Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: Quantitative assessment of outer-leaflet lipid by prothrombinase complex formation

(asymmetry/recognition/macrophage)

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ABSTRACT   Phosphatidylserine (PS) is asymmetrically distributed in mammalian cell membranes, being preferentially localized in the inner leaflet. Some studies have suggested that a disturbance in the normal asymmetric distribution of PS—e.g., PS exposure in the outer leaflet of the cell membrane, which can occur upon platelet activation as well as in certain pathologic red cells—serves as a potent procoagulant surface and as a signal for triggering their recognition by macrophages. These studies suggest that the regulation of PS distribution in cell membranes may be critical in controlling coagulation and in determining the survival of pathologic cells in the circulation. In this paper we describe a sensitive technique, based on PS-dependent prothrombinase complex activity, for assessing the amount of PS on the external leaflet of intact viable cells. Our results indicate that tumorigenic, undifferentiated murine erythroleukemic cells express 7- to 8-fold more PS in their outer leaflet than do their differentiated, nontumorigenic counterparts. Increased expression of PS in the tumorigenic cells directly correlated with their ability to be recognized and bound by macrophages.

Macrophages play an important role as effector cells in host defense against cancer metastasis (1) and viral diseases (2). When appropriately activated, macrophages are able to recognize and destroy a variety of tumorigenic and virus-infected cells, including cells resistant to other host defenses such as T cells and natural killer cells (1), while leaving normal cells unharmed. Macrophage-mediated tumor cell killing has been shown to be independent of such cell characteristics as surface receptors, drug resistance, cell cycle, and metastatic potential (1, 3).

The mechanism responsible for the ability of mononuclear phagocytes to discriminate between normal and pathologic cells is not known. The broad spectrum of target cells susceptible to macrophage-mediated cytolysis might suggest, however, that a uniform surface moiety could be involved in target cell recognition.

An interesting feature of some cell membranes is the asymmetric distribution of membrane phospholipids between the two leaflets of the bilayer (4). In red blood cells (RBC), for example, most membrane phospholipids show some preference for either leaflet, whereas phosphatidylserine (PS) is the only phospholipid that adopts a totally asymmetric distribution, being localized exclusively in the cell's inner leaflet (5–7). Although the mechanisms responsible for maintaining an asymmetric distribution of PS are still unclear, recent evidence suggests that the preservation of PS in the cell's inner leaflet is of central importance in cellular physiology. For example, the exposure of PS that occurs in activated platelets (8) and in sickled RBC (9, 10) regulates

hemostasis by serving as a potent procoagulant surface (11, 12) and as a signal for triggering the recognition of these cells by macrophages (13). Related experiments have shown that artificially generated phospholipid vesicles (14, 15) and RBC that contain an exogenously inserted fluorescent PS analog (16, 17) are bound and endocytosed by macrophages. These studies suggest that the maintenance of PS asymmetry in cell membranes may represent a homeostatic mechanism, the failure of which may lead to alterations in normal cell function.

To determine whether tumor cells express PS in their outer leaflet, tumorigenic Friend murine erythroleukemic cells (MELC) and their chemically differentiated (18) nontumorigenic "normal" counterparts (dMELC) were assessed for PS expression and for their ability to be bound by macrophages. This was accomplished by employing a modified protocol of the PS-dependent prothrombin-to-thrombin cascade assay (19, 20). Our results indicate that tumorigenic, undifferentiated MELC express relatively large amounts of PS in the outer leaflet of their plasma membranes which disappears from the surface upon the cells' differentiation. In addition, the expression of PS correlated directly with the ability of the cells to be bound by macrophages.

MATERIALS AND METHODS

Materials. Activated factor X (factor Xa, 500 units/mg of protein), QAE-cellulose, and octyl-Sepharose 4B were obtained from Sigma. Thrombin and the thrombin-sensitive chromogen S2238 were purchased from Helena Laboratories. Prothrombin (factor II) was obtained from Behring Diagnostics. Dioleoyl phosphatidylcholine, PS derived from bovine brain, and 1-oleoyl-2-{6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl} phosphatidylcholine (NBD-phosphatidylcholine) were purchased from Avanti Polar Lipids. NBD-PS was prepared by phospholipase D-catalyzed base exchange of NBD-phosphatidylcholine (21).

