REVIEW

Changes in Human Red Blood Cells during Aging *in vivo*

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Abstract. The human red blood cells survive in the circulation for 120 days. Density-gradient centrifugation technique is widely used to prepare red cell fractions of different ages. It has been known that various biochemical properties of the red cells change with aging *in vivo*. If the changes in the red cell surface lead to the destruction of senescent cells, it would be of interest to know how these changes are produced concerning the cell ages. Several different mechanisms for the both specific and nonspecific recognition have been considered. The first hypothesis concerning this mechanism demonstrated that desialylated glycoporphins may play the role of recognition signal triggering the elimination of senescent red cells. There was also another hypothesis that demonstrated the red cell removal occurs due to the Fc receptor-dependent phagocytosis of the senescent cells by opsonization with anti-band 3 immunoglobulin G autoantibody. The present review provides a summary of the works in the area of red cell senescence. (Keio J Med 43 (3): 155–163, September 1994)

Key words: red blood cell, aging, senescence, aging mechanism

Introduction

It has been known that the life span of the human red blood cells in the blood stream is approximately 120 days.¹ To clarify the aging mechanism of the red cells *in vivo*, biochemical characterization of the red cells of different ages has been investigated by numerous investigators. In particular, the relationship between the aging of the red cells and the intracellular components and/or the chemical composition of the red cell membranes were investigated.^{2–20}

Today, the majority of the investigations concerning the red cell senescence is focused on the mechanism by which the aging of the cell promotes its destruction. The first hypothesis that the reduction in sialic acid induced a rapid sequestration of the red cells from circulation was proposed to explain the senescence and destruction of the red cells.^{4,21–26} A number of studies have provided the evidence that the IgG antibody that is bound to newly exposed sites on the membrane surface by desialylation would be effective in promoting macrophages' phagocytosis of the old red cells obtained from density separation method.^{26–29}

Kay and coworkers^{30–34} proposed another hypothesis

that one component of the aging process, namely a senescent antigen, might involve proteolytic cleavage of band 3 protein on the red cell membranes.^{31–33} They also mentioned that the old red cells appeared to have autologous serum antibodies bound to their surfaces.^{35,36}

The present review is attempting to summarize the current biochemical researches on the changes and the destruction mechanism of the senescent red cells.

Methods for Age-Correlative Separation of Red Blood Cells

To clarify the age-associated changes of the red cells and the actual process of senescent cell destruction, many investigators have attempted to isolate the different population of the red cells. Density-gradient centrifugation has been a widely used method.^{28,29,37–42} The investigation with radioactive iron labeled cells revealed that the density of human red cells increased with aging.^{6,43} This fact showed that the bottom layer of centrifuged cells (high-density cells) contained preponderantly old red cells, while young red cells were concentrated in the top layer of centrifuged cells (low-density cells). Piomelli and Seaman⁴⁴ showed that the

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density separation, validated by much experimental evidence, remained a most useful technique for the study on the mechanism of red cell aging. Among the density-separation techniques, a simple method which gave reasonably good separation was centrifugation at 30°C, in an angle centrifuge at 39,000 X g.³⁷ The systems were devised for separating the red cells using the discontinuous gradient media of bovine serum albumin,³⁸ arabino-galactane,³⁹ phthalate esters⁴⁰ and polyvinyl-pyrrolidone-coated colloidal silica matrix (Percoll®).^{28,29,41}

Thompson *et al*⁴⁵ adopted the counterflow centrifugation or elutriation technique, which separates the cells primarily on the basis of cell size. It was suggested that this technique provides some degree of age-dependent separation by using labeling studies in human subject.^{45,46}

Marks and Johnson⁴⁷ used a method by alternations in osmotic fragility. Differences in osmotic resistance between the young and the old red cells provided a basis for separating the whole cell populations into different age fractions.

The author reported a new approach to cell separation based on possible variations in surface charge.¹⁴ This method used the filtration of the red cells through alkyl-Sepharose gels with various lengths of alkyl chains. Density-separated cell populations showed slightly different affinities for the octyl-Sepharose 6MB gel.

