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HEALTHY AGING RESEARCH

Age-related changes in erythrocyte membrane sulfhydryl groups and β-D-glucuronidase activity

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Abstract

Background: Aging is associated with oxidative stress but its underlying mechanisms remain poorly understood. The aim of this study was to investigate alterations in human erythrocyte membrane sulfhydryl groups and levels of β-D-glucuronidase during aging.

Methods: The study was carried with a cohort of 52 normal, healthy subjects of both sexes. They were divided into three groups: young, middle-aged and old. Blood samples were taken, and membrane sulfhydryl groups and β-D-glucuronidase activity were measured within 24 hours.

Results: Results showed significantly ($p<0.001$) decreased sulfhydryl group levels as a function of human age. A significant ($p< 0.001$) decrease in erythrocyte membrane β-D-glucuronidase activity was also observed in subjects in the 'old' group.

Conclusions: We conclude that aging is associated with systemic oxidative stress that is able to influence the integrity of cell membranes and their antioxidant capacity. Evaluation of sulfhydryl groups and β-D-glucuronidase activity provides a useful and early indication of structural and functional alterations of the red blood cell membrane during human aging.

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Introduction

Red blood cells and their membranes have always been important in biomedical study due to the important roles they play in various physiological and metabolic processes [1]. Erythrocytes have been increasingly studied because they are the most readily available human cell type. Aging is an inherently complex, progressive physiological alteration of the organism - manifested at the genetic, molecular, cellular, organ and system levels, which ultimately leads to death [2]. Although the fundamental mechanisms of aging are still poorly understood, a growing body of evidence points towards the oxidative damage caused by reactive oxygen species (ROS) as a primary determinant of aging [3]. A certain amount of oxidative damage takes place even under normal conditions; however, the rate of this damage increases during pathological conditions. Increased oxidative stress has been linked to a shortening of life span [2]. Several studies report alterations in the erythrocyte membrane during human aging [4, 5].

Besides oxidative damage of the membrane, aging has a considerable effect on its mechanical properties. Oxidative damage to sulfhydryl (-SH) groups on the membrane may be an important molecular mechanism inducing changes in membrane micro-elasticity or whole-cell deformability under conditions of physiological and pathological oxidative stress [6]. Altered redox balance with increasing age can

potentially affect the activity of any protein, since virtually all proteins contain cysteine and methionine; amino acids that are particularly susceptible to changes in oxidation reduction. Glycohydrolases, including β-D-glucuronidase, present on the plasma membrane of the erythrocytes, have been the subject of much investigation [7, 8] due to their distinct modality of anchoring, non-homogeneous distribution on the membrane micro-domains, and their involvement in specific pathologies. Beta-D-glucuronidase hydrolyzes β-D-glucuronides to glucuronic acid and an aglycone, which may be in the form of an alcohol, another organic acid, an imine, or a thiol compound. Previous work has reported alterations in β-D-glucuronidase activity in red blood cell membranes in various oxidative stress-related pathologies [9, 10].

The role of the human red blood cell membrane during aging remains poorly understood. Evidence has been gathered to suggest that aging is associated with an increase of both ROS generation rates, and the susceptibility of tissues to oxidative damage [11-13]. In addition, it has been shown that oxidative stress is crucial for the development of chronic, degenerative, age-related diseases such as diabetes, atherosclerosis, Alzheimer's disease and hypertension [14, 15]. Recently, several age-associated alterations have been reported in the membranes of human erythrocytes [16-19]. Considering these findings, the aim of this study was to investigate alterations in the human erythrocyte membrane associated with aging - specifically sulfhydryl groups and β-D-glucuronidase levels and their roles in membrane fluidity, integrity, and anti-oxidative capacity.

Methods

Study participants

The cohort for this study was a total of 52 normal, healthy human subjects of both sexes, who were divided into 'young' (n=17; 20 to 40 years), 'middle-aged' (n=20; 41 to 60 years) and 'old' (n=15; >60 years) groups. Subjects were screened for diabetes mellitus, asthma, tuberculosis and other major illnesses. None of the subjects were smokers, or were taking any medication [17]. All participants gave informed consent for their blood samples to be used for the study. The study protocol conformed to the guidelines set by and was approved by the Institutional Ethical Committee.

Venous blood was obtained from healthy volunteers by venipuncture with heparin. The blood was centrifuged at 1800 x g for 10 minutes at 4°C. After removal of the plasma, buffy coat and the top 15% of the layer of packed red blood cells, the remaining red blood cells were washed twice with cold PBS (0.9% NaCl, 10 mM Na_2HPO_4, pH 7.4). Erythrocyte ghosts from leukocyte-free red blood cells were prepared by an osmotic shock procedure [20].

Determination of erythrocyte membrane -SH group content

The number of membrane bound -SH groups were estimated according to the method described in Kitajima *et al.* [21], which is based on the ability of the sulfhydryl group to reduce 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and form a yellow-colored anionic product with an optical density (OD) of 412 nm. The concentration of -SH groups is expressed as nmol/mg protein. The protein content of erythrocyte ghosts was estimated according to Lowry's method, using bovine serum albumin as the standard [22].

