk of opportunistic infections and complications [15,18]. Despite numerous published papers about the changes in the immune response after polytrauma at the cellular and molecular levels, very little is known about the changes in the cytolytic potential of the immunocompetent cells, including expression of cytotoxic molecules such as perforin, granulysin, Fas ligand (FasL), and tumour necrosis factor apoptosis-induced ligand. Natural killer (NK) cells, T lymphocytes, and NK T cells can exert cytotoxic activity to target cells through a mechanism of necrosis or apoptosis. Necrosis is a process that begins by creating pores in the cell membrane, which enables penetration of water into the cell by an osmosis mechanism, resulting in swelling of the cell and its organelles and finally cell death [19–22]. Perforin, or cytolysin, is the main cytotoxic mediator that creates pores and causes necrosis of the cells. Perforin is a glycoprotein with a molecular weight of 66–70 kDa that shows functional homology with components of the lytic complement complex C5–C9. Consequently, there is a potential for perforin to be retained in the granules of the cytotoxic lymphocytes and to become excreted in response to a stimulus [19]. Once released in the immunological synapse, perforin monomers are incorporated into the membrane of target cells and create pores that enable the influx of extracellular fluid and apoptotic molecules (serine esterases, FasL, granulysin) in the target cell [23].

The aim of this study was to analyse and compare the frequency of T lymphocytes, NK and NK T cells and their perforin expression in the peripheral blood of patients with lower limb fracture, thoracic injury, and simultaneous lower limb fracture and thoracic injury.

Patients and methods

Patients

Fifteen patients with lower limb fracture (Group 1), 15 patients with thoracic injury (Group 2), and 15 patients with simultaneous lower limb fracture and thoracic injury (Group 3), admitted to the Emergency Department of Clinical Hospital Rijeka, Croatia, were included in the study. Patients younger than 18 years and older than 65 years, patients with immunological or malignant disease, and those who had an acute infection at admission were excluded from the study. The study was approved by the local ethics committee of Clinical Hospital Centre, Rijeka, Croatia according to the World Medical Association criteria outlined in the declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects". All patients included in the study signed informed consent forms. Twenty healthy sex- and aged-matched volunteers participated in the study as control subjects (Group 0). A total of 20 mL of peripheral blood was sampled from each subject at the time of admission and used for further immunological investigations. Demographic (sex, age) and clinical (injury severity, length of hospitalisation, incidence of infections and complications) data were also recorded for each patient. Injury severity was numerically expressed by the Injury Severity Score (ISS), the most commonly used scale for the anatomical definition of multiple injuries, which is based on indexing the individual anatomical injuries with the Abbreviated Injury Scale, in which the number indicates the injury severity of the isolated body region or organ [24,25].

Isolation of peripheral blood mononuclear cells

A total of 20 mL of peripheral blood was sampled from each patient included in the study and was overlaid onto Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 600 × *g* for 20 minutes. The overlaid cells were collected after centrifugation, washed twice in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Auckland, NZ) and used for further experiments. The viability of the cells was assessed with 0.5 μg/mL propidium iodide per 10^6 cells (Sigma-Aldrich Chemicals, St. Louis, MO, USA) and flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA) analysis, and was over 95%.

Cell surface and intracellular labelling of peripheral blood mononuclear cells

The intracellular and surface labelling methods are described in detail elsewhere [26]. In brief, after isolation, peripheral blood mononuclear cells (2×10^5/sample) were incubated with 10% heat-inactivated human AB serum for 20 minutes at room temperature (RT) to block non-specific Fc receptor binding. After incubation with AB serum, the cells were washed in fluorescence-activated cell sorter (FACS) buffer (140 mM NaCl, 1.9 mM KH_2PO_4, 16.5 mM Na_2HPO_4, 3.75 mM KCl [all from Kemika, Zagreb, Croatia], 0.96 mM Na_2-EDTA [Fluka, Buchs, Switzerland], 1.5 mM NaN_3 [Difco, Detroit, Michigan]) and were fixed with 4% paraformaldehyde (pH 7.4) for 10 minutes at RT. The cells were then washed twice in FACS buffer and permeabilised with saponin buffer (0.1% saponin, Sigma, Poole, Dorset, UK), 2% goat serum in phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 2.87 g $Na_2HPO_4 \cdot 12H_2O$, and 0.2 g KH_2PO_4 [all from Kemika] dissolved in 1 L of distilled water), for 20 minutes at room temperature. Mouse δG9 IgG2b anti-perforin monoclonal antibody or isotype-matched control (mouse MA-21, IgG2b, Biosciences Erembodegen, Belgium) directly conjugated with fluorescein isothiocyanate (FITC) was added to an appropriate cell sample (3 μg diluted in 200 μL saponin buffer) and incubated for 30 minutes at 4°C. After incubation, the samples were washed twice in saponin buffer followed by resuspension in 1 mL FACS buffer. For surface labelling, the combination of CyCrome phycoerithryn-5 (Cy-PE5)-conjugated anti-CD3 (mouse UCHT1, IgG1) and phycoerithryn (PE)-conjugated anti-CD4 monoclonal antibody (mouse RPA-T4, IgG1), Cy-Pe5-conjugated anti-CD3 and PE-conjugated anti-CD8 (mouse RPA-T8, IgG1), or Cy-Pe5-conjugated anti-CD3 and PE-conjugated anti-CD56 (mouse B159, IgG1) were used. Directly conjugated FITC, PE or CY-PE5 mouse isotype-matched antibodies were used as controls. All antibodies were purchased from eBioscience (Erembodegen, San Diego, CS, USA). Labelled samples were fixed in