Changes in Red Blood Cells during Aging *in vivo*

Morphological characterization

The red cells obtained from fresh human blood were fractionated into three fractions, that is, top (low-density), middle and bottom (high-density) layers by the density gradient centrifugation.⁴⁸ When compared with the top layer containing young red cells, the old red cells of the bottom layer showed decreases in mean corpuscular diameter (MCD), mean corpuscular surface area (MCSA) and mean corpuscular volume (MCV), but an increase in mean corpuscular average thickness (MCAT) as shown in Table 1.

The old cells isolated from normal human blood showed a marked reduction in their capacity to deform under shear stress.^{49,50} In addition to the contribution of increased intracellular viscosity, the old cells also appeared to have reduced surface area.^{49,50} It was thought that the deformability loss was mostly due to an increase in membrane rigidity. Whereas MCV decreased linearly with the red cell age, mean corpuscular hemoglobin concentration of the young red cells was lower than that of the old red cells, which indicated that there was no loss of hemoglobin in the red cells during aging.^{3,10}

Nakao *et al*⁵¹ described that the adenosin triphosphate (ATP) level in the red cells decreased gradually with their life-span in the blood vessels. They also suggested that the decrease of the ATP level caused the destruction

Table 1 Characteristics of Human Red Cells of Top (Young) and Bottom (Old) Layers Separated by Density Gradient Centrifugation^{15,48}

Fraction	Top Layer (18.3 ± 1.7%)	Bottom Layer (16.8 ± 2.7%)	Relative Percentage (%) ^a
Density (g/ml)	<1.092	>1.100	
MCD (μm)	8.3 ± 0.9	7.2 ± 1.7	86.7
MCAT (μm)	1.6 ± 0.4	1.8 ± 0.3	112.5
MCSA (μm ²)	134.5 ± 20.6	105.9 ± 13.6	78.7
MCV (μm ³)	85.3 ± 0.9	71.8 ± 0.6	84.2
ATP Level (μmol/10 ¹⁰ cells)	0.81 ± 0.09	0.64 ± 0.12	79.0
G-6-PDH Activity (U/10 ¹⁰ cells)	1.90 ± 0.25	0.63 ± 0.04	33.2
2,3-DPG (μmol/ml cells)	5.74 ± 0.82	4.36 ± 0.64	76.3
Sialic Acid (μg/10 ¹⁰ cells)	135.9 ± 11.9	109.9 ± 5.7	80.9

^a Relative percentage was following equation: (Values of bottom layer red cells/Values of top layer red cells) × 100.

of the red cells *in vivo*. It was demonstrated that ATP levels in the old red cells were significantly lower than those in the young red cells (Table 1). Their study suggested that the viability of the red cells is not based on the shape but on the ATP level.

Intracellular component

Since hemoglobin constitutes the major mass of the red cells, it primarily determine their density.^{3,10} The cell density depends also on the water content of the red cell, which is regulated by the quantity of slowly permeable cytoplasmic constituents, primarily K and Na.¹³ The red cells with the higher-density are thought to represent the senescent population and contain less K and Na than the rest of cell populations. This nature of the red cells prompted the investigations of the transport pathways involved in regulation of intracellular K and Na.

The ATP level,^{4,8,14,15} glucose-6-phosphate dehydrogenase (G-6-PDH) activity^{2,5,7,11,14,15} and 2,3-diphosphoglycerate (DPG) content⁸ decreased in aged cells as shown in Table 1. It was also demonstrated that the activities of intracellular enzymes such as pyruvate kinase,⁶ cholinesterase,^{6,11} glutamate-oxaloacetate transaminase,^{2,9,10} and Na/K ATPase⁹ were significantly lower in the old red cells than in young ones.

