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SENESCENT ERYTHROCYTES: MODIFICATION OF RHEOLOGIC PROPERTIES, ANTIGENIC EXPRESSION AND INTERACTION WITH MONOCYTES

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Abstract Human erythrocytes have a well-defined lifespan of 120 days. Their eventual removal from circulation is a complex process affected by many cellular parameters, making them susceptible to sequestration in the spleen and other organs. The purpose of this study was to investigate putative changes in rheologic properties, antigenic expression and interaction with monocytes of senescent erythrocytes (SE). SE and young erythrocyte (YE) fractions were obtained by differential centrifugation from 20 healthy donor blood samples. Membrane rheomechanic properties (by difractometric method), ABO and MN antigens reactivity and erythrophagocytosis by peripheral monocytes were investigated in each fractions. SE showed a little decrease in the deformability index and an increase of both membrane elastic modulus and surface viscosity. The studies performed indicate a decreased expression in the antigens of both blood group systems studied ($p < 0.01$) and an increased rate of erythrophagocytosis by monocytes in SE compared to YE ($p < 0.01$). The significant modifications in the biomechanic properties of senescent red blood cell membrane and the loss of antigenic expression could lead to physiological phagocytosis.

Resumen ***Eritrocitos senescentes: modificación de las propiedades reológicas, expresión antigénica e interacción con monocitos.*** Los eritrocitos humanos tienen una vida media de 120 días. Su remoción de la circulación es un proceso complejo afectado por diversos parámetros celulares, que conduce al secuestro en el bazo y otros órganos. El objetivo de este trabajo fue investigar modificaciones en las propiedades reológicas, expresión de antígenos eritrocitarios e interacción con monocitos de los eritrocitos senescentes (ES). Se realizó la separación de las fracciones ES y eritrocitos jóvenes (EJ), por centrifugación diferencial de 20 muestras de sangre de dadores voluntarios sanos, para analizar las características de las mismas. En cada una de las fracciones se investigó las propiedades reomecánicas de la membrana (por el método difractométrico), la reactividad de los antígenos de los sistemas ABO y MN y la eritrofagocitosis con monocitos obtenidos de sangre periférica. Se observó en los ES una pequeña disminución en el índice de deformabilidad y un incremento en el módulo elástico de la membrana y en la viscosidad superficial. En esta fracción se obtuvo una expresión disminuida en los antígenos de ambos sistemas ($p < 0.01$) y un aumento de la eritrofagocitosis ($p < 0.01$). Las modificaciones de las propiedades biomecánicas de la membrana, la pérdida de reactividad antigénica y la interacción con monocitos podrían ser utilizadas para evaluar la fagocitosis fisiológica.

Key words: senescent erythrocytes, rheologic properties, antigenic expression, interaction with monocytes

The mammalian erythrocyte has a well-defined lifespan that is genetically pre-programmed and species specific. However, despite rather intense investigation, the biochemical mechanism that determine the red blood cell (RBC) lifespan are not yet well understood and the lack of progress in this field can be directly attributed to the difficulty of isolating aged erythrocytes[1, 3].

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Originating in the bone marrow, the normal human erythrocyte circulates for an average of 120 days. Its eventual removal from circulation is generally believed to be a consequence of declining deformability, making it susceptible to sequestration in the spleen and other organs, wherein the erythrocyte must negotiate extraordinarily narrow passages. The critical determinants of the deformability characteristics of young or senescent erythrocytes have yet to be clearly sorted out. Changes in cell shape, in the viscoelastic properties of the membrane, and in the cytoplasmic viscosity are all potentially involved[4, 6].

The determination of erythrocyte lifespan is a complex process affected by many cellular parameters. Among

the factors that have been proposed to play an important role in erythrocyte senescence, the accumulation on the erythrocyte membrane of autologous IgG has received much attention, because it provides a direct mechanism for removal of senescent erythrocytes via phagocytes[7]. Modification of protein band 3, either by proteolytic cleavage or aggregation, has been suggested to lead to the formation or exposure of an antigenic site resulting in the accumulation of cell surface IgG[8].

Some authors[3, 9, 10] have proposed that a decrease of negative charge on the erythrocyte membrane would significantly reduce the repulsive interaction between red cell and phagocytes and thus facilitate phagocytosis. Specifically, sialic acid, the principal source of membrane negative charge, is lost from membranes of high density red cells and the total surface negative charge is reduced. Desialylation (10-15%) observed in senescent red blood cells decreases the negative charge density and produces membrane alteration responsible for the selective removal of these cells.

