The Apoptosis of Blood Polymorphonuclear Leukocytes in Sickle Cell Disease

Hakan Ozdogu,1 Can Boga,1* Oktay Sozer,1 Nurzen Sezgin,2 Ebru Kizilkilic,1 Erkan Maytalman,1 and Defne Yalcintas1

1Department of Hematology, Faculty of Medicine, Baskent University, Ankara, Turkey
2Department of Biochemistry, Faculty of Medicine, Baskent University, Ankara, Turkey

Background: The apoptosis of human polymorphonuclear leukocytes (PMNs) in patients with sickle cell disease (SCD) is not well understood. The goal of this study was to examine the apoptosis of PMNs in patients with SCD and in controls.

Methods: Flow cytometric quantitation of PMN apoptosis was performed in 17 patients during and after sickle cell vasoocclusive crisis and in 17 healthy volunteers. Plasma nitric oxide concentrations were also measured in patients with SCD.

Results: The mean of annexin-V and annexin-V/PI staining (early and late apoptotic cells) increased to a greater degree in patients with SCD than in healthy controls for patients with SCD during and after vasoocclusive crisis. The mean of PI staining showing dead cells was higher only in patients after SCD crisis than in healthy controls. In the SCD groups during and after vasoocclusive crisis, there was no difference between PMN apoptosis levels. Furthermore, plasma nitric oxide concentrations were not correlated with PMN apoptosis.

Conclusions: There was an evidence that the alteration of blood PMN apoptosis could contribute to the pathogenetic mechanisms of vasoocclusion in patients with SCD. This can be attributed to the effects of numerous inflammatory mediators rather than simply the effects of nitric oxide.

Key terms: sickle cell disease; polymorphonuclear leukocytes; apoptosis; nitric oxide; flow cytometry

Sickle cell disease (SCD) is characterized by ischemia-reperfusion injury and chronic inflammation (1–3). Leukocytosis correlates with clinical severity and early death in patients with SCD (2,4). There is an increasing interest in the role of blood polymorphonuclear leukocytes (PMNs) in the pathogenesis of sickle cell crisis (2,4–6). Blood PMNs are important effector cells in immunity to microorganisms. A balance between the production and death of PMNs is important in maintaining cell numbers within physiologically appropriate ranges (6,7). In the neutrophilic inflammatory state, human PMNs constitutively undergo apoptosis, a process that is critical to the successful resolution of inflammation by the safe removal of effete cells (6–8). There is evidence that PMN apoptosis can be regulated by nitric oxide (NO), the production of which is induced or enhanced by the action of cytokines and other inflammatory factors (6,9–11).

To our knowledge, no association between PMN apoptosis and SCD has been published. In our study, we examined the apoptosis of blood PMNs from patients with SCD in crisis or not in crisis and in samples from healthy controls. We also aimed to demonstrate the effects of NO on PMN apoptosis during and after sickle cell crisis.

PATIENTS AND METHODS

Subjects and Sample Collection

Seventeen patients with sickle cell vasoocclusive crisis (4 women and 13 men; age range, 18–48 years) and 17 healthy volunteers (control group; 5 women and 12 men; age range, 20–27 years) were enrolled in the study. A clinical diagnosis of crisis was made according to the following criteria: widespread pain typically involving the limbs, vertebrae, or ribs that could be ascribed to vascular occlusion and/or ischemic tissue damage, and

