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Eryptosis in hereditary spherocytosis and thalassemia: role of glycoconjugates

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Abstract The present work is aimed to study the mechanism of faster erythrocyte clearance in hereditary spherocytosis (HS), a heterogeneous disorders characterized by alterations in the proteins of the red cell membrane skeleton along with different kinds of thalassemia. The maximum exposure of phosphatidylserine (PS) is found in HS compared to those in both α- and β-thalassemia. Interestingly, in HS more PS exposed cells were found in younger erythrocytes compared to normal and the thalassemics where aged cells showed higher loss of PS asymmetry. Loss of sialic acid and GlcNAc bearing glycoconjugates, presumably the glycophorins, was also found upon aging. The loss of PS asymmetry together with the cell surface glycoproteins mediated by membrane vesiculation, seemed to play key role in early clearance of erythrocytes from circulation following a mechanism similar to HbEβ-thalassemia.

Keywords PS asymmetry · Flow cytometry · Glycoconjugates · Hereditory spherocytosis · Thalassemia

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Abbreviations

HS	Hereditory spherocytosis
HE	Hereditory elliptocytosis
PS	Phosphatidylserine
FITC	Fluorescein-5-isothiocyate
AV-FITC	Fluorescein-5-isothiocyate labeled Annexin V
WGA-FITC	Fluorescein-5-isothiocyate labeled Wheat Germ Agglutinin
PWM-FITC	Fluorescein-5-isothiocyate labeled Poke Weed Mitogen
Ca^{2+}/A23187	1 mM Ca^{2+} with 4 μM of A23187
GlcNAc	*N*-acetyl-D-glucosamine
Neu5Ac	*N*-acetyl-D-neuraminic acid
MFI	Mean fluorescence intensity
SEM	Standard error of the mean
PBS	Phosphate buffered saline containing 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 0.01% NaN_3 pH 7.4

Introduction

Erythrocytes have a lifetime of 120 days in circulation. In most cases, they do not undergo hemolysis, but are removed by phagocytes [1]. Like apoptosis, programmed cell death occurs in erythrocyte, termed as 'eryptosis' [2, 3]. Eryptosis is a caspase independent process [2, 4]. An aging antigen derived from the cleavage of band 3 has been implicated in eryptosis [5–7]. CD47, the integrin-associated protein and a part of Rhesus core complex, also acts as a self-marker on erythrocytes and have some role on spleen-mediated clearance [8]. Erythrocyte membrane is asymmet-

ric in nature with respect to phospholipid distribution between the two leaflets and PS is exclusively found in the inner-leaflet of 'young' erythrocytes under normal circumstances. It has been well accepted that with ageing externalization of PS occurs and that contributes towards removal of erythrocytes via phagocytosis [9, 10]. Cell surface sialic acid level also has some role to play in the survival of erythrocytes [11]. Evidences imply reduction of the level of cell surface sialic acid residues in 'older' erythrocytes in the peripheral blood [12–14]. Again, sialidase treated erythrocytes preferentially adhere to the Kupffer cell in the liver and to the mononuclear cells in the spleen thus mimicking aged erythrocytes [15]. Loss of sialic acid with aging is due to membrane vesiculation, depleting membrane proteins, predominantly the glycophorins [13, 14, 16, 17]. Earlier work from this laboratory showed loss of membrane PS asymmetry in different heterogeneous hemoglobinopathies *e.g.* sickle cell anemia and HbEβ-thalassemia [16].

In the present work, we have studied erythrocytes isolated from patients suffering from HS and HE, type of hereditary red cell disorders with defects in the membrane skeletal protein network [18] along with α-thalassemia and β-thalassemia intermedia. Erythrocytes from HS were found to be maximally PS-exposed compared to the normal, HE, β-carrier, α-thalassemia and β-thalassemia. Cell surface Neu5Ac and GlcNAc levels were reduced by 14% in HS, ~8% in β-thalassemia and ~2.5% in HE erythrocytes upon aging. Taken together, it is evident that loss of PS asymmetry and faster release of microvesicles rich in Neu5Ac and GlcNAc bearing glycoconjugates, presumably glycophorin, together control erythrophagocytosis and the life span of circulating erythrocytes in most of the red cell disorders.

