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The prognostic value of leukocyte apoptosis in patients with severe sepsis at the emergency department

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ABSTRACT

Background and aim: Cell apoptosis in critically ill patients plays a pivotal role in the pathogenesis of sepsis. This study aimed to determine the prognostic value of leukocyte apoptosis in patients with severe sepsis.
Methods: Leukocyte apoptosis was determined by flow cytometry. The values of annexin V, APO2.7, and 7-amino-actinomycin D (7AAD) for each subtype of leukocyte were analyzed in 87 patients with severe sepsis and 27 controls.
Results: The percentages of apoptosis (APO2.7 [%]) in the leukocyte subsets were significantly higher in the patients with severe sepsis than in the controls. The percentages of APO2.7 in leukocyte apoptosis, APO2.7 in lymphocytes apoptosis, and annexin V + 7AAD in monocytes apoptosis were significantly higher in non-survivors than in survivors. Levels of APO2.7 in lymphocytes apoptosis, annexin V + 7AAD in monocytes apoptosis, and serum lactate were all independently predictive of mortality.
Conclusion: Leukocyte apoptosis is significantly higher in patients with severe sepsis. The percentages of late lymphocyte and monocyte apoptosis may be predictive of outcome in such patients. Aside from serum lactate, APO2.7 level in lymphocyte apoptosis is also a useful predictor of outcome on admission to the emergency department.

1. Introduction

Severe sepsis and sequential multiple organ failure are still the leading causes of death worldwide despite advances in medical care [1]. The pathogenesis of multiple organ failure in patients with severe sepsis is a multi-factorial process, but global tissue hypoxia due to an imbalance between systemic oxygen delivery and peripheral oxygen demand plays an important role [2,3]. There is growing evidence that aside from cellular necrosis, dysregulated leukocyte apoptosis may also influence the increasing duration and severity of systemic response to sepsis in critically ill patients, including those with acute respiratory distress syndrome, shock, and trauma [4–12].

Severe sepsis and septic shock represent an over-exuberant host response to an infectious challenge. Neutrophils, monocytes, and macrophages play key roles in the initial reaction and release a variety of cytokines that marshal the immune response. Once the adaptive immune response is awakened, innate immune cells are down-regulated and must be disposed of in a timely and non-injurious manner. Apoptosis represents a key mechanism in this orderly process, but it is not always good. The apoptotic cascade supervenes during the evolution of sepsis in lymphocytes, in tissue macrophages, and in intestinal epithelia and has been associated with organ dysfunction [13–15].

Moreover, the apoptosis of both alveolar epithelial cells and respiratory endothelial cells has been conclusively demonstrated in animals and humans with acute lung injury and acute respiratory distress syndrome [14]. Previous studies also reveal that lymphocyte apoptosis is rapidly increased in the blood of patients with septic shock and this leads to profound and persistent lymphopenia that is associated with poor outcome. The apoptosis of blood monocytes in septic patients is also associated with their final outcome [8,9].

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; 7-AAD, amino-actinomycin D; SOFA, sequential organ failure assessment; PBEF, pre-B cell colony-enhancing factor.

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Previous studies have used only a single method to study apoptosis [8,9,11] or only one leukocyte subset to predict the outcome of sepsis [8,9]. Furthermore, the timing of apoptosis is important and the duration of illness before enrolment is connected to outcome. If such variations follow a standard pattern and temporal relationship, prognostication will be substantially improved.

2. Methods

2.1. Study population and definition

This prospective study on patients with severe sepsis and septic shock was conducted over a 1-year period (January to December 2011). Eighty-seven adult non-traumatic and non-surgical patients at Kaohsiung Chang Gung Memorial Hospital were enrolled. The hospital's Institutional Review Committee on Human Research approved the study protocol and all of the patients provided written informed consent.

All patients aged >18 years consecutively admitted from the ED were screened daily for severe sepsis and septic shock according to the specific criteria defined by the American College of Chest Physicians/Society of Critical Care Medicine. These criteria included suspected or confirmed infection, two or more manifestations of systemic inflammatory response syndrome, and at least 1 sepsis-induced acute organ dysfunction. Patients who met all three criteria were included [16]. For comparison, 27 age- and sex-matched healthy volunteers who received annual physical check-up but without clinical evidence of infection were recruited as controls.

2.2. Clinical assessment and treatment

The medical records were prospectively recorded using pre-existing standardized evaluation forms that included demographic data and the Acute Physiology and Chronic Health Evaluation (APACHE) II score, which was calculated during the first 24 h of admission to assess the severity of organ dysfunction. Basic laboratory tests, lactate concentration, and inflammatory markers (i.e., plasma C-reactive protein and procalcitonin) were taken on ED admission. Data on the source of infection and use of antibiotics were also recorded.

The course of various organ dysfunctions and supportive treatments, including vasoactive and ventilator therapies and renal replacement therapies, were recorded. Physicians evaluated daily the association of existing organ dysfunction and severe sepsis. It was also institutional practice to consult an infectious disease specialist for anti-microbial treatment based on treatment guidelines for different infectious etiologies during the first 24 h.