It was reported that glutathione of intercellular component decreased in aged cells.² The old red cells showed moderately increased amounts of methemoglobin, which was accompanied by a decrease in cytochrome b₅ reductase and its cofactor, cytochrome b₅.¹² The characterization of density-separated cells provided the evidence that the older cells suffered cumulative oxidative damage.^{52,53}

Membrane component

The changes in the carbohydrate content of the human red cell membranes during the aging have been most widely investigated.^{5,6,11,15–18,20,42} Baxter and Beeley⁵ reported that the oldest cell fractions consistently showed a sialic acid content which was approximately by 15% lower than that of the cells at the top of the gradients (young). The study by Yaari *et al*¹² showed that there was significant difference in electrophoretic mobility between the young and the old red cells. The decrease of sialic acid content in the old cell population can be attributed to a reduction in membrane surface area of the red cells.

It was also reported that the other carbohydrate contents, such as neutral sugars and hexosamines, of the cell membranes were significantly higher in the young red cells than in the old ones.^{6,11,15–18,20} From these experimental results, it was presumed that the composition of membrane carbohydrates changed during cell

aging of the human red cells, and that, either glycoprotein or glycolipid, or both types of complex carbohydrate changed.

Lutz and Fehr⁵⁴ reported that the glycophorins extracted from the membranes of the young and the old human red cells had the same sialic acid content when referred to a relative measure of the number of glycophorins. They concluded that the human red cells lost intact glycophorin on the membranes during the red blood cell senescence.

Gattegno *et al*⁶ made a comparative study on the elution patterns in Sephadex G-25 gel filtration of membrane-surface sialoglycopeptides liberated from the young and the old human red cells with either pronase or trypsin. They reported that there were marked differences in the ratio of the sialic acid to neutral hexose of the main fractions of membrane-surface sialoglycopeptides. By authors' previous investigation,¹⁵ the amount of sialoglycopeptide mixture released from the young red cells by trypsin treatment was significantly higher than that from the old ones. The sialoglycopeptide from the young cells showed a greater proportion of sialic acid than that from the old ones.¹⁵

Cohen *et al*⁸ reported that both cholesterol and phospholipid phosphorus decreased similarly with the increasing cell density.

Kadlubowski¹⁹ demonstrated using SDS polyacrylamide gel electrophoresis that the aging *in vivo* of the red cells was virtually associated with significant alterations in the protein complement of the cell membrane.

Blood-group activity and lectin receptor activity

Since the blood group activity and lectin receptor activity of the red blood cells are attributed to the sugar residues in the terminal positions of the carbohydrate chains of glycolipids and/or glycoconjugates on their membranes,⁵⁵ the relationship between the red cell aging and the changes in biological activities of the cells to various anti-sera and lectins were examined by several research groups.^{3,11,20,48,56–60}

Greenwalt *et al*^{3,56} studied the relative agglutinability of the young and the old human red cells with the ABH, I, MN and Rh reagents in an assay method by the continuous-flow autoanalyzer system. They found that the old red cells were more strongly agglutinated by ABH reagents than the young ones. The author⁶⁰ examined the number and distribution density of ABH and MN antigen sites on the surfaces of the young and the old red cells using ¹²⁵I-labeled blood group specific antibodies and lectins. It was found that increases in the distribution density of A₁, B, H, M, N and *Vicia graminea* N antigen sites were observed in the old red cells compared with the young cells. From these results, it was presumed that ABH and MN antigens were not

affected by enzymes including glycosidases and proteinases during the aging process *in vivo*.

Smalley and Tucker⁵⁹ found, using immunoelectron microscopy with hemocyanin-conjugated antiglobulin, that the heterogeneity of the distribution density of blood group A₁ and A₂ antigen site was not age-related.

