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Functional and pathological significance of phospholipid asymmetry in erythrocyte membranes

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Abstract. The normal asymmetric distribution of phospholipids in the plasma membrane is perturbed in erythrocytes from patients with chronic myelogenous leukemia. Since experimentally-produced lipid-symmetric erythrocytes are more interactive with cells of the reticuloendothelial system than are their lipid-asymmetric counterparts, the biological recognition of chronic myelogenous leukemia erythrocytes by the reticuloendothelial system was examined. With one exception, all erythrocyte samples from patients with chronic/benign chronic myelogenous leukemia were more adherent to endothelial cells and more readily phagocytosed by macrophages *in vitro* than were normal erythrocytes. Thus, these naturally occurring pathological erythrocytes display the same dysfunctional intercellular interactions as the laboratory models.

Keywords. Erythrocyte membrane; phospholipid asymmetry; chronic myelogenous leukemia; lipid packing; phosphatidylserine; cellular interactions; reticuloendothelial system; liposomes; annexins.

Introduction

In erythrocytes, the phospholipids of the plasma membrane are asymmetrically distributed across the bilayer (Op den Kamp, 1979). Lysis and resealing of the cells under specified conditions allows production of populations with either lipid-symmetric or lipid-asymmetric membranes (Williamson *et al*., 1985), which have been used to test the functional consequences of loss of asymmetry in erythrocytes. Structurally, lipid-asymmetric erythrocytes have more loosely-packed phospholipids in their exterior leaflet (Williamson *et al*., 1985) and display a more hydrophobic surface (McEvoy *et al*., 1986) than their asymmetric counterparts, besides an increase in the amount of phosphatidylethanolamine and phosphatidylserine (PS) in their outer leaflet. These alterations are associated with changes in the functional characteristics of the cell: lipid-symmetric erythrocytes are more adherent to endothelial cells (Schlegel *et al*., 1985a) and are more readily phagocytosed by monocyte-derived macrophages (McEvoy *et al*., 1986) and cells of the J774A.1 macrophage line (Schlegel *et al*., 1985b) than are lipid-asymmetric cells.

Kumar and Gupta (1983) were the first to recognize that the distribution of membrane phospholipids is altered in erythrocytes from patients with chronic myelogenous leukemia (CML). To determine whether phospholipid packing in the membranes of these pathological erythrocytes was also perturbed, the fluorescent probe merocyanine 540 (MC540), which binds more avidly to loosely packed bilayers (Williamson *et al*., 1983), was applied. Not only did erythrocytes from all CML patients bind increased amounts of dye (Reed *et al*., 1985; Kumar *et al*., 1987),

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Abbreviations used: PS, Phosphatidylserine; CML, chronic myelogenous leukemia; RES, reticuloendothelial system.

but so too did erythrocytes from patients with other myeloproliferative diseases (Reed *et al.*, 1987). These properties of CML erythrocytes predict that their biological recognition may be altered just as in the case of experimentally-produced lipid-symmetric erythrocytes. To test this prediction we have examined the adherence of CML erythrocytes to human endothelial cells and their phagocytosis by J774A.1 macrophages.

Materials and methods

To assess variability among normal samples, blood from multiple normal volunteers was collected and erythrocytes examined in side-by-side assays. As seen in table 1, when the adherence values for 3 samples (1–3) were averaged and

Table 1. Cellular recognition of CML erythrocytes.

Condition	Sample	Adherence ratio[a]	Phagocytosis ratio[a]
Normal			
Pennsylvania[b]	1	1·0	
	2	0·9	
	3	1·1	
	4		1·2
	5		0·8
	6		1·0
	7		0·9
	8		1·1
Texas	1643		1·0
	1679	1·3	
	1680	1·2	
CML			
Benign/chronic	1301	2·9	1·5
	1302	1·5	1·6
	1319		3·2
	1320		2·2
	1321	5·2	2·1
	1322	4·3	1·6
	1330		2·0
	1397	3·1	1·6
	1398	2·4	1·7
	1412	2·0	
	1414	1·7	
	1479		2·8
	1480		4·2
	1571		0·7
Accelerated	1572		1·0
	1573		1·2
Other hematological disorders			
Essential thrombocytosis	1331		1·5
CML Ph^- (BCR^+)	1411		0·9
CMML Ph^-	1478		3·3
AUL	1570		0·9

[a]Assays were performed as described by Pradhan *et al.* (1990).
[b]Individual ratios for samples 1–3, 4–5, 6–8 were calculated against the average value within these groups.

adherence ratios computed against the average for each sample, little variability among samples was seen. The same was true for phagocytosis ratios determined for samples 4 and 5 and 6–8.