Materials and methods

Materials

Peripheral blood samples were collected in EDTA from patients suffering from different red cell disorders, such as HS, HE, thalassemia diagnosed for the first time and normal individuals with written consent (from parents in case of minors). Percoll was purchased from Amersham Biosciences (Uppsala, Sweden). FITC was obtained from Molecular Probes (Eugene, OR). FITC labeled Annexin V was procured from BD Biosciences Pharmingen (San Diego, CA) as Annexin V-FITC apoptosis detection kit I. Wheat germ agglutinin (WGA), Pokeweed mitogen (PWM), Tris base, calcium ionophore (A23187) and Sephadex G-10 ware purchased from Sigma (St. Lewis, MO). All other chemicals were of analytical grade and purchased locally.

Isolation of erythrocytes from whole blood

Human blood samples taken for diagnosis for the first time from patients suffering in various red cell disorders were collected for our study as elaborated earlier [16]. The blood samples used in the present study were taken from patients who had not received any transfusion and were diagnosed for the first time at the Ramakrishna Mission Seva Prathisthan, Kolkata. After removal of plasma, platelet and leukocytes the red blood cells were washed extensively using Dulbecco's phosphate buffered saline (PBS) and suspended in the same buffer.

Young and aged erythrocytes were separated by centrifugation in self-forming percoll gradient as elaborated earlier [16]. The population having buoyant density between 1.088 g/mL and 1.102 g/mL was designated as young erythrocytes and those between 1.102 g/mL and 1.119 g/mL were designated as aged erythrocytes and were collected separately and suspended in PBS.

FITC labeling of WGA and PWM

WGA and PWM were labeled with fluorescein-5-isothiocyanate (FITC), in a buffer containing 0.15 M Na_2CO_3 and 0.35 M $NaHCO_3$, pH ~9.5 by following a procedure discussed in our earlier work [16]. The labeling ratio of fluorescein to WGA and PWM was determined to be ~1 fluorescein per protein molecule.

Annexin V labeling of erythrocytes

Erythrocytes were labeled with annexin V conjugated with FITC (AV-FITC) as per manufacturer's instruction (BD Parmingen, San Diego, CA). Briefly, erythrocytes were suspended in PBS (pH 7.4) supplemented with 2 mM $CaCl_2$ to a final concentration of 1×10^6 cells/ml. AV-FITC binding was analyzed with respect to the control, unlabeled erythrocytes. For binding, the cells were incubated with AV-FITC for 15 min in dark at room temperature.

Lectin labeling of erythrocytes

Erythrocytes and ionophore treated erythrocytes were resuspended in 100 μl of PBS to a concentration of 1×10^6 cells/ml. WGA-FITC was added to the suspension to a final concentration of 10 μg/ml, which is saturating concentration for 1×10^6 cells without causing appreciable hemagglutination (data not shown). The suspension was incubated for 10 min at room temperature in dark. Before flow cytometric analysis, 400 μl of PBS was added in each tube. Labeling procedure of PWM-FITC to erythrocytes is exactly same, only the saturating concentration for 1×10^6 cells was 40 μg/ml.

Flow cytometry

Flow cytometry of AV-FITC, WGA-FITC and PWM-FITC labeled cells were done in FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) to measure one-color fluorescence (FL1) of AV-FITC, WGA-FITC and PWM-FITC [16]. The red cell population was defined by size in forward (FSC) *vs*. side scatter (SSC) dot plots. Events, correlated with intact erythrocytes were analyzed for fluorescence intensity using the same standard settings. For each sample, 25,000 events were acquired and analyzed by CellQuestPro™ software (Becton Dickinson, San Jose, CA). From the FSC *vs*. SSC dot plots, clustered cell populations were selected and analyzed for FL1 histograms.