In the authors' study,⁴⁸ compared with the young red cells, decreases in the number and distribution density of receptor sites for lectins including *Limulus polyphemus* (LPA), *Canavalia ensiformis*, *Triticum vulgaris* (WGA), *Ricinus communis* (II), *Glycine max* (soybean) and *Bauhinia purpurea* on the cell surfaces were observed in the old red cells as shown in Table 2. The number of WGA receptor sites on the cell surface was 2% lower in the old red cells than in the young ones, whereas the distribution density of WGA receptor sites on the cell surface was 27% higher in the old than in the young red cells (Table 2). On the basis of the invariability of the number of WGA receptor sites and the remarkable increase in the distribution density of the same receptor site during aging *in vivo* and considering the marked decrease in cell surface area found in the old red cells, it is probable that the human red cells shrink to reduce their surface areas without being vesiculated during aging *in vivo*. As LPA is a lectin which is capable of binding of sialic acid residues in glycoconjugates,⁶¹ the LPA receptors released from the red cell membranes were presumed to be certain sialic acid-containing glycoconjugates. It is inferred that the human red cell aging is accompanied by elimination of some glycoconjugates which have affinities for these lectins.

Choy *et al*¹¹ reported as a result of examining the activities of the young and the old red cells against some lectins that a decrease of sialyl residue and an increase

of galactosyl and N-acetylgalactosaminoyl residues in the terminal position of the carbohydrate chains of membrane glycoconjugates are observed in old red cells. However, in the authors' study, both the young and the old red cells exhibited very weak binding activity for ¹²⁵I-labeled PNA (*Arachis hypogaea*, peanut).⁴⁸ Skutelsky *et al*⁵⁸ found, from electron microscopic studies of the red cell membrane surfaces, that neither young nor old red cells reacted with ferritin-conjugated PNA. It is known that MN active sialoglycoproteins change into asialoglycoproteins,^{62,63} that is, Thomsen-Friedenreich (T) antigen which can be detected with PNA when sialic acid residues are removed from glycoproteins by action of sialidase.^{58,64} From the authors' result⁴⁸ and the observation by Skutelsky *et al*,⁵⁸ it is implied that the human red cells are not exposed to sialidase during the aging *in vivo*.

Survival of Senescent Red Blood Cells

Role of bound sialic acid on red blood cell membranes

If the changes in the red cell surface lead to the destruction of senescent cells, it would be of interest to know how these changes are produced as the cell ages. Several possibilities were considered, and density-separated cells were examined for the evidence that such processes might have occurred (Table 3). Several different mechanisms including both specific and nonspecific for the recognition were summarized in Table 4.

The old red cells were found to show a significant reduction in the total surface charge and in the sialic acid content.^{10,42} The first possibility to be proposed was that a loss of the highly negatively charged sialic acid residues

Table 2 Number and Distribution Density of Lectin Receptor Sites on Young and Old Human Red Cell Membranes⁴⁸

Population		<i>Limulus</i> <i>Polyphemus</i> (LPA)	<i>Canavalia</i> <i>Ensiformis</i> (Con A)	<i>Triticum</i> <i>Vulgaris</i> (WGA)	<i>Ricinus</i> <i>Communis</i> (II) (RCA-II)	<i>Phaseolus</i> <i>Vulgaris</i> (E) (PHA-E)	<i>Glycine Max</i> (SBA)	<i>Bauhinia</i> <i>Purpurea</i> (BPA)
Young Red Cells	Number of receptor site × 10 ⁶ /cell	0.76 ± 0.09	0.57 ± 0.02	4.10 ± 0.59	1.32 ± 0.35	0.57 ± 0.10	0.33 ± 0.03	0.47 ± 0.03
Old Red Cells	Number of receptor site × 10 ⁶ /cell	0.51 ± 0.03	0.35 ± 0.04	4.00 ± 0.08	0.93 ± 0.39	0.44 ± 0.10	0.18 ± 0.02	0.32 ± 0.01
Relative Percentage (%) ^a		67	61	98	70	77	55	68
Young Red Cells	Distribution density (10 ⁴ μm ²) ^b	0.57 ± 0.06	0.42 ± 0.02	3.00 ± 0.46	0.98 ± 0.26	0.42 ± 0.08	0.25 ± 0.02	0.35 ± 0.02
Old Red Cells	Distribution density (10 ⁴ μm ²)	0.48 ± 0.03	0.33 ± 0.04	0.38 ± 0.08	0.88 ± 0.37	0.42 ± 0.09	0.17 ± 0.02	0.30 ± 0.01
Relative Percentage (%) ^c		84	79	127	90	100	68	86

Value represents mean ± S.D. of ten experiments.

