Flow cytometric analysis of total lymphocyte apoptosis, a potential prognostic assay for sepsis

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Abstract. Intensive research has identified the number of apoptotic lymphocytes as a reliable marker of sepsis. This study determined whether the number of apoptotic lymphocytes can be used as a prognostic marker of patients with severe sepsis. A prospective study comprising 30 patients was conducted with severe sepsis grouped according to 14-day mortality of 15 patients who each were survivors and nonsurvivors. The number of apoptotic lymphocytes was calculated using flow cytometry with a PerCP-labeled anti-CD45 monoclonal antibody, FITC-labeled Annexin V, and propidium iodide. Subjects characteristics were recorded, and the number of apoptotic lymphocytes was calculated. The mean percentages of apoptotic lymphocytes in the survivor and nonsurvivor groups were significantly different (0.992% [SD = 0.44%] and 1.5853% [SD = 0.57%], respectively, p = 0.004). The cutoff value of apoptotic lymphocytes for predicting prognosis was 0.97%, AUC = 0.791 (CI 95% 0.631–0.951), 86.7% sensitivity, and 60% specificity. Kaplan–Meier analysis yielded a hazard ratio of 0.182 (95% CI 0.041–0.814, p = 0.026). These data provide compelling evidence that the number of apoptotic lymphocytes in patients with severe sepsis predicts nonsurvivors and that this cutoff value can be used manage patients with severe sepsis.

1. Introduction

Sepsis is a clinical condition with high mortality rate, which is related to a patient’s response to severe infection. Sepsis affects one-third of patients treated in intensive care units [1]. The surviving sepsis campaign states that sepsis is a systemic inflammatory response syndrome (SIRS) to unproven or proven infections, which can progress to severe and septic shock [2,3]. Surveillance data from Cipto Mangunkusumo Hospital from January 2014 to July 2015 reveal that among 1963 patients who died, 1231 (62.7%) were treated for sepsis [4].

Hotckiss et al. suggested that 10% of lymphocytes undergo apoptosis in patients with sepsis, and Boomer et al. found that apoptosis of lymphocytes occurs from the first 24 h after the onset of sepsis, with a decrease in inflammation after seven days [5,6]. Markers associated with apoptosis were discovered by Bossy-Wetzel and Green when they found that phosphatidylserine (PS) residues on the outer portions of cell membranes of apoptotic cells can be detected using Annexin V. Cells that undergoing early apoptosis are positively stained by Annexin V and negatively stained with propidium iodide (PIs). While cells that undergoing late apoptosis or necrosis are stained by Annexin V and PIs [7]. In contrast, viable cells are not stained by Annexin V and PIs [8].
2. Methods

2.1. Study design
This was a prospective analysis of descriptive data to evaluate a prognostic test for sepsis. The study was conducted at the Clinical Pathology Department, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital in collaboration with the Internal Medicine Department of the Faculty of Medicine, Universitas Indonesia. The research was conducted between January and February 2016. The subjects included adult patients with severe sepsis who were treated in the Emergency Department and the Intensive Care Unit of the RSCM.

2.2. Sample analyses
This study analyzed 3 ml of venous blood taken from the cubital vein that was collected into tubes containing the anticoagulant K3EDTA. The inclusion criteria were as follows: ≥18 years of age with a diagnosis of severe sepsis lasting ≤72 h. (If the patient was referred from another hospital, the diagnosis of severe sepsis was established ≤72 h from the patient’s initial admission date.) The patient or the patient’s family agreed to submit to the research protocol and signed an informed consent agreement. We excluded patients with underlying disease that increased mortality or those infected with human immunodeficiency virus.

Consecutive sampling was performed and the size of the sample population was calculated using a hypothetical test sample formula based on the average numbers of two independent populations. The number of samples was 25, with 15 samples from the group of patients with sepsis who survived 14 days of treatment and 10 samples from the group of such patients who did not survive 14 days of treatment. The minimum sample size was 30, and each of the 15 samples represented survivors and nonsurvivors.