The extent of PS asymmetry was estimated with respect to the control sample, not treated with AV-FITC, the autofluorescence. The selection marker (M1) was set such that less than 0.2% of the cells were included in this region and data were expressed as percent binding of annexin V [19]. The extent of lectin binding was estimated from the mean fluorescence intensity (MFI). For WGA-FITC, we have used a second dot plot with SSC *versus* FL1 and gated the region representing the single cells for analysis. WGA caused agglutination of erythrocytes leading to formation of larger multicellular aggregates. The WGA concentration was kept low to minimize agglutination.

Results

The PS exposed subpopulation, defined by the percent binding of AV-FITC in the mixture of both young and aged erythrocytes in peripheral blood, was the lowest in 19 normal samples (1.78±0.07) and the maximum in 8 HS samples (6.65±0.98) shown in Fig. 1. The PS exposed subpopulation in case of 18 samples of β-thalassemia intermedia (3.32±0.45) and three samples of α-thalassemia (5.26±0.97) was in between the normal and HS levels. The PS exposed subpopulation in 4 HE samples was however, comparable with those of 10 samples of β-carrier and normal volunteers. The errors associated with the percent annexin V binding were estimated as the standard error of mean (SEM) and were further subjected to the two-tail Student's *t*-test. No significant changes were found in β-carrier and HE. For β-thalassemia $P<0.05$ and for the rest of HS and α-thalassemia the P value was <0.0001.

Binding of WGA-FITC and PWM-FITC to normal and β-thalassemic erythrocytes are shown in Fig. 2 as representative cytograms, both for aged and younger cells. The pattern of the cytograms with a major peak in combination with a number of small peaks was observed in case of WGA-FITC treated erythrocytes, particularly from normal individuals. β-thalassemics appeared to be drastically

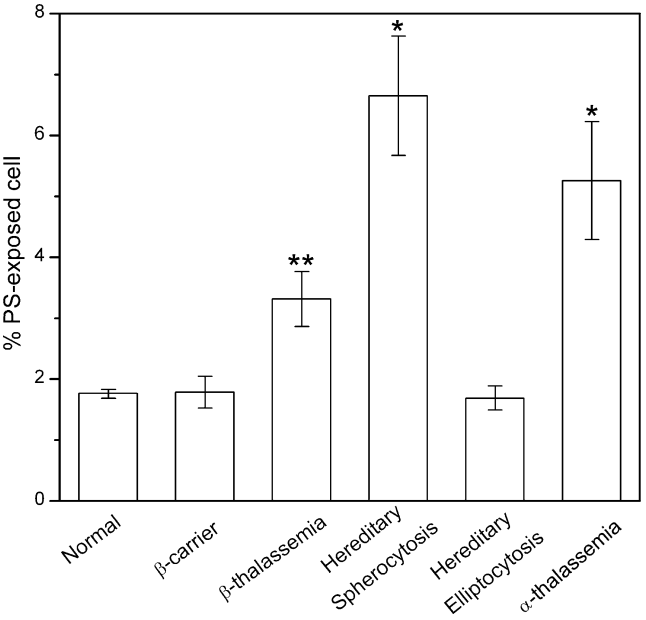

Fig. 1 The extent of loss of PS asymmetry in various red cell disorders. The *bar* indicates mean observed value for 4–18 samples and *error bar* indicates ± SEM. The statistical significance was tested using paired two-tailed student's *t*-test with $p<0.0001$ (*) and $p<0.05$ (**) except for β-carrier and hereditary elliptocytosis, which are not statistically significant

different (Fig. 2a, c). Overlay plots of the cytograms (Fig. 2) between aged and younger cells clearly indicate loss of both Neu5Ac and GlcNAc bearing glycoconjugates from the cell surface of the aged cells. The extent of PS exposure of the aged erythrocytes was found to be higher than the younger erythrocytes in normal red cells (Table 1), which is supported by earlier work [9]. However, this trend was opposite in HS as also found in HbEβ-thalassemia [16]. Figure 3a shows the percent PS exposed cells to be appreciably higher in HS compared to those in β-thalassemia intermedia and the younger cells were much more PS exposed than the aged cells in HS when in β-thalassemia intermedia, aged cells showed higher PS exposure as seen in normal and β-carriers.

