Increased Erythrocyte Phosphatidylserine Exposure in Sickle Cell Disease: Flow-Cytometric Measurement and Clinical Associations

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Increased exposure of phosphatidylserine (PS) in erythrocytes has been postulated to contribute to the pathophysiology of sickle cell disease because of its possible effects on blood coagulation, cell adhesion, and cell clearance. We developed a flow-cytometric assay to measure exposure of PS on the outer face of the erythrocyte membrane based on addition of fluorescein-annexin V to whole-blood specimens. The assay correlated linearly with binding of 125I-annexin V (r² = .95, n = 125 samples). Normal donors (n = 30) showed virtually no annexin-positive cells (0.34% ± 0.18% for 24-hour old samples). In contrast, annexin V binding was above the upper limit of normal in 96% of 205 specimens from 17 adult sickle cell and 2 β-thalassemia patients; the mean percentage of annexin-positive cells was 2.98% ± 2.00% (range, 0.4% to 12.0%). Values varied substantially over time for some patients, and mean values varied between patients. The percentage of annexin-positive cells always decreased after transfusion (11 events in 6 patients), and out of proportion to the amount of blood transfused. In conclusion, increased exposure of PS on a subpopulation of erythrocytes in vivo is a virtually universal feature of sickle cell disease, and its measurement may be useful to evaluate clinical status and response to therapeutic measures such as blood transfusion.

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Materials and Methods

Specimens and patients; reagents. Blood was drawn by venipuncture into evacuated tubes containing EDTA anticoagulant. Normal samples were drawn from 30 healthy adult male and female volunteers. Specimens from adult patients with sickle cell disease were surplus portions of EDTA-anticoagulated whole blood samples obtained during routine clinical care and sent to the clinical laboratory for routine hematology studies over a 7-month period. Samples stored at 4°C were accepted for assay up to 48 hours after being drawn. Of the sickle cell samples, 34 were less than 6 hours old at the time of assay; 143 were ~24 hours old; and 28 were ~48 hours old. Of the 19 patients studied, 17 had sickle cell disease and 2 had β-thalassemia; their ages ranged from 22 to 35 years; 10 were male and 9 were female. A stabilized human blood preparation (4CP Normal Control) and fluorescent microspheres (Standard-Brite) were obtained from Coulter Corp (Hialeah, FL); anti-CD71 (mouse monoclonal) and affinity-purified, phycoerythrin-labeled goat polyclonal antibody against mouse IgG were from Immunotech (Westbrook, ME).

Purification and labeling of annexin V. Native annexin V was purified from human placenta. Recombinant annexin V was prepared by cytoplasmic expression in Escherichia coli by modifications of our previous vector and method. E coli strain BL21(DE3) containing plasmid pET12a-PAP1 was grown to saturation overnight. Bacteria were then sonicated in 50 mmol/L TrisHCl pH 7.2, 10 mmol/L CaCl₂; after centrifugation, annexin V bound to bacterial membranes was released by resuspending the pellet in 50 mmol/L TrisHCl pH 7.2, 20 mmol/L EDTA. Residual bacterial membranes were removed by centrifugation and the supernatant dialyzed against 20 mmol/L TrisHCl pH 8.0. The dialysate was applied to a Mono Q column (Pharmacia, Piscataway, NJ) and eluted with a gradient of 0 to 1 mol/L NaCl in the same buffer. Recombinant annexin V eluted at approximately 0.22 mol/L NaCl. The purified protein was concentrated, dialyzed against 50 mmol/L HEPEs pH 7.4, 100 mmol/L NaCl, and stored in aliquots at ~70°C. The final yield was approximately 45 mg/L of culture, with a purity of 98% to 99% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophore.

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sis. Native and recombinant annexin V were labeled with FITC (fluorescein 5-isothiocyanate; Molecular Probes, Eugene, OR), purified to yield a fraction containing a single mole of fluorescein per mole of protein, and quantitated by absorbance at 494 nm, as all described. Ion-exchange purification of FITC-annexin V substantially improved the sensitivity of the flow-cytometric assay. Recombinant annexin V was used for most of this study because it was easier to produce than placental annexin V; direct comparison showed that recombinant and native annexin V behaved identically in the flow-cytometric assay.