2.3. Diagnosis of sepsis
The diagnosis of sepsis was based on clinical features, laboratory results (number of leukocytes, urine analysis) and radiography (thorax radiograph), which were acquired from patients' medical records. Infections included pneumonia, urinary tract infection, intra-abdominal infections, central nervous system infections, and skin or soft tissue infections. The diagnosis of sepsis was based on a modification of the international sepsis definitions of the Society of Critical Care Medicine, European Society of Intensive Care Medicine, American College of Chest Physicians, American Thoracic Society, and Surgical Infection Society [16] as follows: 1. SIRS parameters. Body core temperature; fever >38.3 °C, hypothermia < 36 °C; heart rate >90 beats/min; breathing frequency >30 breaths/min; leukocytosis, leukocytes >12,000/µL; or leukopenia, leukocytes < 4,000/µL; or normal leukocyte counts with >10% immature forms; plasma C-reactive protein (CRP) >5 mg/L; and plasma procalcitonin >0.05 ng/mL. 2. Organ dysfunction parameters. Arterial hypoxemia (P_{A}O_{2}/FiO_{2} < 300); acute oliguria, urine output 0.5 ml/kg/h or 45 mmol/L for ≥2 h, despite adequate fluid resuscitation; creatinine increase >0.5 mg/dL; coagulation abnormalities, international normalized ratio >1.5 or activated partial thromboplastin time >60 s; ileus, negative bowel sounds; thrombocytopenia, platelet count < 100,000/µL; hyperbilirubinemia, total plasma bilirubin >4 mg/dL or 70 mmol/L; hyperlactemia >3 mmol/L; blood pressure, hypotension with systolic blood pressure (SBP) < 90 mmHg or decreased SBP >40 mmHg; and changes in mental status (Glasgow Coma Scale < 15). Severe sepsis was suspected if the assays were positive for two SIRS parameters and two organ dysfunction parameters.

2.4. Flow cytometric detection of apoptotic lymphocytes
Annexin V fluorescein isothiocyanate (FITC)-PI staining. The structure of the membrane of an apoptotic cell changes so that the PS in the inner bilayer membrane is exposed on the cell surface and can bind Annexin V, which is detected by the FITC reporter [9]. During late apoptosis and in cells undergoing necrosis that is caused by the disruption of the integrity of the cell membrane, PI enters the cells and stains the DNA [10]. The analysis was performed using BD CellQuest PRO software. The population of lymphocytes was identified by gating (R1) the side-scatter vs CD45. Lymphocyte populations were identified as those with low SSC and high CD45 values (Figure 1).
Figure 1. Lymphocyte populations.

The patterns of Annexin V and PI staining of these lymphocyte populations were then analyzed. The percentages of apoptotic lymphocytes in quadrants II and IV were calculated using QuadrantStat provided with CellQuest Pro (Figure 2).

Figure 2. Percentages of apoptotic lymphocytes.

3. Results
3.1. Subjects' characteristics
Four people were excluded from this study because they were released before the 14-day treatment period and could not be contacted thereafter. Subjects' characteristics are summarized in Table 1.

Table 1. Subjects' characteristics

| Characteristics                  | Survivors | Nonsurvivors | Total  |
|----------------------------------|-----------|--------------|--------|
| Sex, n (%)                       |           |              |        |
| Men                              | 8 (26.7)  | 6 (20)       | 14 (46.7) |
| Women                            | 7 (23.3)  | 9 (30)       | 16 (53.3) |
| Age, mean (SD)                   | 44.8 (17.1)| 54 (13.9)    | 49.4 (16) |
| DM, n (%)                        |           |              |        |
| Yes                              | 1 (3.3)   | 3 (10)       | 4 (13.3) |
| No                               | 14 (46.7) | 12 (40)      | 26 (86.7) |
| Infection source, n (%)          |           |              |        |
| Pneumonia                        | 9 (30)    | 9 (30)       | 18 (60) |
| Digestive tract                  | 1 (3.3)   | 4 (13.8)     | 5 (16.7) |
| Central nervous system           | 1 (3.3)   | 1 (3.3)      | 2 (6.7) |
| Skin and soft tissue             | 3 (10)    | 1 (3.3)      | 4 (13.3) |
| Urinal tract                     | 1 (3.3)   | 0 (0)        | 1 (3.3) |
| Age >65 years, n (%)             |           |              |        |
| Yes                              | 2 (6.7)   | 3 (10)       | 5 (16.7) |
| No                               | 13 (43.3) | 12 (40)      | 25 (83.3) |
| Malignancy, n (%)                |           |              |        |
| Present                          | 4 (13.3)  | 3 (10)       | 7 (23.3) |
| Not present                      | 11 (36.7) | 12 (40)      | 23 (76.7) |

SD = standard deviation.
3.2. Determination of lymphocyte apoptosis
The Shapiro–Wilk test of normality was performed to evaluate the percentage of apoptotic lymphocytes of each group. The \( p \) values of normally distributed data for the survivors and nonsurvivors were 0.445 and 0.127, respectively. The mean numbers of apoptotic lymphocytes of the survivor and nonsurvivor groups were 0.992\% (standard deviation [SD] = 0.44\%) and 1.5853\% (SD = 0.57\% \( p = 0.004 \)).