WGA shows specificity for two types of *N*-acetylated sugars, *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-neuraminic acid (Neu5Ac) those are present on the red cell surface as glycoconjugates [20–22]. WGA binds to erythrocyte surface and binding was inhibited in the presence of 5 mM Neu5Ac (data not shown). PWM shows specificity for only GlcNAc residues on the erythrocyte glycoconjugates [23]. Binding of both lectins to erythrocytes decreased upon ageing. Flow cytometric analysis of WGA (Fig. 3b) and PWM binding patterns of the older and younger erythrocytes (Fig. 3c) for β-thalassemia and HS indicate changes in the cell surface glycoconjugates of erythrocytes. The same trend is also followed in case of HE (Table 1) indicating the loss of Neu5Ac and GlcNAc bearing

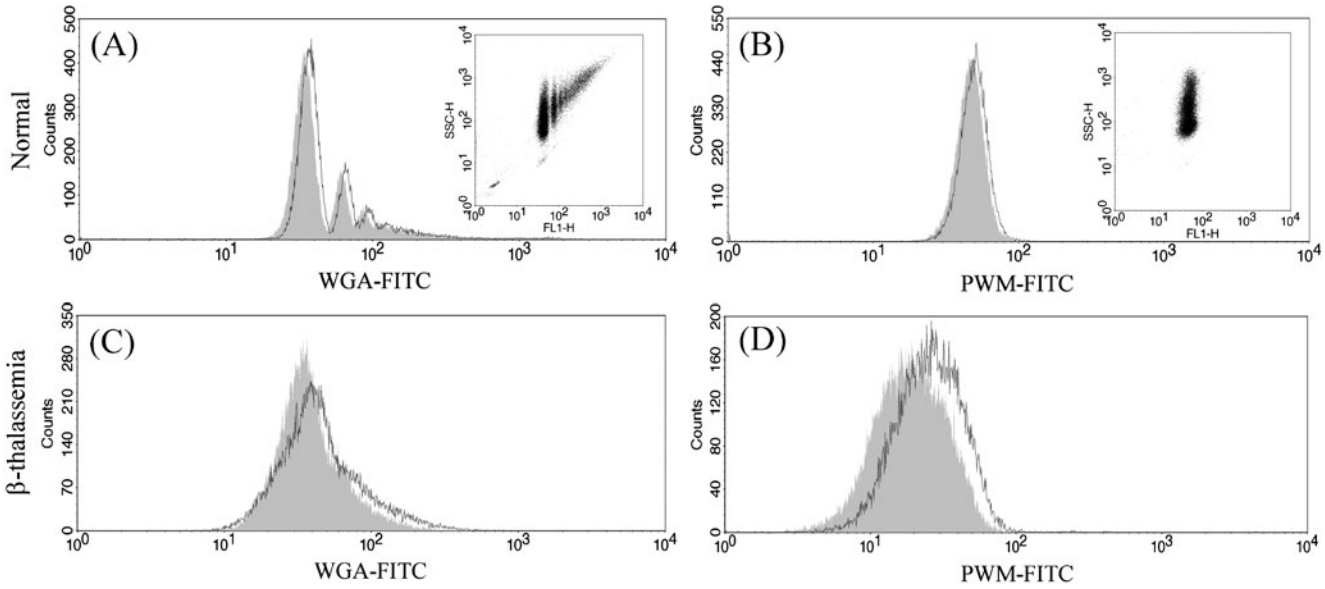

Fig. 2 The extent of lectin binding to normal (**a** & **b**) and β-thalassmic (**c** & **d**) erythrocytes. Overlaid cytograms representing aged red cells (*filled area*) and younger red cells (*black line*) treated with WGA-FITC and PWM-FITC indicating shift of MFI towards lower value. Inset of (**a**) indicates dot-plot of WGA-FITC treated erythrocytes where several populations of large multicellular aggregates are visible. Inset of (**b**) indicates dot-plot of PWM-FITC treated erythrocytes showing no such aggregates

glycoconjugates from the erythrocyte membranes upon aging.