Erythrocyte binding assay. Binding of 125I-annexin V to erythrocytes was measured as described. For flow cytometry, whole blood was diluted 1:100 (3 to 5 × 10⁷ erythrocytes/mL) to a final volume of 0.25 mL in a buffer consisting of 10 mmol/L HEPES-Na pH 7.4, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L MgCl₂, 1 mmol/L NaH₂PO₄, 5 mmol/L glucose, 5 mg/mL bovine serum albumin (fatty-acid free), and 2.5 mmol/L CaCl₂. 100 nmol/L FITC-annexin V was added, the sample incubated for 10 to 15 minutes at room temperature and an aliquot directly aspirated into a Coulter XL flow cytometer for analysis (excitation wavelength 488 nm, emission wavelength 505 to 545 nm). Each day, the instrument was first standardized with a solution of fluorescent microspheres (Coulter Standard-Brite) to give a mean fluorescence intensity of 64.8. Fifty thousand cells were then monitored in a gate of forward- and side-scatter values set to include all erythrocytes. Cells were counted as annexin-positive if they had a mean fluorescence of at least 5.6; this threshold value was determined in initial studies based on analysis of freshly drawn samples from normal donors. The flow cytometer’s software was used to calculate the percentage of annexin-positive cells; the mean fluorescence of these cells; and the heterogeneity of fluorescence intensity of positive cells (full-peak coefficient of variation). For two-color analysis, whole blood was diluted 1:800 in the same buffer as above but without CaCl₂; incubated with anti-CD71 (30 minutes at 4°C); washed twice; incubated with phycoerythrin-goat-antimouse antibody (30 minutes at 4°C); washed twice; and incubated with FITC-annexin V and 2.5 mmol/L CaCl₂, as described above. Flow cytometry was performed as described above, with phycoerythrin fluorescence monitored at 560 to 590 nm.

RESULTS

Assay development and validation. Figure 1 shows typical flow cytometer histograms for normal and sickle cell samples. The normal sample showed a single peak with a low mean fluorescence intensity, attributed to non-specific binding of FITC-annexin V. Sickle cell samples showed the same peak, but in addition a second smaller population of annexin-positive cells with a mean fluorescence of 11.2 (see Table 1); this value is approximately seven times the mean fluorescence of normal cells; the mean fluorescence of these cells; and the heterogeneity of fluorescence intensity of positive cells (full-peak coefficient of variation). For two-color analysis, whole blood was diluted 1:800 in the same buffer as above but without CaCl₂; incubated with anti-CD71 (30 minutes at 4°C); washed twice; incubated with phycoerythrin-goat-antimouse antibody (30 minutes at 4°C); washed twice; and incubated with FITC-annexin V and 2.5 mmol/L CaCl₂, as described above. Flow cytometry was performed as described above, with phycoerythrin fluorescence monitored at 560 to 590 nm.

Nature of the annexin-positive cell population. Examination of blood samples by fluorescence microscopy showed that normal samples lacked annexin-positive cells, whereas sickle cell samples had positive cells in approximately the same proportion as measured by the flow-cytometric assay. Some annexin-positive cells had the morphology of normal...
Fig. 1. Typical flow-cytometer histograms for binding of FITC-annexin V to erythrocytes. (A) Normal donor; (B) sickle cell patient; (C) positive control (Coulter 4CPlus) with high level of PS exposure; (D) normal erythrocytes treated with 1 μmol/L A23187 at 37°C for 24 hours before assay.

erythrocytes, whereas some cells were elongated, with the appearance of irreversibly sickled cells. The fluorescence was evenly distributed around the cell membrane, and was not present intracellularly, in agreement with results reported for A23187-treated normal erythrocytes.

The relative age of the annexin-positive cells was assessed by two-color analysis with antibody to CD71 (the transferrin receptor; a marker for reticulocytes). The following results (mean ± SD) were obtained for a group of nine sickle cell samples: 2.13% ± 1.66% positive for annexin V; 5.39% ± 4.35% positive for CD71; and 1.21% ± 0.92% positive for both annexin V and CD71. Thus, some but not all annexin-positive cells are still young enough to be expressing the transferrin receptor, and only some young erythrocytes are positive for annexin V binding.

Analysis of samples from normal volunteers and sickle cell patients. Freshly drawn samples from normal volunteers showed virtually no annexin-positive cells (Table 1). Binding increased very slightly over the subsequent 48 hours for samples stored at 4°C, and somewhat more for samples stored at room temperature or 37°C. These results are consistent with previous findings with the 125I-annexin V binding assay.