*Correspondence to: Can Boga, MD, Department of Hematology, Baskent University, Adana Teaching and Medical Research Center, Dadoğlu Mahallesi Serin Evler Sokak 39, No. 6, Yureğir, Adana, Turkey.
E-mail: drcanboga@hotmail.com
Received 3 July 2006; Accepted 29 September 2006
Published online 16 November 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.b.20160
symptomatic relief from that pain after the administration of analgesic medication (4). The medical history of each subject was recorded, and all subjects underwent a physical examination. Painful episodes of crisis, previous cerebrovascular accidents, bone necrosis, hepatomegaly, and the need for transfusion were noted. Seropositivity for hepatitis B or hepatitis C virus and peak levels of serum alanine aminotransferase, bilirubin, and creatinine were also documented. Chest radiography and ultrasonographic examination of the abdomen were performed. Information about the use of hydroxyurea was noted, as was the need for automated red-cell exchange therapy to control vasoocclusive crisis. Blood samples were collected during painful vasoocclusive crises and after those crises had ended (during the steady state, which was defined as being pain free and without an acute clinical event in the 30 days before sampling). A peripheral blood sample from each subject was collected with a 21-gauge needle and was placed into heparinized tubes. After having been subjected to immediate centrifugation at 1000 \( \times \) g for 10 min at 4 \( ^\circ \) C, plasma samples were frozen and were then stored at −30 \( ^\circ \) C until they were used for NO analysis.

The exclusion criteria for study groups were active infection, cirrhosis, severe organ dysfunction, cancer, pregnancy, and treatment with hydroxyurea. Patients with autoimmune disease responsive to corticosteroid therapy were also excluded. All subjects provided informed consent. The institutional review board of the Baskent University in Ankara, Turkey, approved the study. Measurements of quality control were made with the Biorad External Quality Assurance Service (Bio-Rad Laboratories, NSW, Australia).

**Flow Cytometry Protocol**

This evaluation is based on the early redistribution of phosphatidylserine from the inner to the outer layer of the cell membrane of apoptotic cells. During early apoptosis, a cell will stain with annexin V, which has a selective affinity for phosphatidylserine, but not with propidium iodide (PI). Propidium iodide stains the nucleus of cells with ruptured membranes. During late apoptosis, living cells will stain with annexin V and PI, but dead cells will stain only with PI (9,12).

Annexin V-fluorescein isothiocyanate (FITC) staining was performed with the annexin V-FITC apoptosis detection kit (ImmunoTech, Beckman Coulter, Marseille, France) according to the manufacturer’s instructions. Briefly, the cell samples were washed with ice-cold culture medium or PBS after having been subjected first to 5 min of centrifugation at 300g at 4 \( ^\circ \) C and then to erythrocytic lysis by means of ammonium chloride lysing solution. The supernatant was discarded, and the cell pellet was resuspended in ice-cold diluted binding buffer to 5 \( \times \) \( 10^{7} \)–5 \( \times \) \( 10^{8} \) cell/mL. The tubes were kept on ice. One microliter of annexin V-FITC solution and 5 \( \mu \)L of dissolved PI were added to the cell sample, which was then analyzed by means of either flow cytometry or fluorescence microscopy. Measurement was performed within 15 min by a 4-color flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, FL) with EXPo 32 ADC software (Beckman Coulter).

Peripheral blood cells were observed by forward scatter/side scatter (FS/SS) to determine their size and granularity. A monoclonal antibody (anti-CD13) conjugated with phycoerythrin (PE-CD13, Beckman Coulter, Marseille, France) was used to identify PMNs. Blood PMNs that were larger and more granulated than lymphocytes and were positive for CD13/SS were selected in the ungated dot plot panel (Fig. 1a). Annexin V-FITC staining was identified in fluorescent-1 and PI staining in fluorescent-4 (Fig. 1b). Cells were identified as follows: early apoptotic PMNs if they were positive for marker annexin V-FITC but negative for PI, late apoptotic PMNs if they were positive for annexin V-FITC and PI, dead PMNs if they were negative for annexin V-FITC and positive for PI, and intact PMNs if they were negative for annexin V-FITC and for PI. Early apoptotic PMNs, late apoptotic PMNs, and dead PMNs were expressed as a percentage of the total PMN count.

**Measurement of Nitric Oxide Level**

Because NO is a very labile molecule, its direct measurement in biological samples is very difficult. In aqueous solution, NO reacts with molecular oxygen and accumulates in the plasma as nitrite (NO\(_2^-\)) and nitrate (NO\(_3^-\)) ions. The stable oxidation end products of NO, NO\(_2^-\), and NO\(_3^-\) can be readily measured in biological fluids and have been used in vitro and in vivo as indicators of NO production. Therefore, plasma nitrite concentration was accepted as an index of NO. To enable total nitrite detection, deproteinized plasma was treated with copperized cadmium granules to reduce NO\(_3^-\) to NO\(_2^-\). Nitrite concentrations were quantified by a colorimetric assay based on the Griess reaction (13).