^a Relative percentage was following equation: (Number of receptor sites of old red cells/Number of receptor sites of young red cells) × 100.

^b Distribution density was following equation: Number of receptor site/mean corpuscular surface area of cell membrane.

^c Relative percentage was following equation: (Distribution density of old red cells/Distribution density of young red cells) × 100.

Table 3 Summary of Reported Alterations in Aged Red Cells

Alterations	References
Morphological Characterization	
Mean corpuscular diameter	48
Mean corpuscular average thickness	48
Mean corpuscular surface area	48
Mean corpuscular volume	10, 48
Intracellular Component	
Glucose-6-phosphate dehydrogenase (G-6-PDH)	2, 5, 7, 11, 14, 15
Pyruvate kinase	6
Cholinesterase	6, 11
Glutamate-oxaloacetate transaminase	2, 9, 10
Na/K ATPase	9
Cytochrome b ₅ reductase	12
Superoxide dismutase	52
Glutathione peroxidase	53
Glutathione reductase	53
Glutathione	2
K and Na	13
Adenosin triphosphate (ATP)	4, 8, 14, 15
2,3-diphosphoglycerate (DPG)	8
Hemoglobin	3, 10
Membrane Component	
Total surface charge and sialic acid	5, 42
Carbohydrates	6, 11, 15, 16, 17, 18, 20
Cholesterol and phospholipid	8
Blood-group Activity and Lectin Receptor Activity	
Blood group activity	3, 20, 56, 59, 60
Lectin receptor activity	11, 48, 57, 58

Table 4 Signals Appearing on Red Cell Membrane

Signal Suggested	References
Desialylation of Sialoglycoprotein	4, 23, 24, 25, 26
Exposure of Galactosyl Residues	11, 26, 27, 28, 29, 66
62 K Glycoprotein	33, 34
Band 3 Protein Oligomers	36
IgG Antibodies Bind to Band 3 Protein	30, 31, 32, 33, 34, 35, 36, 72, 73

from membrane glycoproteins reduced the repulsive interaction between the red cells and the phagocytes, thus permitting ingestion of the changed red cells.^{23–26} This hypothesis was suggested to a new concept of the role of carbohydrates in regulating the serum survival time of the plasma glycoproteins by Ashwell and Morell⁶⁵ in 1974. They reported that a galactose was exposed as the terminal nonreducing sugar of protein-linked carbohydrate chains by treating the native protein with neuraminidase, and served as a specific determinant for the hepatic recognition of the sialic acid-deficient molecules (Fig 1(A)).

The study by Jancik *et al*^{23,24} was carried out to determine the physiological significance of sialyl residues to the viability of the red cells in the circulation. They

reported that the release of bound sialic acid on the red cell membranes by neuraminidase treatment led to a significant reduction of the lifetime of these cells in circulation as shown in Fig 1(B). These experimental results led us to believe that the diminished surface charge may play a role in the removal of the senescent red cells from the circulation.

Choy *et al*¹¹ suggested that the newly exposed galactose and galactosamine residues in the desialylated glycoproteins may serve as recognition signals triggering the elimination of the senescent red cells from circulation. Alderman and coworkers^{28,29} concluded that age related antigenic determinants present on the senescent human red cells were exposed by desialylation of the major sialoglycoprotein component of the red cell membrane.

Fig 1 Plasma survival time of labeled asialoglycoproteins (A),⁶⁵ and radioactivity profiles in the whole blood after reinjection ⁵¹Cr labeled human red blood cells (B).²⁴ C: control, NT: neuraminidase-treated red blood cells.