A total of 205 samples from sickle-cell patients were assayed (Fig 4). Almost all samples had an elevated number of annexin-positive cells: 96% were above a value of 0.7%, which is the approximate upper limit of normal for 24-hour-
old specimens. Sickle cell samples (n = 10) also showed a slight increase in annexin-positive cells when stored at 4°C (4.33% ± 2.74% at 24 hours, v 4.70% ± 2.80% at 48 hours). However, because this change was very slight compared with the total variation in the sickle cell population, samples drawn in EDTA anticoagulant and stored at 4°C were accepted for assay up to 48 hours after being drawn. The mean fluorescence also varied somewhat in this group of patients, but much less so than the percentage of annexin-positive cells (Fig 4, inset). There was no overall correlation between the values of mean fluorescence and the percentage of annexin-positive cells (Fig 4, inset). The annexin-positive cell peak was fairly broad, as indicated by a mean value of 89% ± 24% for the full-peak coefficient of variation. The mean percentage of annexin-positive cells differed between patients, and values often varied over time for individual patients (Fig 5; and see Fig 7). In most instances the observed variation is much greater than the variability of the assay (SD = 0.25%), indicating that the observed differences reflect underlying biological variation.

Correlation of clinical variables with assay results. For patients where clinical information was available, a review was performed to determine if any clinical variables could explain the observed variation in annexin-positive cells within and between individuals. For a given patient, blood transfusion was the most clearly identified factor (Fig 6): in every case where the assay was performed both before and after blood transfusion, there was a decrease in the percentage of annexin-positive cells. In all but two instances the decrease was out of proportion to the amount of blood transfused, indicating that it was not solely due to dilution of sickle cell erythrocytes. However, for the study group as a whole over a 6-month period, there was no significant correlation between average values of annexin-positive cells and the patients’ average hematocrits (r² = .05) or with the number of units transfused in a 6-month period (r² = .06). Thus, the number of annexin-positive cells may be useful in evaluating an individual patient’s response to transfusion therapy, but there is probably not an absolute value of this variable that predicts the need for transfusion for patients in general.

Figure 7 shows serial values over time for two individual patients. Patient 1 had received no medical care for several months, and returned for treatment of tibial ulcers at time zero. His percentage of annexin-positive cells decreased rapidly after initiation of transfusion therapy, and remained low for the remainder of the study period. Patient 10 received two exchange transfusions to treat episodes of priapism. Samples obtained before and after the second exchange transfusion showed that the percentage of annexin-positive cells decreased to near-normal levels after the transfusion. For these two patients, episodes of pain did not clearly correlate with changes in the level of annexin-positive cells. For the study group as a whole, it was not possible to systematically assess the correlation between frequency or severity of sickle cell crisis and the number of annexin-positive cells, partly because complete clinical data on pain episodes and medical
visits were not available for all the patients studied, and partly because blood samples were often not available during intercritical periods.

Three of the patients (nos. 13, 17, and 18 in Fig 6) had suffered strokes in childhood; the remaining patients had no history of stroke. The mean percentage of annexin-positive cells in these three patients was higher than in the patients without a history of stroke, but this difference did not reach statistical significance.

DISCUSSION

Assay development and validation. Flow cytometry with annexin V provides a highly sensitive assay for exposure of PS in erythrocytes, which can be performed directly on whole-blood specimens. The precision of the assay is about 0.25% (expressed as the SD of the percentage of annexin-positive cells). The major variables in the binding assay (incubation time and the concentrations of calcium, FITC-annexin V, and cells) were optimized to provide a simple

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Fig 4. Distribution of values in the sickle cell population (n = 205 samples). The lines show cumulative frequency distributions for sickle cell samples (■) and 30 normal samples (24 hours old) (○). (Inset) Frequency distribution of mean fluorescence of annexin-positive cells for the same set of sickle-cell samples.

Fig 5. Values of annexin V binding for individual patients. Results obtained on all individual specimens from each patient studied are plotted (▲). The adjacent bars show the mean ± SD for each patient. All patients have sickle cell disease except patients 15 and 16, who have β-thalassemia.
yet reliable assay that can be performed in 15 to 30 minutes in a clinical laboratory setting. The results obtained here are generally in agreement with those obtained with a similar procedure on purified erythrocytes. Thus, if this assay is used for clinical purposes in the future, it should be relatively straightforward to obtain results that are reliable over time and comparable between different centers and laboratories.

Previously we showed that 125I-annexin V could be used to measure exposure of PS in erythrocytes. The very close linear correlation ($r^2 = .95$) between the radioligand and flow-cytometric assays therefore provides strong evidence for the validity of the flow-cytometric assay. The annexin V flow-cytometric assay also correlates linearly with the ability of red blood cells to support prothrombinase activity. The properties of erythrocyte-annexin V binding observed in this study are also consistent with a number of other studies of

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Fig 6. Effect of blood transfusion. The percentage of annexin-positive cells is shown at the indicated times before and after transfusion; each patient is represented by a different symbol. Each pair of points represents a single transfusion with the indicated number of units.

