Analytical Biochemistry 393 (2009) 111–116

Contents lists available at ScienceDirect

Analytical Biochemistry

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

A flow cytometry approach for quantitative analysis of cellular phosphatidylserine distribution and shedding

Inna Freikman [a], Johnny Amer [b], Israel Ringel [a], Eitan Fibach [b,*]

[a] Department of Pharmacology, School of Pharmacy, Hebrew University of Jerusalem, Jerusalem 91120, Israel
[b] Department of Hematology, Hadassah–Hebrew University Medical Center, Jerusalem 91120, Israel

ARTICLE INFO

Article history:
Received 19 April 2009
Available online 14 June 2009

Keywords:
Red cells
Thalassemia
Flow cytometry
Phospholipids
Shedding

ABSTRACT

Phospholipids are asymmetrically distributed across the membrane of all cells, including red blood cells (RBCs). Phosphatidylserine (PS) is mainly localized in the cytoplasmic membrane leaflet, but during RBC ageing it flip-flops to the external leaflet—a process that is increased in certain pathological conditions (e.g., β-thalassemia). PS externalization in RBCs mediates their phagocytosis by macrophages and removal from the circulation. PS is usually measured by flow cytometry and is reported as the percentage of cells with external PS. In the current study, we developed a novel two-step flow cytometry procedure to quantitatively measure not only the external PS but also the intracellular and shed PS. In this method, PS is first bound to fluorescent annexin V, and then the residual nonbound annexin is quantified by binding to PS exposed on apoptotic cells. Using this method, we measured 1.1 ± 0.2 and 0.12 ± 0.04 μmol inner and external PS, respectively, per 10^7 normal RBCs. Thalassemic RBCs demonstrated increased PS externalization (1.7-fold) and shedding (11-fold) that was accompanied by lower intracellular PS (31%). These results suggest that quantitative flow cytometry of PS could have a diagnostic value in evaluating the pathology of RBCs in hemolytic anemias associated with increased PS externalization and shortening of the RBC life span.

Phospholipids are asymmetrically distributed across the membrane of all cells, including the red blood cells (RBCs)[1] [1]. Aminophospholipids such as phosphatidylserine (PS) are mainly localized in the cytoplasmic leaflet of the membrane, whereas lipids with a choline head (e.g., phosphatidylcholine) are mainly present in the outer leaflet [2]. Changes in this asymmetry are one of the hallmarks of apoptosis of nucleated cells. For example, PS flip-flops to the external leaflet following induction of apoptosis. Although, mature enucleated RBCs do not undergo classical apoptosis, they experience PS externalization, which is thought to be a major signal for phagocytosis by macrophages and removal from the circulation [3]. This process occurs during physiological RBC ageing (senescence), but it is increased in certain pathological conditions such as β-thalassemia [1]. Moreover, it has been shown that a fraction of the external PS undergoes shedding into the extracellular milieu [4,5].

Externalization of PS is usually estimated by staining cells with fluorochrome-conjugated annexin V (AV) and determining the percentage of positive (AV^+) cells by flow cytometry (FCM) [6,7]. Although, FCM is a readily available methodology in research and clinical laboratories, this method is applicable to populations containing a large fraction of AV^+ cells (e.g., cells exposed to an apoptosis-inducing agent). However, in vivo, only a small fraction of any cell population is AV^+ at a given time, making their determination statistically unreliable. In addition, this procedure does not yield information regarding the inner PS, which is not exposed on the outer surface of the cells, or on the PS shed into the surrounding medium. Moreover, the procedure provides relative comparison rather than absolute quantitative values.

In the current study, we developed novel quantitative FCM methodologies to measure the external PS as well as the intracellular and shed PS from RBCs and showed that in thalassemia there is an increased externalization and shedding of PS that is accompanied by decreased intracellular PS. These results suggest that quantitative FCM measurement of PS could have diagnostic value in evaluating the pathology of RBCs in hemolytic anemias associated with reduced RBC life span.