Preparation of Phospholipid Vesicles. Stock solutions of lipid in chloroform were mixed at the indicated mole ratios, dried under N_2, and resuspended in Tris/NaCl buffer (50 mM Tris, pH 7.8/120 mM NaCl) to a final concentration of 0.5 mg/ml. Small unilamellar vesicles were produced by sonicating the lipid suspension with a probe ultrasonicator (Heat Systems) under N_2 at 4°C for 30 min. The vesicles were centrifuged at 30,000 × g for 30 min to remove titanium fragments.

Isolation of Factor V. Factor V was isolated from bovine plasma by a modification of the procedure of Nesheim et al. (22). Four liters of fresh, heparinized bovine whole blood was centrifuged at 4000 × g to remove cells. The plasma was

Abbreviations: MELC, murine erythroleukemic cell(s); dMELC, chemically differentiated MELC; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; PS, phosphatidylserine; RBC, red blood cell(s).
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mixed with concentrated $BaCl_2$ (to yield 80 mM) and poly-ethylene glycol 8000 (4% final concentration) at 4°C for 30 min. The resulting precipitate was removed by centrifugation at 5000 × g for 30 min at 4°C. The supernatant was applied to QAE-cellulose anion-exchange resin at 4°C. Factor V was eluted from the resin with 25 mM Tris, pH 7.4/5 mM $CaCl_2$/1 mM benzamidine and concentrated by ammonium sulfate precipitation (60% saturation). The precipitate was dissolved in buffer (20 mM Tris borate, pH 8.3/1 mM $CaCl_2$/0.8 M NaCl) and applied to an octyl-Sepharose 4B column (2.5 × 20 cm) preequilibrated with the solubilization buffer. After the column was washed with the application buffer, the protein was eluted with the same buffer without NaCl. The eluted protein was concentrated by ammonium sulfate precipitation (70% saturation) and the precipitate was redissolved in the elution buffer. The protein was then applied to a Sephadex G-200 gel filtration column (2 × 50 cm) equilibrated with the solubilization buffer. Protein was monitored by absorbance at 280 nm. Factor V, which was eluted in the void volume, yielded a single 330,000-dalton polypeptide band by SDS/PAGE analysis. Factor V was activated by incubation with catalytic amounts of thrombin (0.02 unit) for 3 min at 37°C.

Cells. A tumorigenic tetraploid Friend MELC line (cell line 179) was obtained from A. Deisseroth (M. D. Anderson Cancer Center). MELC were grown in RPMI-1640 supplemented with glutamine, fetal bovine serum (10%), nonessential amino acids, and vitamins. Differentiation was initiated by addition of 5 mM hexamethylenebisacetamide as described (23). After 5 days of incubation, the cells were collected and the fraction of differentiated cells was assessed for accumulation of hemoglobin by benzidine staining (24). This procedure routinely yielded >95% differentiated (hemoglobin-positive) cells (dMELC). The tumorigenic and nontumorigenic phenotypes of the MELC and dMELC were verified by their ability to produce tumors in DBA/2 mice. Subcutaneous injection of 0.5 × 10^6 MELC produced 1-cm tumors in 5 out of 5 mice within 2 weeks, whereas the dMELC produced a palpable tumor in 2 out of 5 mice after 3 weeks.

Prothrombin-Converting Activity Assay. Vesicles or cells (MELC or dMELC) at various concentrations were incubated at 37°C with the following "prothrombinase complex cocktail," which contains the necessary components to catalyze the conversion of prothrombin to thrombin: 6 mM $CaCl_2$, factor Va (0.2 nM), factor Xa (0.2 unit), and prothrombin (0.8 unit) in Tris/NaCl buffer (total volume, 600 μl). After 3 min the reaction was stopped by the addition of EDTA (15 mM final concentration). The thrombin-dependent chromophore S2238 was then added (to 0.4 mM) and the rate of chromogen formation was monitored at 405 nm with a Gilford Response spectrophotometer employing appropriate kinetics software. The initial rates of prothrombin-converting activity, which is directly proportional to the amount of PS present on the catalytic (cell or vesicle) surface, were determined from the slopes of absorbance.