Fig 7. Serial values for two individual sickle cell patients. "P" indicates an episode of painful crisis requiring medical attention. Partial exchange transfusion is indicated by the number of units removed followed by the number of units given; a complete exchange transfusion is indicated by "X."
the binding of annexin V to cell surfaces based on both
radioligand and flow-cytometric methods. All of
these studies are consistent with the view that annexin V
binds to anionic phospholipids (primarily PS) exposed at the
extracellular face of the plasma membrane; the binding is
calcium-dependent, of nanomolar affinity, and fully reversible;
and there is no evidence for intracellular uptake or
irreversible binding of ligand. Thus, the evidence that
annexin V is a valid measure of extracellular PS exposure
is by now conclusive.

**Altered membrane phospholipid asymmetry in sickle cell
erythrocytes.** It has long been postulated that increased PS
exposure could contribute to the pathophysiology of sickle
cell disease. This study provides some further observations in
support of this view. First, it is now clear from this and
another study that a small subpopulation of abnormal eryth-
rocytes accounts for the previously observed increase in annexin V
binding to sickle cell erythrocytes. Second, the
abnormal erythrocytes contain a substantial density of ex-
posed PS: the mean fluorescence of the abnormal cell popula-
tion (Fig 4) is approximately 20% to 50% of the value ob-
tained for cells with completely randomized membranes,
such as ionophore-treated or preserved cells. Given the heter-
ogeneity of the abnormal cell population, a small fraction
will contain levels of exposed PS equal to randomized cell
membranes (see Fig 1B). Because PS accounts for about
14% of erythrocyte membrane phospholipids, this would
correspond to an average level of 3% to 7% PS in the extrac-
cellular face of the membrane. Because of the highly throm-
bogetic nature of exposed PS, and the positive dependence of coagulation reactions on the percentage of PS in the mem-
brane, these two facts together suggest that even a small
number of abnormal erythrocytes could have a substantial
thrombogenic effect, particularly if they are concentrated at
a site of vascular occlusion. This may contribute to a
hypercoagulable state observed in sickle cell anemia and
β-thalassemia, and other erythrocyte disorders, such as
polycthemia vera, that are associated with an intravascular
thrombosis. In this regard, a correlation between the number
of annexin-positive cells and the level of prothrombin frag-
ment 1.2 in sickle cell patients has recently been reported. However, at this point there is no definite evidence that
higher levels of annexin-positive cells represent a risk factor for clinical thrombosis.

Evidence from animal models indicates that presence of
PS at ≥3% of total phospholipids is sufficient to trigger reticuloendothelial clearance of intravenously injected lipos-
omes. This threshold is exceeded for the annexin-positive
cell population of this study, as noted above. Although liposomes lack protein and are not functionally
Equivalent to erythrocytes, our results are consistent with the
view that increased exposure of PS in sickle cell erythrocytes
may contribute to their shortened life span.

**Other clinical aspects.** Flow cytometry analysis can pro-
vide several parameters for the cell population under study.
For sickle cell patients, the parameter that showed the great-
est overall range of variation was the percentage of annexin-
positive cells, ranging from a low of 0.4% to a high of
12.0%; this parameter also gave the best separation between
normal and abnormal populations. The mean fluorescence
of the annexin-positive cells and the heterogeneity (width)
of this peak varied less, and gave less separation of normal and abnormal populations (Fig 4, Table 1). Therefore, we
used the percentage of annexin-positive cells as the primary variable for clinical-correlative studies.

This study confirms, in a much larger dataset, that in-
creased exposure of erythrocyte PS in vivo is a constant feature of sickle cell disease. However, there is a substan-
tial degree of variation in values between patients, and some-
times within a given patient over time (Figs 5 and 7). Blood
transfusion clearly reduces the percentage of annexin-positive
cells (Figs 5 and 7), and this effect is not simply due to
dilution of sickle cells with normal cells. It may be due
to a part in suppression of erythropoiesis, in part to faster
clearance of abnormal erythrocytes, and perhaps in part to
amelioration of the process that causes the patients’ erythro-
cytes to expose abnormal levels of PS in the first place. If
larger studies confirm our observations, this assay might be
useful in monitoring transfusion therapy in individual pa-
tients. We were not able to assess systematically whether assay values correlated with the frequency or severity of sickle cell crisis because the study design did not allow systematic sampling of patients when they were clinically
well, nor did it capture all clinical encounters at all sites.
Thus, there may well be additional clinical variables affect-
ing the percentage of annexin-positive cells that remain to
be identified. It is also worth remembering that we studied
adult patients, so these results do not necessarily apply to
children.

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