The study of Danon and Marikovsky²⁷ suggested that the binding site for antibody on the senescent human red cells might have been the Thomsen-Friedenreich antigen, which was unmarked when sialic acid residues were removed from the membrane glycoprotein, and/or glycophorin.

There were many of the investigations^{6,15-18,20} saying that the old red cells decreased in contents of sialic acid accompanying with the various carbohydrates such as fucose, galactose, mannose, N-acetylgalactosamine and N-acetylglucosamine of the membranes. Lutz and Fehr⁵⁴ reported that the glycophorins extracted from membranes of the young and the old red cells have the same sialic acid content. From these chemical findings,^{6,15-18,20} it seemed that the human red blood cell aging is accompanied by the elimination of some sialoglycoconjugates.

Binding of autologous antibody on senescent red blood cell membranes

The effect of sialic acid on the red cells survival were extensively studied. It was found that the reduction in sialic acid induced a rapid sequestration of the red cells from the circulation. This mechanism might have mean that the macrophage interact with the cytophylic

antibodies bound with the component of the newly exposed membrane sites by desialylation. Aminoff and his colleagues^{25,26,66} have suggested that the selective removal of sialic acid generated specific recognition sites that promoted phagocytosis. The results of their studies with human red cells suggested that the interaction between the old red cells and the liver macrophages leading to phagocytosis was able to be blocked by carbohydrates that had a β -galactosyl structure. The glycopeptide prepared from the old red cells by trypsin treatment had a terminal β -galactosyl residue that was absent in preparations from the young cells. It was proposed that the generation of the peptide on the surface of the aging red cell provided a signal that was recognized by a lectin-like receptor on macrophages, thereby promoting phagocytosis by an antibody-independent mechanism.

Thus the surface recognition may provide the ultimate signal for cell destruction, but the appearance of the signal on the cell surface may be associated with a degradation of intercellular function. Other studies with rats provided firm evidence that desialylated red cells and high-density red cells were removed by different mechanisms.⁶⁷ An extensive characterization of density-separated red cells failed to provide definitive evidence for a direct role for metabolic failure in the

destruction of senescent cells.

En(a-) red cells lack the major sialoglycoprotein (glycophorin A) which carries the MN blood group determinants.^{68–71} En(a-) cells have less than one-half the normal sialic acid content, but there is no clinical abnormality.⁶⁸ Tanner and Anstee⁶⁸ reported that band 3 from En(a-) cells contained greater amounts of galactose and N-acetylglucosamine. Gahmberg *et al*⁶⁹ reported that band 3 contained two oligosaccharides, and that the more complex oligosaccharide had a higher molecular weight in En(a-) cells than in normal cells. Lutz and Fehr⁵⁴ reported that glycophorins A and B isolated from membranes of young and old red cells had the same sialic acid contents. These experimental results also indicated that the desialylated red cells and the old red cells were removed by different mechanisms.

Thus it was suggested that there existed a similar lectin-like receptor mechanism in human, and that there were observations by others implying an antibody-dependent mechanism for phagocytosis of senescent human red cells. Kay *et al*^{30–34} reported that one of the mechanisms of the red cell removal was the Fc receptor-dependent phagocytosis of the senescent cells opsonized with anti-band 3 immunoglobulin G (IgG) autoantibody. The senescent antigen to which anti-band 3 IgG antibody binds on the cells is thought to be a 62000-dalton fragment of band 3 molecules.^{33,34} Lutz *et al*^{35,36} suggested the binding of anti-band 3 IgG antibody to the red cells treated with diamide and the antigen to be the cross-linked band 3 molecules. Beppu *et al*⁷² had shown that anti-band 3 IgG antibody bound to the red cell oxidatively damaged by iron ion catalyst, and suggested involvement of disulfide formation of the membrane proteins in the generation of the senescent antigen. They also suggested that naturally occurring IgG autoantibody against band 3 glycoprotein of human red cell membranes recognized the red cells modified with oxidizing as well as the senescent red cells, and that the antigenic determinants of band 3 are located in sialylated poly-N-acetyllactosaminyl sugar chains.⁷³

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