Samples from patients were shipped overnight on ice from M. D. Anderson Cancer Center, Houston, Texas and examined within 24 h of collection. Normal samples, against which patient samples were compared, were collected in Pennsylvania identically and on the same day as patient samples and stored overnight on ice. However, to assure the veracity of these normal samples, several samples collected from normal volunteers in Texas were compared against normal samples collected in Pennsylvania and expressed as adherence ratios. As seen in table 1, neither adherence or phagocytosis ratios differed much from unity for any of these samples.

Results and discussion

Erythrocytes from 14 different patients with Philadelphia chromosome positive, benign/chronic CML, all undergoing therapy, were examined in the study. Because endothelial cells were never passaged more than twice before use, they were not always available for assays, accounting for the smaller number of adherence ratios obtained. However, as seen in table 1, all samples which could be examined were more adherent than normal controls. Because J744A.1 macrophages are a continuous line, they were always available for phagocytosis assays. As seen in table 1, with the exception of sample 1571, all phagocytosis ratios were greater than any value obtained with normal erythrocytes. Perhaps surprisingly, the phagocytosis ratios for the only two patients in accelerated CML, or blast crisis, deviated little from the norm. In addition to the samples from CML patients, a limited number of samples from patients with other hematological disorders were examined. One patient with Philadelphia chromosome negative chronic myelomonocytic leukemia showed a markedly elevated phagocytosis ratio, and one patient with essential thrombocytosis had a somewhat increased ratio. A Philadelphia chromosome negative, but breakpoint chromosome region positive, CML patient and a patient with adult undifferentiated leukemia had ratios within the normal range.

These results indicate that with one exception CML erythrocytes from all patients showed increased adherence to endothelial cells and increased phagocytosis by macrophages. It would have been of interest to determine whether the samples with the highest phagocytosis ratios also had the highest adherence ratios. Unfortunately, adherence ratios were not obtained for the samples which had the highest phagocytosis ratios. It would also be of interest to determine whether the presence of the Philadelphia chromosome is required for increased adherence and phagocytosis, and whether these altered properties of erythrocytes are present in a wider range of hematological disorders. These and other questions can be pursued in future studies of a larger patient population.

The affector mechanisms responsible for maintenance and loss of phospholipids asymmetry, and the effector mechanisms responsible for enhanced cellular recognition following loss are under intensive investigation in numerous laboratories. With regard to effector mechanisms, several signals have been proposed for recognition of lipid-symmetric erythrocytes by cells of the reticuloendothelial system (RES): loosened packing of surface phospholipids (McEvoy *et al*., 1986) and exposure of PS on the outer surface of the cell (Tanaka

and Schroit, 1983; Schroit *et al*., 1985). Since these properties cannot be dissociated in lipid-symmetric erythrocytes, liposomes modeled after the erythrocyte membrane which circulate for extended periods in mice were used to test these alternatives separately. By systematically altering the composition of the liposomes and assessing the effects on their ability to remain in circulation, it was concluded that both tight packing of phospholipids and exclusion of PS from the surface are required to prevent enhanced clearance by the RES (Allen *et al.,* 1988).

The relative importance of the cytoskeleton and the aminophospholipid translocase as affector mechanisms is being closely scrutinized (see, for example, Williamson *et al*., 1987; Middelkoop *et al*., 1989). In CML erythrocytes, a possible role for the cytoskeleton in the perturbations of phospholipid distribution has emerged from the finding of abnormalities in the architecture of the proteins underlying the membrane of these cells (Basu *et al*., 1988). Another potential contributing factor has been uncovered in investigations of the finding that elevation of intracellular Ca^{2+} levels in normal erythrocytes results in loss of phospholipid asymmetry (Williamson *et al*., 1985; Chandra *et al*., 1987). The past few years have seen the discovery of a new class of Ca^{2+}-sensitive, potentially regulatory proteins in the annexins, which bind to membranes in a Ca^{2+}-dependent fashion (Burgoyne and Geisow, 1989). If annexins are involved in maintenance and loss of lipid asymmetry, their levels and/or distribution might be altered in CML erythrocytes. We have recently established that a 67 kDa annexin is present in the cytoplasm of normal human erythrocytes and is translocated to the membrane in the presence of Ca^{2+}. This 67 kDa protein, and also other proteins of 35 and 38 kDa which react with antisera to annexins, are present in CML erythrocytes as well. However, the 67 kDa protein is found on the membranes of the CML cells even in the absence of Ca^{2+} (Fujimagari *et al*., 1990). Whether the abnormal amount and distribution of annexins play a role in the perturbations of lipid asymmetry in CML membranes is currently under investigation.

In summary, the use of both laboratory and naturally occurring pathological models in which phospholipid asymmetry has been perturbed has allowed insight into the mechanisms of maintenance and loss of phospholipid asymmetry and the functional consequences of the failure of those mechanisms.

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