3.3. Determination of the cutoff value of lymphocyte apoptosis
To determine the cutoff value of lymphocyte apoptosis for predicting the prognosis of patients with sepsis, we performed receiver operating characteristic curve analysis. The area under the curve (AUC) was 0.791 (95\% confidence interval [CI] 0.631–0.951). The ROC curve shows cutoff values of the number of apoptotic lymphocytes with prognostic significance. The cutoff value of 0.97\% was selected based on sensitivity, specificity, and AUC values of 86.7\% (95\% CI 62.12–96.26), 60\% (95\% CI 35.75–80.18), and 0.791 (95\% CI 0.631–0.951), respectively.

3.4. Kaplan–Meier analysis
Kaplan–Meier analysis and hazard ratio (HR) calculations using values higher and lower than the cutoff of 0.97\% for the sepsis group was performed. The Kaplan–Meier analysis fulfilled the proportional hazards assumption, which means that the incidence of deaths between groups was not significantly different (HR 0.182 (95\% CI 0.041–0.814), \( p = 0.026 \)).

3.5. External variables
The Bivariate analysis did not reveal a significant association of survival with age or malignancies (\( p = 1 \)).

4. Discussion
The subject population of the present study included 53.3\% women and 46.7\% men. In contrast, studies by Tamayo E et al. and Schroeder et al. included 65\% and 70\% men, respectively [11,12]. The mean number of subjects studied here was 49.4 ± 16 compared with 68.3 ± 16 and 59 (30–76) studied by Tamayo E et al. and Schroeder et al. respectively [11,12]. In the present study, the percentage of subjects with diabetes mellitus was 13.3\% compared with those reported by Zhao et al. and Marino et al. (25.6\% and 34.7\%, respectively) [13,14]. The percentage of elderly subjects in the present study was 16.7\% compared with 70.1\% studied by Zhao et al. [13]. The percentages of subjects with malignancies were 23.3\%, 24.6\%, and 12.9\% in the present study, Zhao et al. and Marino et al. respectively [13,14]. The differences are likely explained by the numbers of test samples as follows: 30, 501, and 101 in the present study, Zhao et al, and Marino et al [13,14]. The percentages of patients with pneumonia, which was the most frequent infection, were 60\% and 43.4\% in the present study and the study of Wang et al. respectively [15].

The average numbers of apoptotic lymphocytes apoptosis were 1.29 ± 0.58 and 34.4 ± 1.6\% in the present study and that of Schroeder et al. respectively [12]. Schroeder et al. stimulated lymphocytes with a monoclonal antibody against CD-3 (OKT-3) [12], which increased the binding of Annexin V to apoptotic lymphocytes through an unknown mechanism. The results of select studies of apoptosis in patients with sepsis are summarized in Table 2.

Table 2. Selected studies of the association of apoptotic lymphocytes and sepsis.

| Reference       | Location   | n  | Cells                  | Marker                  | Mortality | Apoptosis (%) |
|-----------------|------------|----|------------------------|-------------------------|-----------|---------------|
| Tamayo et al.   | Spain      | 80 | Neutrophils            | Anexin V and 7-AAD      | 28 days   | 14.8 ± 13.4\% |
| Schroeder et al.| Germany    | 10 | Lymphocytes (stimulated by OKT-3) | Annexin V and PI         | -         | 34.4 ± 1.6\%  |
| Present study   | Indonesia  | 30 | Lymphocytes            | Annexin V and PI        | 14 days   | 1.29 ± 0.58\% |


A HR of 0.182 may define patients with < 0.97% apoptotic lymphocytes as those with a probability of death that is 82% lower compared with that of patients with severe sepsis with ≥0.97% apoptotic lymphocytes. Here we show that patients with sepsis with < 0.97% apoptotic lymphocytes survived for an average 12.8 days compared with 8.3 days for such patients with an apoptotic lymphocyte count ≥0.97%.

5. Conclusions
It is concluded that flow cytometric analysis of apoptotic lymphocytes in patients with severe sepsis can serve as a predictor of prognosis. The number of apoptotic lymphocytes in severe sepsis patients can be used to predict nonsurvivors based on 14-day mortality, with moderate AUC. The apoptotic lymphocytes cut-off value of 0.97% can be used as a cut-off for severe sepsis patient management.

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