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SHORT COMMUNICATION

Clearance of senescent erythrocytes: Wheat germ agglutinin distribution on young and old human erythrocytes

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To add an additional aspect to the process of recognition and removal of senescent human erythrocytes from the circulation, the binding of wheat germ agglutinin (WGA) to separated young, old and sialidase-treated human erythrocytes is evaluated with the immune-electron microscopical method. WGA/gold conjugate binding to old erythrocytes was lower (27%) than to young erythrocytes and even lower following treatment with sialidase (82%), exhibiting a clustered, non-continuous labeling pattern in all three erythrocyte populations, thus showing a possible redistribution of WGA binding sites. The decrease in bound WGA/gold particles correlates well with the previously reported decrease in surface sialic acid on old erythrocytes. The binding of WGA/gold are indicative of the changes occurring on erythrocyte membrane surfaces when interacting with different agglutinins.

Keywords: **erythrocyte, aging, WGA, electron microscopy, agglutination, distribution**

Introduction

Human erythrocytes survive for 120 days in the circulation, a process in which every day about one percent of the cells are "recognized" as senescent erythrocytes, leading to their ultimate and rapid clearance. In search for a mechanism trough which senescent erythrocytes are recognized and cleared from the circulation, a large number of investigations have approached various aspects in the multifactor process of erythrocyte aging [1–6]. In an early review [7] we raised some of those aspects which might be relevant in the process of recognition and removal of senescent erythrocytes from the circulation. Whether the structural changes in the erythrocyte membrane, the cytoskeleton, membrane sialic acid and other carbohydrate moieties, the surface negative charge, membrane antigenic determinants, lectin-binding sites, complement receptors and others are responsible for the clearance is unclear. The list of factors is still growing and the search for a definitive explanation continues.

We showed that young erythrocytes are agglutinated by soybean agglutinin (SBA) at a higher rate than old ones and the labeling density of SBA-ferritin on surfaces of young erythrocytes is about twice as high as that of old ones [8]. Sialidase treatment caused a about three times as high labeling density for young and about six times as high as for old erythrocytes. We also showed that with peanut agglutinin (PNA), neither young nor old erythrocytes agglutinate and that no binding of PNA-ferritin labeled lectin was observed prior to treatment of erythrocytes with sialidase [9].

Glycophorin, a sialic acid–rich glycoprotein of the human erythrocyte membrane represents major binding sites for some of the lectins [10]. Wheat germ agglutinin (WGA) binds specifically to N-acetylglucosamine and N-acetylneuraminic acid [11]. Quantitative assays of the binding of WGA to the erythrocyte membrane, have been performed [12], but electron microscopical observations on the distribution of their binding sites have not been reported yet. Therefore, it is of interest to show in this study how the WGA-binding sites on the surface are actually distributed in young and old erythrocytes.

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Material and methods

Blood was collected from healthy volunteer donors (blood group O, Rh^+) by venipuncture into heparinized test tubes. Young and old erythrocytes were separated by the differential flotation method [13].

Enzymatic treatment: A 10 percent suspension of separated young and old erythrocytes in PBS were incubated with 50 U/ml of protease-free sialidase (neuraminidase) from *Vibrio Cholerae* (Behringwerke AG) at 37°C for 60 min and then washed twice in PBS.

Immunochemistry and electron microscopy: Two ml of a 10% suspension of separated young and old human erythrocytes, untreated or treated with sialidase, prefixed with paraformaldehyde, was labeled with WGA/gold 10 nm (WGA/G10) particles (Sigma) for 15 min at room temperature. Unbound WGA/gold was removed by three washes with PBS, then fixed with 2 percent glutaraldehyde and postfixed with 1 percent osmium tetroxide. The samples were embedded in Epon 812 and thin-sectioned with a Sorvall ultramicrotome. Micrographs were taken with a JEM 100B electron microscope at 80 kV. Counts of WGA/gold particles were performed on micrographs of perpendicularly sectioned young, old and sialidase treated erythrocyte membranes at a final magnification of 100,000 (see table). Statistical analysis of variance 2 tailed-p Student *t*-test was applied.