NBD-PS/RBC Analysis. RBC (2 × 10^7 cells per ml) in Hepes buffer (20 mM Hepes, pH 7.4/145 mM NaCl/5 mM KCl/10 mM glucose) were incubated with 2 mM pyridyldithioethylamine for 30 min at 4°C, washed, and incubated with various amounts of NBD-PS for 1 hr at 37°C. The RBC were washed in cold Hepes buffer and the amount of cell-associated NBD-PS was quantified by fluorescence (25). The fraction of PS in the outer leaflet was determined by its ability to be removed by "back-exchange" with acceptor vesicles (25). Aliquots of the NBD-PS-containing RBC were analyzed for PS-dependent prothrombin-converting activity as described above, except that the concentration of factor Va was increased to 15 nM to attain the required degree of sensitivity.

Binding of MELC and dMELC to Activated Macrophages. Macrophages were obtained from the peritoneal cavity of 8-

to 10-week-old C57BL/6 female mice 4 days after intraperitoneal injection of 2 ml of thioglycolate medium (Difco). The cells were washed several times with Hanks' balanced salt solution and plated (5 × 10^5 cells per well) into 200-mm^2 tissue culture plates (Costar). After incubation for 2 hr at 37°C, the nonadherent cells were removed by washing, and the adherent macrophages were incubated overnight with bacterial lipopolysaccharide (1 μg/ml) and recombinant γ-interferon (10 units/ml) (26) in RPMI-1640 medium containing 5% fetal bovine serum.

MELC, dMELC, and normal mouse RBC were washed, resuspended to 10^7 cells per ml in RPMI-1640 containing 10% fetal bovine serum, and labeled with ^{51}Cr [10 μCi (0.37 MBq) per ml] for 30 min at 37°C. The cells were washed with the same medium, and 250 μl (2.5 × 10^6 cells) were added to the washed macrophage monolayers. At various intervals the cultures were washed for 10 sec with a constant stream of buffer through an 18-gauge needle to remove unbound target cells. The fraction of target cells bound by the macrophages was determined by solubilizing the cells with 1% SDS and assessing the radioactivity of the lysates by scintillation counting.

Determination of Surface Area. Cell surface areas were determined by three independent techniques: sequential thin sections, determination of cell diameters, and forward light scatter.

Sequential Thin Sections. MELC and dMELC were fixed in cacodylate buffer containing glutaraldehyde (3%) and paraformaldehyde (2%) and were postfixed with osmium tetroxide and uranyl acetate (1%). The samples were washed with water, dehydrated with graded ethanol solutions to propylene oxide, and embedded in Polybed (Polysciences). The blocks were polymerized, cut into serial sections (100 nm), mounted on Formvar-coated one-hole grids, and stained with uranyl acetate and lead citrate. Section thickness was determined with an Aus Jena interference microscope (G. Hirasaki, Shell Oil, Houston, TX). Electron micrographs of all sections were made at ×300 (JEOL 1200-EX transmission electron microscope at 100 kV) to identify a series of complete cross-sectioned cells. Selected cells were photographed at ×4000–7000 magnification, which required 75–150 photomicrographs per cell. Image analysis was performed on an IBAS Image Analyzer (Zeiss) using commercially available software. The image was captured and enhanced to provide sufficient contrast between the cell and background. A gray scale was superimposed on the enhanced image and the cell perimeter was delineated. The surface area of the entire cell was calculated from the formula $\Sigma_{i=1}^{n-1} 0.5 (P_i + P_{i+1}) H$, where P and H are the thin-section perimeters and thickness, respectively.

Cell Diameters and Forward Light Scatter. Cell diameters were determined at ×40 magnification. The images were electronically captured and digitized, and the diameters (D_{max}) were obtained as described above. Forward light scatter was measured with an EPICS C instrument (Coulter) calibrated with beads of defined sizes. For these methods, surface areas were calculated under the assumption that the cells were perfect spheres.