2.3. Blood sampling and assessment of leukocyte apoptosis

Blood samples were collected on presentation to the ED (Day 1). Follow-up blood samples were obtained on Days 4 and 7 after admission. All blood samples were collected by venipuncture of forearm veins from both the study group and controls. All flow cytometry assays were performed within 1 h after blood extraction to ensure that the results were as close as possible to an *in vivo* situation.

Flow cytometry assay using APO 2.7 antibody for detecting apoptosis.

Fixed amounts of blood were diluted 1:5 with PBS, and 100 μL was stained with 10 μl of each of the following: fluorescence-conjugated monoclonal antibodies against CD45-phycoerythrin (PE)-Cy5 (clone J33), CD61-fluorescein isothiocyanate (FITC; clone SZ21), and APO 2.7-PE (clone 2.7A6A3; Immunotech). The samples were titrated at saturating concentrations. The CD45-PE-Cy5 antibody reacted with the CD45 family of trans-membrane glycoproteins, expressed on the surface of all human leukocytes, and a pan-leukocyte marker. The CD61-FITC antibody was a pan-platelet marker that reacted with the GPIIb/IIIa complex (fibrinogen receptor).

The APO 2.7-PE antibody reacted with a 38-kDa mitochondrial membrane protein (7A6 antigen), which was detectable on non-permeabilized cells in the late apoptotic state [17].

Annexin staining, relevant to early apoptosis, produced similar results but was rejected for questionable reliability under fixation conditions, with formaldehyde clearly biasing the staining results. Mouse immunoglobulin G-PE was a control for non-specific staining, but it did not differ from the APO2.7-PE signal on platelets. Thus, each subject was used as its own control without changing the sample tube. After 30 min of incubation in the dark at room temperature, the stained samples were diluted with 0.5 ml of FACSFlow (Becton Dickinson).

Flow cytometry was performed immediately after staining using an Epics XL flow cytometer (Beckman Coulter) and the CellQuest software. Five thousand CD45-PE-Cy5 + cells per sample were acquired in a combined forward and side scatters, and deep-red FL4 fluorescence (CD45-PE-Cy5) leukocyte gate. Another 5000 CD61-FITC + cells per sample were acquired in a combined forward and side scatters, and green FL1 fluorescence (CD61-FITC) platelet gate to define the negative control threshold for the measurement of apoptosis, so that each subject was its own control.

2.3.1. Annexin V-FITC 7-AAD Fluorescence-Activated Cell Analysis

Membrane phosphatidyl-serine was detected by annexin-V using a commercially available kit (Boehringer Mannheim). The PBS-washed leukocytes were incubated with annexin V-FITC and 7-amino-actinomycin D (7-AAD) for 15 min at room temperature according to the manufacturer's guidelines. Samples were transferred to 5-ml polypropylene tubes, diluted with 900 μl Hanks' balanced salt solution, and placed on ice before flow cytometry analysis. The samples were analyzed using an Epics XL flow cytometer (Beckman Coulter) and CellQuest software. Fifteen thousand events were counted per sample. Low-fluorescence debris was gated-out of the analysis. Leukocyte subtypes were identified according to their CD45 expression intensity and were divided into neutrophils, monocytes, and lymphocytes. From here on, white blood cells (WBC) represented total leukocytes.

Annexin V-FITC staining was identified in fluorescent-1 and 7-AAD staining in fluorescent-4. The cells were identified as follows: early apoptotic cells if they were positive for marker annexin V-FITC but negative for 7-AAD; late apoptotic cells if they were positive for annexin V-FITC and 7-AAD; dead cells if they were negative for annexin V-FITC but positive for 7-AAD; and viable cells if they were negative for annexin V-FITC and 7-AAD.

2.3.2. Apoptosis of lymphocytes

Fixed amounts of blood were diluted 1:5 with PBS and 100 μl was stained with 10 μl of each of the following: fluorescence-conjugated monoclonal antibodies against CD4-phycoerythrin (PE)-Cy5, CD19-fluorescein isothiocyanate (FITC), and CD8- phycoerythrin (PE). Each sample was further stained with annexin V-FITC, 7-amino-actinomycin D (7-AAD), or APO 2.7-PE (clone 2.7A6A3; Immunotech, Marseille, France) and titrated at saturating concentrations. The samples were then transferred to 5-ml polypropylene tubes, diluted with 900 μl of Hanks balanced salt solution, and placed on ice before flow cytometry analysis. The samples were analyzed using an Epics XL flow cytometer (Beckman Coulter) and CellQuest software. Fifteen thousand events were counted per sample. Lymphocyte sub-types were identified according to their surface antigen (i.e., CD4, CD8, or CD19) expression intensity. A database coordinator was responsible for monitoring all of the data collection and entry.

All of the tests were performed in a quality-controlled central laboratory at Chang-Gung Memorial Hospital. Concentrations of CRP were determined by enzyme immunoassay (EMIT; Merck Diagnostica), while PCT was measured using a time-resolved amplified cryptate emission technology assay (VIDAS; bioMerieux). Serum lactate levels were measured using a serum-based assay catalyzed by lactate oxidase (UniCel Integrated System; Beckman Coulter INS).