Results and discussion

The binding of WGA/gold particles as visualized in the electron microscope correlates well with that of fluorescently tagged WGA on young and old and sialidase treated erythrocytes as reported by Bratosin et al. [12]. In the present study we employed the immuno-gold methodology to determine the number and distribution of WGA-specific binding sites in young, old and sialidase treated erythrocytes.

A key role for the sialic acid of the erythrocyte membrane in the determination of the life span of erythrocytes was postulated [14], and confirmed later by various investigators [15–18]. Enzymatic removal *in vitro* of sialic acid by sialidase results in the unmasking of the penultimate β-galactosyl residues of erythrocyte membrane glycoconjugates and the exposure of membrane-glycoprotein-specific lectin binding sites. Like *in vitro* enzymatic desialylation, physiological *in vivo* desialylation occurring in aging population of erythrocytes, is believed by many investigators to play a crucial role in determining the life span of erythrocytes.

Evaluation of counted WGA/gold particles from twenty thin-sectioned membranes, each for old (Figure 1A), young (Figure 1B) and sialidase treated (Figure 1C) erythrocytes, clearly shows a 27% decrease of bound WGA/gold particles per unit membrane on old erythrocytes and a more significant decrease of 82% on sialidase-treated, both young and old erythrocytes (Table 1).

The labeling and distribution of WGA/gold particles on the membrane surface of erythrocytes is shown in Figures 1 A–C,

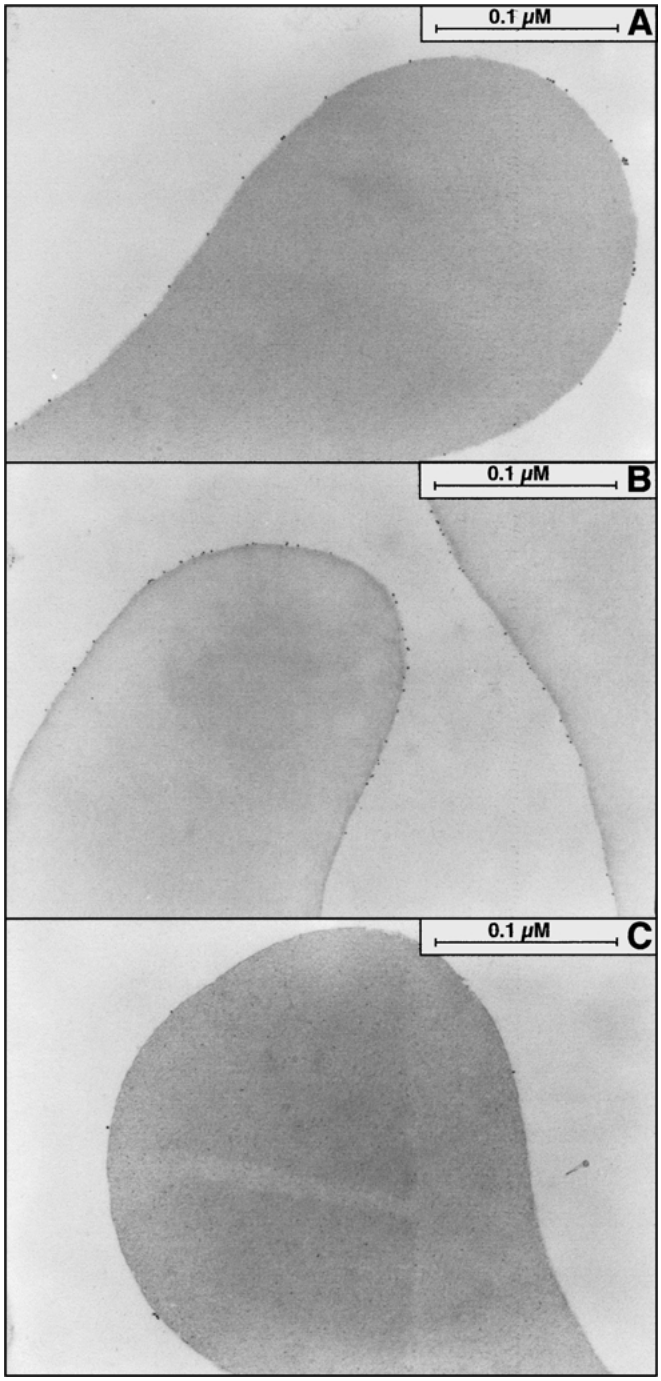

Figure 1. Labeling of human erythrocyte membranes with WGA/gold particles: Old (A), Young (B) and sialidase (Nase)-treated–young erythrocyte (C) were labeled with WGA/gold particles as described in "Material and Methods".