WGA-FITC binding to erythrocytes led to the formation of multicellular aggregates indicated by appearance of several peaks in the cytogram (Fig. 2). The first major peak represents single cell population and the rest corresponding to WGA-induced cross-linked erythrocytes. The extent of reduction of WGA binding between younger and older erythrocytes in β-thalassemic individuals was ~8% from young to old cells compared to ~14% for HS patients (Fig. 3b). The PWM binding to aged erythrocytes was also found to be reduced compared to the younger ones, both in the β-thalassemia and HS cells (Fig. 3c). The extent of reduction of PWM binding between younger and aged erythrocytes, was ~17% for β-thalassemia and ~13% for HS.

Discussion

The erythrocyte membrane is asymmetric in nature with respect to the aminophospholipid, PS, which almost exclusively remains in the inner leaflet of the bilayer membrane. During *in vivo* aging PS externalization occurs and triggers an "eat-me" signal to the phagocytes [9, 10]. The low abundance of PS exposed subpopulation of erythrocytes in the circulation ensures longer lifetime of normal erythrocytes. The PS exposed subpopulation in normal aged cell is high compared to the younger cell, as summarized in Table 1. However, an opposite trend is found in case of HS, also found earlier in case of HbEβ-thalassemia, where younger cells are highly PS exposed compared to normal [16]. The extent of PS exposed cells analyzed from 18 samples of β-thalassemia intermedia, mixture of both young and old erythrocytes isolated from peripheral blood, was 3.32±0.45, significantly lower ($P<0.05$) than 5.26±0.97 in α-thalassemia.

Defects in the protein–protein interactions among the membrane skeletal proteins *e.g.* spectrin, ankyrin and band 3 could be one of the key reasons for the loss of PS asymmetry in the younger erythrocytes of HS [24]. This is also seen, however, to a much lesser extent, in erythrocytes isolated from HE individuals, where defects in spectrin and protein 4.1 have been observed [25]. In an

Table 1 Extent of PS exposed subpopulation in the aged and younger cell population and their sialic acid and GlcNAc content in various red cell disorders

Red cell disorders	% PS-exposed cell		WGA-FITC (MFI)	
	Aged cell	Young cell	Aged cell	Young cell
Hereditary Elliptocytosis	1.39±0.04	1.69±0.20	14.27±0.41	14.66±0.46
Normal	2.32±0.39[a]	1.76±0.07[a]	35.40±1.20	37.04±1.47

[a] Taken from [16]