Materials and methods

RBCs, RBC supernatants, and human serum

Peripheral blood samples of both normal donors and β-thalassemic patients were obtained from counting vials after all diagnostic

* Corresponding author. Fax: +972 2 6423067.
E-mail address: fibach@yahoo.com (E. Fibach).

[1] *Abbreviations used:* RBC, red blood cell; PS, phosphatidylserine; AV, annexin V; AV^+, AV-positive; FCM, flow cytometry; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; PMT, photomultiplier tube; MFI, mean fluorescence intensity; FITC, fluorescein isothiocyanate; FIA, fluorescence inhibition assay; FSC, forward light scattering; SSC, side light scattering; BSA, bovine serum albumin; NMR, nuclear magnetic resonance.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.06.011

laboratory tests were completed. The relevant clinical aspects of these patients were summarized recently [8]. Most patients are treated by frequent blood transfusions; their blood samples were obtained prior to transfusion (i.e., at least 3 weeks following the previous transfusion). Informed consent was obtained in all cases according to the Helsinki committee regulations.

Blood (2.5 ml) was centrifuged for 5 min at 200*g*. RBCs were washed twice with phosphate-buffered saline (PBS) and analyzed immediately. In some experiments, the washed RBCs were incubated in PBS at approximately 5×10^9/ml for 30 min at 37 °C, and following centrifugation (1700*g* for 5 min) the supernatants were collected and kept at −80 °C until analysis.

Serum was separated from blood samples obtained without anticoagulants. After clot formation, the blood was centrifuged for 10 min at 3000*g* and the supernatant was collected and stored at −80 °C until analysis.

HL-60 cells

HL-60 cells, a human myeloid leukemia cell line, were maintained by subculturing twice weekly at approximately 1×10^5/ml in alpha minimal essential medium supplemented with 10% fetal calf serum (both from Biological Industries, Beit-Hamek, Israel) and incubating at 37 °C in a humidified atmosphere of 5% CO_2 in air. For experiments, unless otherwise indicated, the cells were maintained for 6 days without changing the medium.

Flow cytometry

Cells were analyzed by a fluorescence-activated cell sorter (FACScalibur, Becton Dickinson, Immunofluorometry systems, Mountain View, CA, USA). Instrument calibration and settings were determined using CaliBRITE 3 beads (Becton Dickinson). The cells were passed at a rate of approximately 1000/s with saline serving as the sheath fluid. A 488-nm argon laser beam was used for excitation. The sensitivity of the photomultiplier tubes (PMTs) was set by adjusting their voltage for each set of experiments. The percentage of positive cells and the mean fluorescence intensity (MFI) were calculated by FACS-equipped CellQuest software. In each assay, unstained cells served as negative control.

Measurements of PS

The direct method

Approximately 10^6 washed RBCs were resuspended in Ca-binding buffer (IQP, Groningen, Netherlands) and stained with excess (5/100 μl) fluorescein isothiocyanate (FITC)–AV (IQP) for 30 min at 37 °C (Fig. 1A). The RBCs were then washed with 5 ml of the same buffer and analyzed by FCM (Fig. 1B). Under these conditions, AV binds to the outer (exposed) PS. AV^+ RBCs were defined as RBCs having AV fluorescence above the fluorescence (>60) of the negative control (unstained RBCs).

Total cellular PS was measured following permeabilization of the washed RBCs for 2 min at 39 °C with 50% ethanol in PBS. The cells were then washed with and resuspended in Ca-binding buffer, stained with FITC–AV (5/100 μl) (Fig. 1C), and analyzed by FCM (Fig. 1D). Under these conditions, AV binds to the outer (exposed) PS as well as to inner (intracellular) PS.