RESULTS

Generation of PS Calibration Curves by Using Small Unilamellar Vesicles of Defined Compositions. The determination of surface PS is based on the PS-initiated cleavage of prothrombin to thrombin by factor Xa in the presence of factor Va and Ca^{2+} (19, 20, 27). The thrombin produced then cleaves an appropriate substrate to a chromogenic product. Since the components of the prothrombinase complex are maintained constant and in excess, the concentration of the catalytic PS surface is rate-limiting. The results presented in Fig. 1 indicate that the rate at which the chromophore is produced is directly proportional to the amount of available
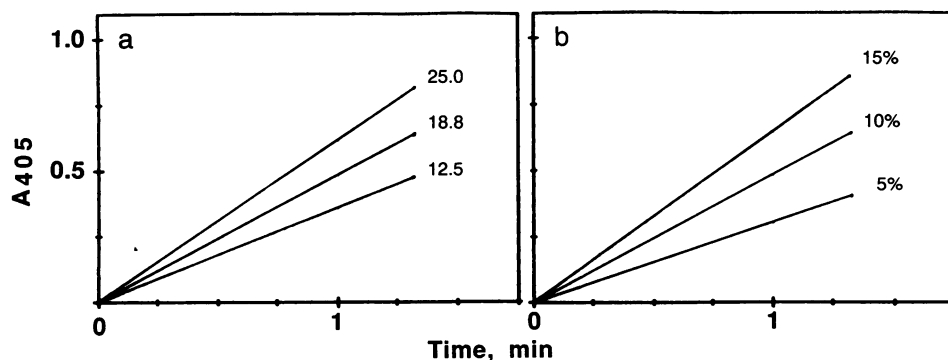
FIG. 1. Kinetics of prothrombin activation by standard phospholipid vesicle preparations as a function of phospholipid concentration (*a*) and PS density (*b*). The prothrombinase complex was formed, and the rate of thrombin-catalyzed chromophore production was assessed in real time as described in *Materials and Methods*. (*a*) Various amounts (12.5, 18.8, and 25.0 nmol) of 10 mol % PS in dioleoyl phosphatidylcholine. (*b*) Constant amount (18.75 nmol) of total lipid containing 5, 10, or 15 mol % PS.

(outer-leaflet) PS. Thus, similar rates were obtained (based on the absolute amounts of PS present) when increasing amounts of vesicles containing 10 mol % PS were used (Fig. 1*a*) and when the total lipid concentration was kept constant but with increasing densities (mol %) of PS (Fig. 1*b*). This result demonstrates that, under these conditions, the rate of thrombin production is independent of PS density and particle number. Analysis of initial rates of thrombin production versus vesicle surface area [2.6×10^8 μm^2/nmol of lipid (28)] produced a double-reciprocal plot that displayed a linear relationship (>0.99 correlation) of rate to surface area (Fig. 2*a*). At 5, 10, and 15 mol % PS, different rates with varying *y* intercepts (V_{max}) but common *x* intercepts were obtained. A plot of V_{max} versus the mole density of PS in these standard vesicle preparations shows a direct relationship between the maximum velocity of thrombin production and the amount of PS present on the membrane surface (Fig. 2*b*). Therefore, by measuring the initial rates of thrombin production by a membrane of unknown PS density but of known surface area, the V_{max} of the catalytic surface can be assessed and the density of PS determined by comparison to an appropriately generated standard (vesicle) curve.

Determination of Cell Surface PS in RBC Containing Known Amounts of Exogenously Inserted Lipid. The results presented above suggested that it should be possible to directly determine the amount of PS on cell surfaces by comparison to artificially generated vesicles containing known amounts of PS. To verify this, defined amounts of exogenous NBD-PS were inserted into pyridyldithioethylamine-treated RBC [to eliminate its translocation to the inner leaflet (25)]. The rates of thrombin production for these RBC increased linearly with increasing NBD-PS localized in the outer leaflet. Quantita-

tive assessment of the density of NBD-PS exposed on the outer leaflet of these cells was determined from a calibration curve generated as described in Fig. 2. Independent analysis of the density of NBD-PS by the prothrombinase assay (ordinate) showed a linear relationship with the actual amount of available PS as determined by fluorescence (abscissa, Fig. 3). These results indicate that, under these conditions, the prothrombinase assay detects only PS localized in the outer leaflet and that there are no other RBC surface components that can serve as the required catalytic surface, since thrombin production could not be detected in untreated (no NBD-PS) RBC.