The purpose of this study was to investigate putative changes in some biological properties of senescent erythrocytes such as rheologic characteristics, antigenic expression and interaction with monocytes. Such changes are probably due to the erythrocyte aging process and can be considered as responsible for their sequestration and subsequent destruction in the spleen.

Material and Methods

Preparation of RBCs:

Blood samples were drawn by venipuncture from 20 hematologically normal volunteer donors, collected into 10 ml vacutainers containing 1 ml of 3.8% sodium citrate and centrifuged at 1 000 g for ten minutes, to concentrate the red cells to a hematocrit of 80%. Density separation was effected by short-duration, high speed centrifugation of these concentrated (0% hematocrit) red cells at 10 000 g for 15 minutes. Following centrifugation, the top and bottom 10% fractions were removed and designated as the young and old cells, respectively. The cells were then washed twice in isotonic phosphate-buffered saline (PBS, 0 005 ml/l KH_2PO_4 + Na_2PHO_4, pH 7.40, 290 ± 5 mosm/kg) plus 10 mmol/l dextrose. After the second wash, the cells were resuspended either in plasma or saline to perform rheologic studies, antigenic reactivity and interaction with monocytes.

Rheologic studies

Rheological measurements of young and senescent erythrocytes were performed with an Erythrodeformeter[11]. This device applies the diffractometric method on red blood cells undergoing a definite fluid shear stress. Deformability index (DI) is calculated from photometric readings performed on the elliptical diffraction pattern generated by the shear elongated cells. Recorded curves of creep and recovery of shear deformed cells are used in the calculations of membrane rheological properties[12]: relaxation time (t_c), membrane elastic modulus (μ) and membrane surface viscosity (η_m). 100 μl of packed young and senescent erythrocytes were resuspended in 4 ml of Polyvynilpyrrolidone (PVP 360-Sigma, MW 360 000) disolved in PBS. The viscosity of this suspending medium was 22 ± 0.5 mPa.s at 23°C as measured in a Wells-Brookfield DV-III coneplate viscometer. The Erythrodeformeter was operated at room temperature, controlled and maintained within the range 23 ± 0.5°C. The lower disk rotation was fixed at 60 rpm for static determinations to give a shear rate of $1257s^{-1}$ and a shear stress of 27.6 Pa. Light intensity corresponding to the major axis of the elliptical pattern was registered during the start and the stop of the lower disk rotation to obtain creep and recovery data respectively. Photometric signal was digitalized and stored in an A/D converter. 256 readings were performed at constant time intervals over a total scanning time of 110 ms to obtain the creep curve. The same number of readings was taken during 350 ms to obtain the recovery curve. All data were stored in the memory unit of the A/D converter. After storage completion of both curves, the stored data were retrieved over the A/D converter as a curve of cell deformation versus time. Retrieval in digital form were transferred to the PC and processed according to a pre-programmed numerical process.

Antigenic reactivity

Antisera and Lectin: Anti-A, anti-B, anti-M y anti-N commercial sera were used. Lectin were obtained from *Ulex europaeus.*

Titre and Score: Standard two-fold serial dilutions of antisera or lectin, depending on the red blood cell phenotype, were prepared to obtain their titre and score with both young and senescent erythrocytes.

Percentage of agglutination: Experimental Procedure[13]: 0.1 ml of the appropiate two-fold serial dilution was mixed with 0.1 ml of either young or senescent erythrocytes. Each tube was then carefully rotated during 30 seconds and left for 10 minutes at the optimal antigen-antibody reaction temperature. Following this period of sensitization the tubes were centrifuged 1 minute at 80 g in order to accelerate agglutination. Such low speed was chosen to avoid the formation of false agglutinates which should be disaggregated prior to the resuspension in glucose and the subsequent photometric reading. After centrifugation, 4 ml of gluclose solution (29% w/v of glucose powder, Sigma G-5000, in PBS) were added to each tube and then stoppered and carefully reversed for 4 or 5 times to resuspend agglutinated and free cells. The glucose solution is a medium of high density and viscocity in which the reaction product (agglutinates) remains in stable suspension without settling during the period while photometric readings are taken and recorded. Immediately afterwards, each tube was placed into the spectrophotometer and the optical extinction (E) was measured at 410 nm. A suspension of free cells without agglutinins was used for each double dilution series as a control suspension. It was considered as 0% agglutination in the photometric readings. This control suspension was prepared by mixing 4 ml of standard glucose solution, 0.1% of standard cell suspension and 0.1 ml of PBS. The percentage of agglutination (AG%) was calculated as:

$$100 - [(E_i - E_1)/E_0 - E_1)] \times 100$$

where E_i is the absortion from the analyzed tube, E_1 is the absortion of the reagent blank and E_0 is the absortion of the control suspension.