The data were statistically analyzed with SPSS software (Statistical Package for the Social Sciences, version 11.0, SPSS, Chicago, IL). The percentages of PMNs that were stained with annexin V, annexin V/PI, PI and the values of plasma NO were expressed as the mean \( \pm \) SD. The data for annexin V, annexin V/PI, PI staining, and NO were compared with the paired \( t \) test. Correlations between quantitative parameters were tested by Spearman’s rho test. A \( P \) value < 0.05 was considered significant.

**RESULTS**

**Characteristics of Patients with SCD**

Two of the patients with SCD (12%) had a history of a cerebrovascular event, and 7 (41%) had avascular bone necrosis. The median of the need for transfusion was 1 unit per year (range, 0–3 units per year). Six subjects (6%) exhibited HBsAg or anti-HCV positivity. The following median values were identified: alanine aminotransferase peak level, 67 IU/L (range, 50–126 IU/L); bilirubin peak level, 8.4 \( \mu \)mol/L (range, 4.8–16.6 \( \mu \)mol/L); and cre-
atine peak level, 1.2 μmol/L (range, 0.8–1.6 μmol/L). All patients had been pretreated with an average of two drugs (usually zinc and folic acid).

**Study Groups and Blood PMN Apoptosis**

The mean annexin-V staining value that indicated early apoptotic PMNs was higher in patients with SCD than in healthy controls (4.13% ± 3.23% vs. 0.58% ± 0.25%, P = 0.002 for patients with SCD during vasoocclusive crisis and 5.90% ± 6.96% vs. 0.58% ± 0.25%, P = 0.001 for patients with SCD after vasoocclusive crisis). A similar result was observed in the mean of annexin-V/PI staining (late apoptotic cells) when patients with SCD were compared with healthy controls (0.40% ± 0.43% vs. 0.11% ± 0.09%, P = 0.026 for SCD during vasoocclusive crisis and 0.86% ± 1.13% vs. 0.11 ± 0.09%, P = 0.018 for SCD after vasoocclusive crisis). The mean of PI staining that showed dead cells did not differ in control subjects and patients with SCD in crisis (0.18% ± 0.31% vs. 0.019% ± 0.032%, P > 0.05), but the mean increased in patients with SCD after crisis when compared with healthy controls (0.19% ± 0.0.18% vs. 0.019% ± 0.032%, P = 0.003). When SCD groups were compared during and after vasoocclusive crisis, there was no difference between the annexin-V, annexin-V/PI, and PI staining of PMNs (P > 0.05).

PMNs with the typical features of apoptosis (early apoptotic, late apoptotic, and dead PMN) were shown in (Figs. 2a–2c).

**Effect of Plasma NO Level on the Apoptosis of PMNs**

The mean NO values were 21.19 ± 8.95 μmol/L during vasoocclusive crisis and 19.75 ± 10.39 μmol/L after vasoocclusive crisis. No statistically significant difference was found during and after vasoocclusive crisis with respect to plasma NO concentration levels (P > 0.05). We did not find a significant correlation of NO concentrations and neutrophil apoptosis levels during and after vasoocclusive crisis (P > 0.05).
DISCUSSION

In patients with SCD, vasoocclusive crises are the major cause of morbidity and mortality (1,2). The increased binding of sickled erythrocytes to the vascular endothelium and endothelial activation are the important contributory factors (2–5). There is an increasing interest in the role of blood PMNs in the pathogenesis of SCD. The association of PMNs and an increased risk of death, severe acute pain, and marrow necrosis with PMN infiltration is well known (3–6,14). In patients with SCD, the binding of PMNs to the endothelial surface leads to PMN extravasation into tissues at sites at which inflammatory cytokines are produced (3–5,15). Inflammatory cytokines and other inflammatory factors such as chemokines and immunoglobulin-like molecules induce the expression of ligands (eg, intercellular adhesion molecule-1, E-selectin, and P-selectin) on the endothelium, and these ligands are capable of binding PMNs to the endothelium by the action of several adhesion molecules (eg, CD11a, CD11b, CD11c/CD18, L-selectin, and CD15) (3–5,15,16). An elevated level of PMNs in the blood, the adhesiveness of PMNs to the endothelium, the extravasation of PMNs into tissues, and the aggregation of PMNs in patients with SCD can reduce blood flow and, in association with sickled erythrocytes, can cause microvascular occlusion and crisis (1,5,16).

