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 ß-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 ß-thalassemia; their ages ranged from 22 to 35 years; 10 were male and 9 were female. A stabilized human blood preparation (4CPloy 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 dialysand 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-
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.\textsuperscript{27} 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 \textsuperscript{125}I-annexin V to erythrocytes was measured as described.\textsuperscript{17} For flow cytometry, whole blood was diluted 1:100 (3 × 10\textsuperscript{7} 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 MgCl\textsubscript{2}, 1 mmol/L NaH\textsubscript{2}PO\textsubscript{4}, 5 mmol/L glucose, 5 mg/mL bovine serum albumin (fatty-acid free), and 2.5 mmol/L CaCl\textsubscript{2}. 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 CaCl\textsubscript{2}; 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 CaCl\textsubscript{2} 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 the nonspecific peak present in normal samples. A commercially available stabilized whole-blood control pool ("4CPlus") showed high levels of positive cells with a high mean fluorescence intensity (37 ± 3). This material provided a convenient and stable positive control for the assay. Binding of FITC-annexin V to all of these sample types was always totally reversible upon addition of EDTA to the reaction mixture, confirming the calcium dependence of the observed annexin V binding. Normal erythrocytes treated in vitro with the calcium ionophore A23187 at 1 mmol/L and 37°C as described\textsuperscript{15} showed a time-dependent increase in the percentage of annexin-positive cells which was maximal by 24 hours. The mean fluorescence of the positive cells was 43 ± 1.6 at 24 hours. A preparation of erythrocyte ghosts showed a single peak with a mean fluorescence of 52 ± 15.

Major assay variables were optimized as follows. Binding of FITC-annexin V to erythrocytes was negligible below 1 mmol/L CaCl\textsubscript{2}, and then increased progressively over the range from 1 to 10 mmol/L (Fig 2). However, the percentage of annexin-positive cells plateaued at 2.5 mmol/L CaCl\textsubscript{2} when gates were set as described in Materials and Methods. Because values of CaCl\textsubscript{2} above 2.5 mmol/L can induce artifactual exposure of PS during incubation in vitro,\textsuperscript{13} a calcium concentration of 2.5 mmol/L was therefore chosen for subsequent experiments. Binding of FITC-annexin V to erythrocytes was very rapid; the mean fluorescence was nearly maximal within 30 seconds of addition of FITC-annexin V, and remained constant for at least 60 minutes thereafter. An incubation time of 10 to 15 minutes was chosen for convenience and consistency. Addition of FITC-annexin V over the concentration range of 10 to 100 nmol/L showed progressive increases in mean fluorescence until saturation was reached around 100 nmol/L, consistent with previous results with \textsuperscript{125}I-annexin V.\textsuperscript{15} Titration of 100 nmol/L FITC-annexin V with 4CPlus erythrocytes showed that the mean fluorescence stayed approximately constant up to a level of 5 × 10\textsuperscript{7} cells/mL in the binding reaction, after which it decreased because of progressive depletion of free FITC-annexin V; however, the percentage of positive cells stayed constant up to a value of 20 × 10\textsuperscript{7} cells/mL. Thus, when whole-blood samples are assayed at 1:100 dilution (ie, ~3 to 5 × 10\textsuperscript{7} cells/mL), variation in cell number will have no significant effect on the percentage of annexin-positive cells.

The flow cytometric assay was further validated by direct comparison with the \textsuperscript{125}I-annexin V binding assay for a total of 125 samples from normal volunteers and sickle cell patients (Fig 3). An excellent linear correlation (\(r^2 = .95\)) was observed between the two assays throughout the range tested. Thus, if one first converts the flow-cytometric data into a measure of average binding per cell for the whole cell population present, results of the two methods can be directly compared. Also, dilution of a sickle cell sample in normal whole blood in increments from 0% to 100% showed a linear correlation between the percentage of abnormal blood added and the percentage of annexin-positive cells detected (Fig 3, inset).

The precision of the assay was determined by replicate analyses of samples. On a single day, three samples were analyzed 20 times to determine the number of annexin-positive cells, with the following results (expressed as mean ± SD): normal blood sample, 0.12% ± 0.041%; sickle-cell blood sample, 4.84% ± 0.24%; 4CPlus control, 98.7% ± 0.095%. The 4CPlus control was also assayed on 32 separate days, yielding a value of 98.7% ± 0.27%. Corresponding values cannot be determined for natural blood samples, because of the progressive increase in PS exposure that occurs over time for blood stored in vitro.\textsuperscript{19}

**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
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-
peak was fairly broad, as indicated by a mean value of underlying biological variation. 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 times after being drawn.

For the mixture.

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 ($r^2 = .05$) or with the number of units transfused in a 6-month period ($r^2 = .06$). 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

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**Table 1. Flow-Cytometric Values for Normal and Sickle Cell Samples**

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Annexin-Positive Cells (%)</th>
<th>Mean Fluorescence (arbitrary units)</th>
<th>Full-Peak Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-fresh</td>
<td>30</td>
<td>0.19 ± 0.12</td>
<td>9.4 ± 2.5</td>
<td>80 ± 31</td>
</tr>
<tr>
<td>Normal-24 h</td>
<td>30</td>
<td>0.34 ± 0.18</td>
<td>12.4 ± 3.8</td>
<td>106 ± 28</td>
</tr>
<tr>
<td>Normal-48 h</td>
<td>30</td>
<td>0.48 ± 0.14</td>
<td>14.6 ± 4.1</td>
<td>119 ± 19</td>
</tr>
<tr>
<td>Sickle cell</td>
<td>205</td>
<td>2.86 ± 2.00</td>
<td>11.2 ± 2.8</td>
<td>89 ± 24</td>
</tr>
</tbody>
</table>

Normal samples were stored at 4°C and assayed at the indicated times after being drawn.
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
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. Therefore, 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 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...
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 erythrocytes accounts for the previously observed increase in annexin V binding to sickle cell erythrocytes. Second, the abnormal erythrocytes contain a substantial density of exposed PS: the mean fluorescence of the abnormal cell population is approximately 20% to 50% of the value obtained for cells with completely randomized membranes, such as ionophore-treated or preserved cells. Given the heterogeneity 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 extracellular face of the membrane. Because of the highly thrombogenic nature of exposed PS, and the positive dependence of coagulation reactions on the percentage of PS in the membrane, 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 polycythemia vera, that are associated with a thrombotic tendency. In this regard, a correlation between the number of annexin-positive cells and the level of prothrombin fragment 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 phospholipid is sufficient to trigger reticuloendothelial clearance of intravenously injected liposomes. This threshold is exceeded for the annexin-positive cells of most sickle cell patients in 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 provide several parameters for the cell population under study. For sickle cell patients, the parameter that showed the greatest 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 increased exposure of erythrocyte PS in vivo is a constant feature of sickle cell disease. However, there is a substantial degree of variation in values between patients, and sometimes 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 in part to suppression of erthropoiesis, in part to faster clearance of abnormal erythrocytes, and perhaps in part to amelioration of the process that causes the patients’ erythrocytes 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 patients. 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 affecting 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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