Determination of β-D-glucuronidase activity

A fluorimetric assay of β-D-glucuronidase (E.C. 3.2.1.31) was performed [7], using 4-methylumbelliferyl-β-D-glucuronide as a substrate. 50 µl of ghost preparation was briefly incubated in a final volume of 250 µl, containing 25 µl of 50 mmol/l citric acid-sodium phosphate buffer at pH 6.2, and 175 µl of 4-methylumbelliferyl-β-D-glucuronide dissolved in water. The mixture was incubated in a shaker bath at 37°C for 60 minutes. The reaction was stopped and fluorescence developed by adding 750 µl of an alkaline solution (0.2 mol/l glycine-NaOH buffer containing 0.125 mol/l NaCl at pH 10.75). The control incubation mixtures (blanks) were set up using incubation mixtures lacking the ghost sample. These were incubated separately and added immediately before stopping the reaction. The enzyme activity is expressed as µU/mg protein.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA). Data are presented as means±S.D. A confidence level of $p < 0.05$ is considered statistically significant.

Results

Like most biological membranes, the erythrocyte plasma membrane is extremely rich in proteins and lipids. Because of this, erythrocyte membrane proteins are primary targets for ROS [23]. This study found that membrane -SH group content was significantly decreased ($p < 0.001$) in subjects in the 'old' group compared to those in the 'young' group (Fig. 1). Beta-D-glucuronidase enzymes present at the erythrocyte membrane are differentially susceptible to oxidative stress, according to their presence in different domains of the membrane. We also report significantly ($p < 0.001$) decreased β-D-glucuronidase activity as a function of increasing human age (Fig. 2).

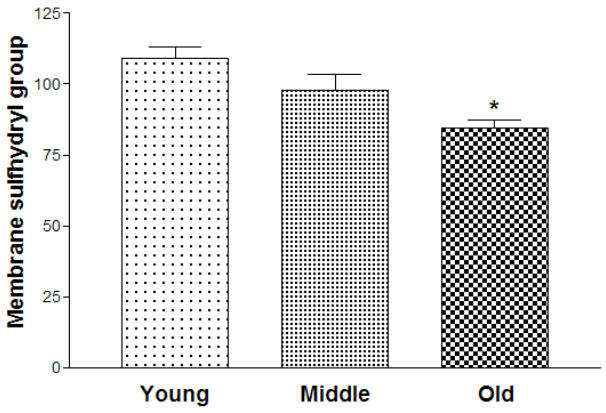

Figure 1. Erythrocyte membrane sufhydryl (–SH) group level as a function of human age

The concentration of –SH group is expressed as nmol / mg protein. Values represent mean ± S.D. * $p < 0.0001$ as compared to young.

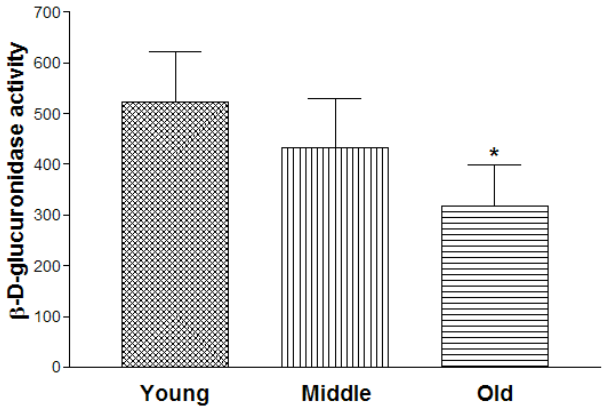

Figure 2. Erythrocyte membrane β-D- glucuronidase activity as a function of human age

The enzyme activity is expressed as μU / mg protein. Values represent mean ± S.D. * $p < 0.001$ as compared to young.

Discussion

Our results distinctly show that subjects in the 'old' group (those aged over 60 years) are more prone to oxidative stress. This, in turn, may cause a profound decrease in erythrocyte membrane fluidity, and an imbalance in redox status. It has been hypothesized that decreased levels of membrane -SH groups and β-D-glucuronidase activity during aging may be due to increased generation of ROS, which leads to oxidative damage and alters membrane fluidity. Oxidative modification of erythrocyte membrane -SH groups and β-D-glucuronidase activity is very significant in view of the important role of these components in transport, enzyme activity, membrane fluidity and integrity. In fact, the results of this study are in agreement with our previous reports on the transport functions across erythrocyte membranes, which are affected during human aging [24-26].

Red blood cell membranes are rich in proteins and polyunsaturated fatty acids (PUFA). For many years, membrane redox research has focused on the peroxidation of lipids, but due to their relatively high abundance it is now recognized that cell membrane proteins and enzymes are the main targets for cellular oxidants. Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cellular function, and changes in protein redox homeostasis are among the major molecular mechanisms leading to endothelial

dysfunction [27]. A recent study of Indian populations of different age groups revealed an age-dependent decline in intracellular reduced glutathione (GSH) concentration, which correlated with total plasma antioxidant capacity [12]. Gil *et al.* reported a slow but significant decline in intra-erythrocyte GSH concentration versus increasing age in European subjects [28]. Similar observations were also reported by Erden-Inal *et al* [29]. Human erythrocytes are rich in -SH functions, and Reglinski *et al*. previously emphasized the importance of these groups in maintaining overall cellular redox balance [30]. Beta-D-glucuronidase is present in the erythrocyte membrane in two forms; one weakly associated, and another firmly anchored [7]. Based on the results reported in the present study, it can be hypothesized that a decrease in membrane fluidity may cause the loss of the weakly associated form. Decreased β-D-glucuronidase activity will lead to the alterations in membrane fluidity observed in subjects in the 'old' group [31].

Our results are in agreement with previous studies, which demonstrated that aging is associated with a systemic oxidative stress able to influence the integrity of cell membranes and antioxidant capacity [12, 17]. This study shows that the evaluation of membrane SH groups, and of β-D-glucuronidase activity, provides useful and early indications of antioxidant capacity, and structural and functional alterations of the red blood cell membrane.

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