Apoptosis is genetically programmed cell death (12). The derangement of the apoptotic process contributes to the pathogenesis of a wide variety of diseases, such as cancer, degenerative diseases of the nervous system, heart disease, and vascular diseases (17). Many investigators have focused on understanding the role of altered apoptotic cell death in the immune system and the tissue injury that are characteristic of inflammatory diseases (7–10,16). Immune cells (blood PMNs, macrophages, dendritic cells, and lymphocytes) that are involved can exhibit apoptotic changes under inflammatory conditions (7,18,19). Mediators such as steroids, tumor necrosis factor, NO, C5a, and the Fas ligand appear to contribute to apoptotic changes, and their effects are tissue- and cell-population selective (10,20,21). Delayed PMN death is a general feature of neutrophilic inflammation (6). Therefore, we established the theory that in patients with sickle cell vasoocclusive crisis, PMN apoptosis may be important in regulating the inflammatory process by controlling PMNs numbers and thus activity.

We demonstrated that PMN apoptosis (the staining of annexin V, annexin V/PI, and PI in PMNs) increased in patients with SCD (in steady state or in crisis) when compared with controls, but being in crisis had no effect on PMN apoptosis when compared with the steady-state phase. The degree of the induction of PMN apoptosis is expected to be high enough to prevent clinically evident vasoocclusion. However, a delay in expected PMN death might be associated with PMNs that accumulate in injured or inflamed vessels during vasoocclusive crisis.

The explanation of our findings was based on the following information from the literature: (a) Studies have revealed increased levels of IL-1, IL-4, IL-6, IL-8, IL-10, and TNF-alpha in the serum of patients with SCD in the steady state (15). Among those cytokines, IL-6 and TNF-alpha in particular exert an inhibitory effect on the normal apoptotic process (21,22), and those cytokines may play a mediating role in the dysregulation of PMN apoptosis. (b) Inflammatory mediators such as endotoxic lipopolysaccharide, complement factor 5a, and human granulocyte-macrophage colony-stimulating factor all markedly inhibit the rate of neutrophil apoptosis and prolong the functional life span of neutrophils (20). (c) It has been suggested that the generation of free heme inhibits human neutrophil apoptosis (13). High levels of free heme are found in pathologic states of increased hemolysis, such as sickle cell vasoocclusive crises, and might be a possible inhibitory mechanism in one of the representative pathways of apoptosis. (d) Oxidative radicals, which generate continuously in patients with SCD, might be associated with increased PMN apoptosis (2,9). In contrast, antioxidants such as NO can modulate PMN apoptosis during vasoocclusive crises (9,10). According to the previously mentioned observations, NO, which is a critical factor in the pathophysiologic process of SCD and is now used in patients with SCD (particularly those with acute lung injury), has a marked influence on PMN apoptosis (9–11). However, in our study, NO seemed to exert no effect on PMN apoptosis in the development of vasoocclusive crises, which suggests that vasoocclusion is a complex pathophysiologic condition and that the results of in vivo observations might differ from those of in vitro studies. The sum of these results suggests that any factor leading to an alteration in the normal PMN apoptotic process (eg., a change in the degree of endothelial activation or in the adhesiveness of red cells or neutrophils) might be associated with vasoocclusion in patients with SCD.

To our knowledge, our study is the first to investigate the association of blood PMN apoptosis and SCD. Our data showed that PMN apoptosis may play an important role in the development of ischemic complications in people with SCD. The study results may facilitate the development of new agents for the treatment of SCD.

LITERATURE CITED