Determination of PS on MELC, dMELC, and RBC. The rates of thrombin production initiated by standard vesicle populations, MELC, dMELC, and mouse RBC were determined simultaneously. Reciprocal plots of surface area versus initial rates of thrombin production all yielded common *x* intercepts. The density of PS on the cell surface was then calculated based on the results obtained from a standard vesicle curve generated as described in Fig. 2. Since the calculated densities are critically dependent on cell surface area, the data presented in Table 1 were calculated by using surface areas obtained by three independent techniques (see *Discussion*). All of the data indicate that undifferentiated, tumorigenic MELC express significantly more outer-leaflet PS than do their "normal" differentiated counterparts irrespective of the method used to determine surface area. Averaging all of the data shows that MELC express 7- to 8-fold more PS in their outer leaflet than do dMELC. As expected (5–7), PS could not be detected in the outer leaflet of normal mouse RBC.
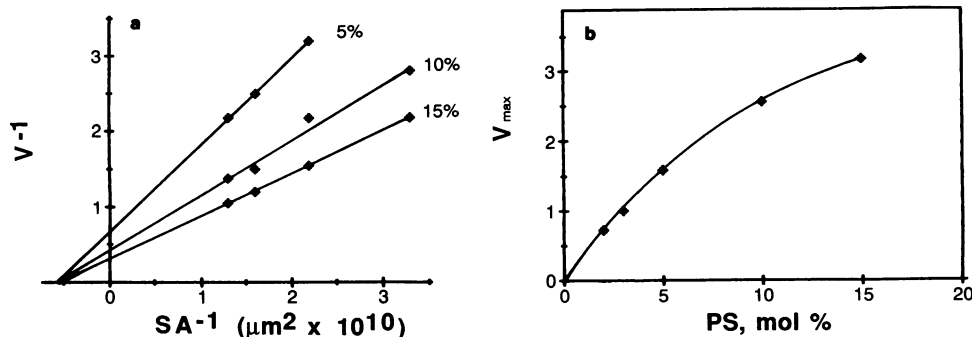

FIG. 2. Thrombin production by standard phospholipid vesicle preparations. (*a*) Double-reciprocal plot of PS-dependent prothrombin activation. Initial rates (*V*) of thrombin production by vesicles (6.25, 12.5, 18.75, and 31.3 nmol of total lipid) containing 5, 10, or 15 mol % PS were plotted as a function of particle surface area (SA). The surface area of the vesicles was estimated to be 2.6×10^8 μm^2/nmol of lipid based on the parameters discussed by Mason and Huang (28). (*b*) Maximum velocity (V_{max}, ΔOD/min) of thrombin production was determined from the *y* intercepts of double-reciprocal plots generated as in *a* and plotted as a function of PS density (mol %).
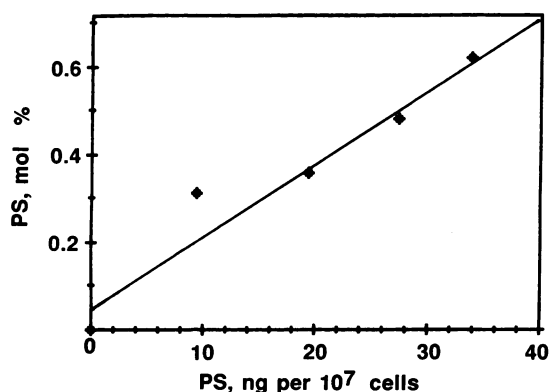
FIG. 3. Analysis of cell surface PS by prothrombinase complex formation and fluorescence. Pyridyldithioethylamine-treated RBC containing increasing amounts of PS in their outer leaflet were assessed for their ability to activate prothrombin. The rates of thrombin production were converted to PS density (mol %) by using a standard curve generated as described in Fig. 2. The calculated densities were then plotted as a function of the amount of outer-leaflet PS determined by direct fluorescence assay of NBD-PS. Best fit was obtained by linear regression analysis of the five data points (correlation of 0.96).