The indirect method

PS was measured quantitatively by the two-step fluorescence inhibition assay (FIA) (Fig. 2). The outer PS of intact RBCs or the total PS of RBC lysates and supernatants (or human serum) were first bound to FITC–AV (step 1), and then the residual nonbound AV was quantified by binding to PS exposed on apoptotic HL-60 cells that served as an indicator reagent (step 2). The HL-60 cell fluorescence in step 2 was reciprocally proportional to the amount of PS in step 1.

For outer PS measurement, intact RBCs were washed with and suspended in Ca-binding buffer and then stained with 1 μl/ml FITC–AV at 37 °C for 20 min. Following centrifugation (1700*g*), the cells were discarded and the spent buffer was collected (step 1). This residual nonbound AV was measured by binding to 6-day-old HL-60 cells containing approximately 30% apoptotic PS-

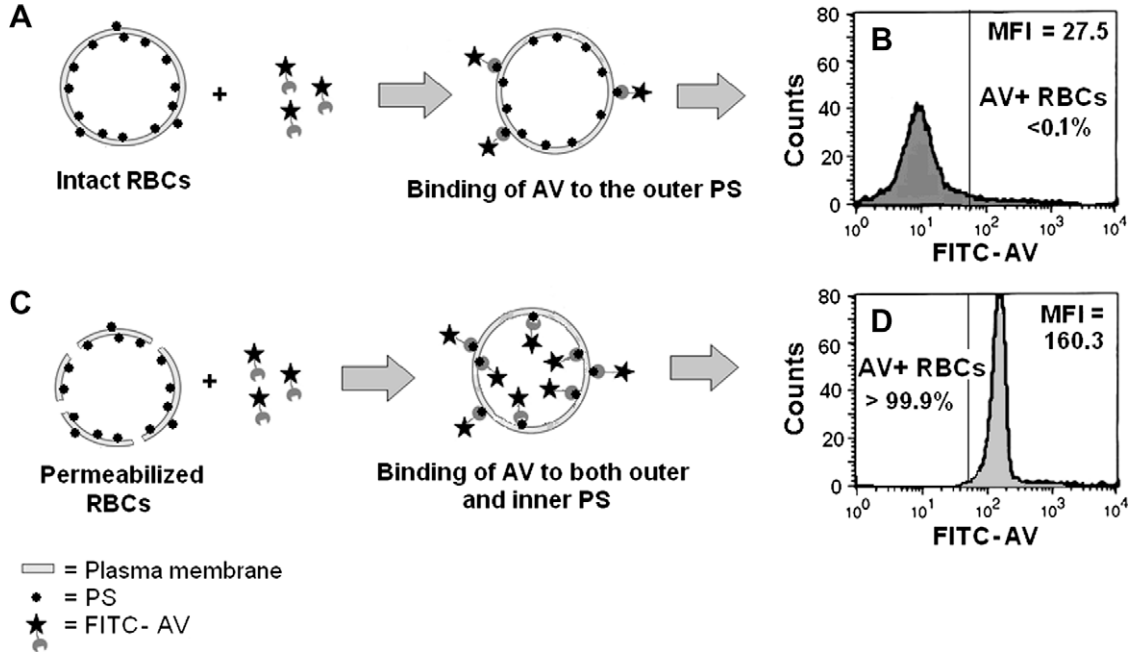

Fig. 1. Direct measurement of PS distribution in RBCs. RBCs were incubated with PBS alone (intact RBCs) (A) or 50% ethanol (permeabilized RBCs) (C) for measurement of their outer and total PS, respectively, as described in Materials and methods. The RBCs were then washed with Ca-binding buffer, incubated with 5 μl of FITC–AV, and analyzed by FCM. FITC–fluorescence histograms of intact RBCs (B) and permeabilized RBCs (D) in a representative sample are shown. The MFI of the total intact RBC population was 27.5, with less than 0.1% of the cells being FITC positive (AV^+) (B). After permeabilization, more than 99.9% of the cells became AV^+ with an MFI of 160.3 (D).