though not continuous and dense as the labeling with Cationized Ferritin (CF) or Colloidal Iron (CI), both cationic labels for surface negative charges as displayed on young and old erythrocytes [21–23]. Still the distribution of WGA/gold particles clearly presents a different characteristic for young and old as

Table 1. Labeling density of young, old and sialidase (Nase)-treated erythrocytes

	WGA-Gold[1]		
Sample	*Mean no. of particles/μm $\pm$ SD*	*Particles/μm (% decrease)*	*p value*[2]
Young erythrocytes	46.50 $\pm$ 2.44		
Old erythrocytes	33.85 $\pm$ 2.08	27	<0.00001
Sialidase-treated erythrocytes	8.35 $\pm$ 1.38	82	<0.00001

Indicated values are mean of 20 erythrocytes $\pm$ S.D.
[1]Distribution of WGA/gold particles on the membrane surface of erythrocytes.
[2]Significance of difference between young, old or sialidase-treated erythrocytes, as determined by a student's test.

well as for sialidase (Nase) treated erythrocytes. The number of WGA/Gold particles per unit membrane is shown in Table 1.

Evidence for structural modification in old erythrocytes has been shown in various biophysical parameters [22,253] as well as the loss of membrane surface sialic acid in the aging erythrocyte [24]. In this study the WGA-Gold binding pattern shows that different lectins react differently with human erythrocytes with respect to their binding ability to young and old erythrocytes, untreated or treated with sialidase, as reported earlier [8,9]. Sialic acid is the major factor, which determines lectin reactivity. Nevertheless, all observations demonstrate a difference of each lectin reativity to young and old erythrocytes.

This correlates well with the results obtained from agglutination measurements of young, old and sialidase-treated erythrocytes with the cationic poly-L-lysine. Agglutination was higher for old erythrocytes (not shown) due to lower mutual negatively charged repulsion forces [25]—which correlates well with their decrease in total amount of surface sialic acid as compared with young erythrocytes. Young and old erythrocytes treated with sialidase, which removed virtually all their surface sialic acid, did not agglutinate at all with poly-L-lysine (not shown). Both young and old sialidase-treated erythrocytes show a low rate of agglutination with WGA (not shown).

The data on WGA binding to old and young erythrocytes presented in this study is one more supportive evidence to the multifactorial process in the search for the clearance mechanism of senescent erythrocytes from the circulation. The cause or causes for the loss of surface sialic acid are not yet clear. One early theory was that erythrocytes in their long journey of 120 days passing several times a day narrow capillaries , become towards the end of their life span smaller and spherical, less deformable i.e. more rigid and as such the sheer stress will cause the release of membrane microvesicles, and thus susceptible to sequestration [26–28].That WGA binding is lower in old than in young erythrocytes due to desialylation was also shown by using WGA-FITC (fluorescin isothiocyanate) [29].

To conclude, loss of sialic acid may account for loss of cell surface glycoproteins, particularly sialoglycoproteins, causing a loss of about 10% of sialic acid from sialoconjugates which may result from release of sialoglycopeptides, promoted by proteolytic enzymes. This in turn may allow for the remaining anionic complexes i.e. antigens or lectin receptors to regroup more readily into clustered non-continuous binding sites thus leaving shorter or longer portions of erythrocyte membrane unlabeled as demonstrated here. Such a regrouping of lectin receptors has been observed on malignantly transformed cell types, producing clusters of attachment of lectin/ferritin labeling, compared with continuous uniform labeling on normal non-transformed ones [30–32]. Interestingly, it seems that in both aging erythrocytes and in cell malignant transformation, an increased membrane fluidity might be responsible for the clustered binding of WGA as demonstrated in the electron micrographs of erythrocytes labeled with WGA/gold particles.

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