Macrophage Binding of Tumor and Normal Cells. To determine whether tumorigenic, PS-expressing MELC and not their "normal" dMELC or mouse RBC counterparts selectively bind to macrophages, the ability of ^{51}Cr-labeled target cells to bind to activated mouse peritoneal macrophages was determined. The results of these experiments indicate that increased adherence of tumorigenic cells to macrophages (Fig. 4) is associated with increased exposure of PS (see Table 1). Although this finding does not indicate direct recognition of PS on target cells by macrophages, it does suggest that PS may be involved in this process.

DISCUSSION

To determine the quantity of PS on the outer surface of intact cells, we exploited the PS-dependent prothrombinase complex cascade. Although other techniques are available, all of them are destructive and may induce alterations in membrane asymmetry. For example, direct derivatization with fluorescamine (29) or picrylsulfonic acid (7) requires lipid extraction, and the amount of derivatized product may be obscured by high backgrounds due to unreacted intracellular lipids. Similarly, hydrolysis by phospholipase A_2 is nonspecific and may perturb bilayer structure by the generation of large amounts of endogenous lysolipids. In contrast, the PS-dependent prothrombinase assay is nondestructive and specific for PS expressed only on the available catalytic surface.

Analysis of the kinetics of thrombin production in relation to the absolute amounts of PS present on the outer leaflet of synthetically generated standard vesicles and RBC containing exogenously supplied PS demonstrated that the prothrombinase-complex assay can determine PS densities in the

Table 1. Density of PS in the outer leaflet of MELC and dMELC

	MELC		dMELC	
Method	Surface area, μm^2	PS density, mol %	Surface area, μm^2	PS density, mol %
Diameter	632	13.1 ± 4.6	430	1.6 ± 1.2
Thin sections	710	11.8 ± 3.5	213	2.6 ± 1.7
Cell sorter	1134	14.4 ± 8.4	531	1.0 ± 0.8
Average	825 ± 220	13.1	391 ± 133	1.7

PS mole percentages represent the average from 4–6 individual trials.
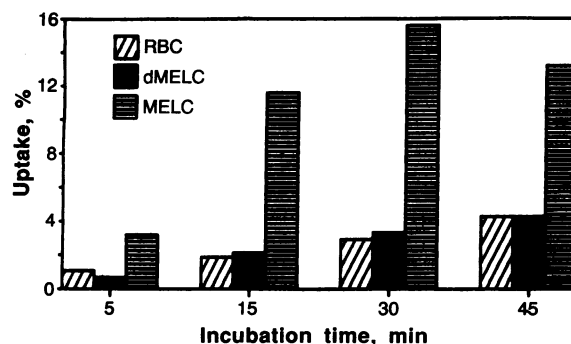

FIG. 4. Binding of MELC, dMELC, and RBC by activated mouse peritoneal macrophages. ^{51}Cr-labeled target cells were incubated for the indicated periods at 37°C with macrophages activated with lipopolysaccharide and γ-interferon.

plasma membrane of cells. However, accurate quantitative measurements are critically dependent on cell surface area. Table 2 shows the amounts of PS calculated from the measured densities in relation to two different estimates of cell surface area. It can be seen that although density determinations are linearly proportional to the actual amounts of PS present in the outer leaflet (see also Fig. 3), the calculated amounts of PS are dependent on the surface area used. If one employs a surface area of 256 μm^2 [based on the data of Bar *et al.* (32) and Demel *et al.* (33); see Table 2 legend], excellent agreement between the independent measurements is obtained.