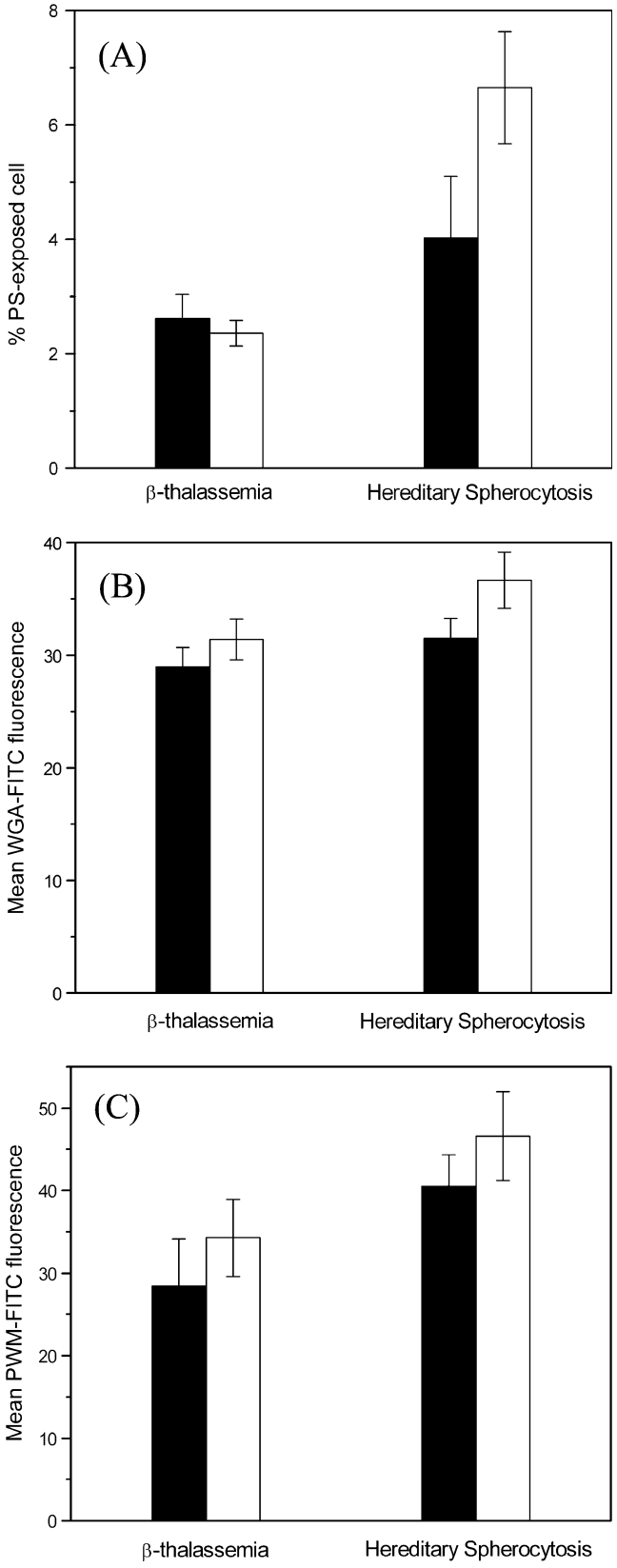

Fig. 3 Histogram representations of (**a**) %PS exposing cells in the aged and younger population of β-thalassmic (n=18) and HS (n=8) red cells along with their (**b**) WGA-FITC and (**c**) PWM-FITC binding efficiency. The aged cells are represented by filled bar and the younger cells are represented by *empty bars*. The *bar* indicates mean observed value ± SEM

earlier report, no PS-exposed subpopulations were detected in red cells from patients of HS and HE [26]. Our results are supportive towards that only in case of HE but not in agreement in case of HS. The HS patients we have studied, perhaps carry dominant mutations in ankyrin, β-spectrin and band 3, drastically affecting PS asymmetry [24]. We have earlier reported higher spectrin binding affinity of α-globin chain compared to β-globin and enhanced yield of spectrin-globin-hydrogen peroxide cross-linked complexes in thalassemia [27, 28]. Earlier work from our laboratory also indicated loss of red cell PS asymmetry in different cases of hematological malignancies [29].

Lowering of sialic acid levels on the erythrocyte surface with ageing is common and has been observed earlier [12–15]. Among the cell surface glycoconjugates, reduction in the sialic acid levels have been mostly correlated with the glycophorins, in particular, with senescence and eryptosis [13, 17]. The extent of lowering of the glycoconjugates, probed by WGA and PWM has also been found in HS, HE and the thalassemics compared to normal (Fig. 3 and Table 1). The changes measured in eight samples of HS and four samples of HE, after performing two-tailed Student's t-test, were not significant. However, the trend in the change in MFI, shown in logarithmic scale (Fig. 2), has been found to be consistent in every sample we have analyzed. Moreover, reduction of both Neu5Ac and GlcNAc levels upon treatment of Ca^{2+}/A23187 inducing vesiculation (not shown), indicated shedding of microvesicles from cell surface predominantly rich in the sialylated glycoprotein, glycophorins [16, 19]. Recent reports have suggested that, these microvesicles containing hemoglobin trapped inside and glycophorins on the surface are rapidly removed from the blood stream by Kupffer cells [30]. Also, the sialic acid content of glycophorins of the thalassemic erythrocytes were found to be ~25% lower than the same from normal [31]. Taken together, the reduction of both Neu5Ac and GlcNAc in the major glycoconjugate of the erythrocytes, presumably the glycophorins could be mediated by membrane vesiculation and are implicated in case of HS leading to faster shedding of microvesicles leaving the highly PS exposed erythrocytes accessible to the phagocytes or reticuloendothelial cells. This also explains the cause of survival of younger erythrocytes in normal individuals, where the cell surface glycophorins mask the exposed PS causing hindrance to phagocytic recognition [17].

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