Clearly, a PS density of 15% (Table 1) seems to occupy an extremely large fraction of the cell surface. However, as discussed above the accuracy of the calculated PS densities is dependent on cell surface areas. Unfortunately, most techniques used for assessing this parameter are estimates, since they do not take into account irregularities of the cell surface. In an attempt to overcome this problem the surface areas of sequential thin sections were assessed. Surprisingly, the areas obtained by the different techniques were quite similar (Table 1). However, as shown in Table 2, reasonable assessments of cell surface area can be obtained by determining the amount of cellular lipid. Since the fraction of total cell lipid comprising the plasma membrane of MELC is known (34), one can also estimate the surface area based on lipid analysis. Determination of the phospholipid phosphate content of MELC (30) revealed 10^{10} lipid phosphates per cell. Assuming that 50% of the plasma membrane lipid resides in the outer leaflet [19% of the total cell lipid (34)] with a

Table 2. Surface area dependence of PS quantity in RBC

PS,* ng per 10^7 cells	PS density,† mol %	PS, ng per 10^7 cells	
		192 μm^2‡	256 μm^2§
9.5	0.31	12.2	16.2
19.4	0.36	14.2	18.9
27.5	0.48	18.9	25.2
33.8	0.62	24.4	32.6

*The amount of PS in the outer leaflet of RBC was determined by fluorescence.

†PS mole percentages were obtained from the experiment summarized in Fig. 3.

‡Total phospholipid (30) and cholesterol (31) content was determined on 10^{10} RBC ghosts extracted with $CHCl_3$/MeOH/0.1 M HCl (2:2:1.8, vol/vol). The surface area of the cell was determined by using the following values: 0.67 fmol of lipid phosphate per cell with a mean surface area of 60 × 10^{-8} μm^2 (28) and 0.66 fmol of cholesterol per cell with a mean surface area of 38 × 10^{-8} μm^2 (32).

§The surface area calculated as described above was corrected by a factor of 1.33 based on the arguments of Demel *et al.* (33) concerning the contribution of membrane proteins on the cell surface areas presented by Bar *et al.* (32).

cholesterol/phospholipid ratio of 0.624 (35), one obtains a surface area of 1591 μm^2. If one takes into consideration the argument that these calculations comprise only 75% of the surface due to the presence of proteins (33), the surface area increases to 2121 μm^2 and effectively reduces the densities shown in Table 1 by a factor of 2–3. Calculations based on this surface area reveal that $\approx$25% of the PS in the cells' plasma membrane resides in the outer leaflet, which is in good agreement with the data of van der Schaft *et al.* (36). In contrast, if we use the areas shown in Table 1, 75% of the membrane PS would reside in the outer leaflet. While it is possible to speculate on the accuracy of all these determinations, the density of PS cannot be accurately calculated without appropriate techniques to quantify true cell surface areas. On the other hand, differences in the relative abundance of PS between different cells can be determined.

We have shown that undifferentiated MELC display 7–8 times more PS than do their differentiated counterparts. Although we have no evidence for the fate of the outer-leaflet PS, it could be relocated to its normal inner-leaflet location or removed from the cell during enucleation (36). Indeed, the asymmetrical distribution of PS is already established in the emerging reticulocyte (36). The decrease in the amount of PS from the outer leaflet of dMELC coincides with other membrane rearrangements that occur upon differentiation (18). Of particular interest is the appearance and reorganization of spectrin (37, 38) and band 4.1 protein (38), which have been implicated in maintaining PS in the cells' inner leaflet (39, 40).

Experiments to determine the propensity of MELC and dMELC to be bound by macrophages revealed a direct correlation between expression of PS in the outer leaflet and binding. Although macrophages avidly bind liposomes (14, 15) and cells (13, 16, 17) that express PS on their surfaces, we do not know whether the differences in binding observed in this system are directly due to expression of PS. The possibility that recognition is due to a shift in PS distribution/equilibrium is intriguing, although this conclusion requires further experimentation. This hypothesis is, however, somewhat supported by results that have shown qualitative differences between the expression of aminophospholipids in different B16 melanoma variants (41).

Clearly, many changes occur in the plasma membrane of differentiating cells. Whether the decrease in outer-leaflet PS or other membrane alterations serve a function critical to the differentiated cell remains to be determined. One can speculate, however, from its unique property of residing preferentially on the inner leaflet of normal cells, in conjunction with the pathological consequences upon its exposure in the outer leaflet, that the redistribution of PS is necessary for the emerging reticulocyte to survive in the circulation.

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