Interaction with monocytes

Pheripheral blood monocytes were obtained through their glass-adhering property. The adhered cells (90% monocytes according to the morphological criteria with May Grünwald

Giemsa method and the presence of peroxidasa and esterase) were overlayered with 0.5% different suspensions (n = 20) of senescent and young erythrocytes in 20% serum AB with Hank's solution. The mixture of cells was then incubated for 3 hours at 37°C, and thereafter the unbound erythrocytes were washed out and the cells on the glass were fixed with methanol, stained by the May Grünwald Giemsa method and observed under the light microscope. Two hundred cells taken from different glass spots were analyzed to determine the percentage of monocytes with phagocytosed and adherent red cells (active phagocytic cells, APC). Negative and positive controls were performed simultaneously, using unsensitized and IgG anti-Rh sensitized red cells[14].

These results indicate a decreased antigenic expression in the population of senescent eythrocytes of both blood group systems studied.

The interaction of senescent erythrocytes with peripheral blood monocytes reflects a signifative increase in the percentage of active phagocytic cells when compared to young erythrocytes. The percentage of active phagocytic cells with senescent erythrocytes was lower than those obtained with in vitro sensitized red blood cells but higher than those with normal red blood cells (Table 4).

Results

Data obtained from the rheologic studies are expressed in Table 1. Senescent cells showed only a little decrease in the deformability index. However an increase of both, membrane elastic modulus and surface viscosity have been observed in senescent erythrocytes when compared with young erythrocytes.

Creep curves (Fig. 1) obtained with young and senescent erythrocytes show a higher slope of the ascending part for young red blood cells than for senescent ones. This implies a slower deformation (higher elastic modulus, higher rigidity) of senescent erythrocytes. The elongation step between the begining and the end of these curves is lower in senescent than in young red blood cells owing to a lower deformability of senescent erythrocytes. Relaxation curves (Fig. 2) obtained with senescent erythrocytes descend more quickly than that obtained with young red blood cells, as a consequence, the relaxation time is lower in old cells.

The assays performed to evaluate ABO system antigens expression (A, B and H antigens) showed a significative decrease in titre, score and percentage of agglutination values obtained with senescent erythrocytes ($p < 0.01$) (Table 2).

Data from studies performed to evaluate the M and N antigens expression also showed a significative decrease in titre values ($p < 0.01$) obtained with senescent eythrocytes (Table 3).

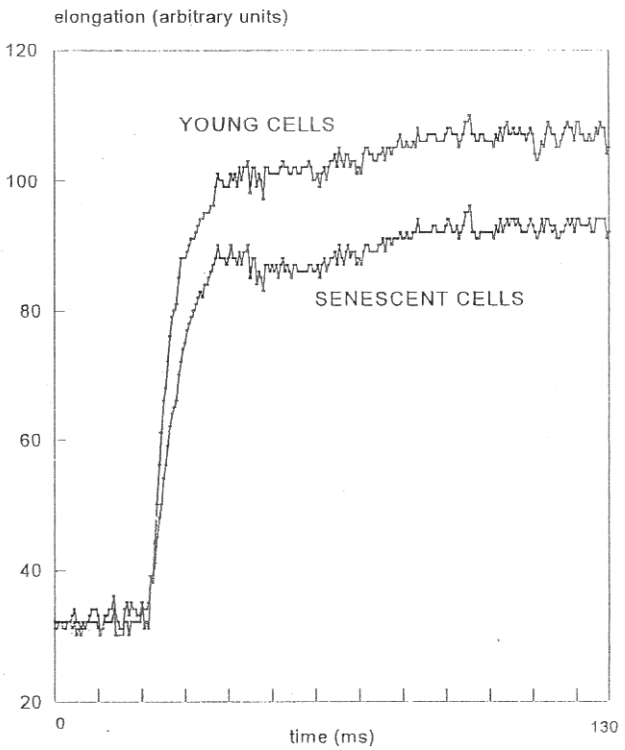

Fig. 1.– Creep curves obtained with young and senescent red blood cells.

TABLE 1.– *Hemorheologic parameters*

	YE	SE
t_c	72.06 ± 16.18 msec	53.69 ± 13.60 msec
DI	0.699 ± 0.03	0.672 ± 0.03
μ	3.46 ± 1.70 x 10^{-3} dynes/cm	6.13 ± 2.97 z 10^{-3} dynes/cm
η_m	2.37 ± 1.00 x 10^{-4} dynes.sec/cm	3.20 ± 1.37 x 10^{-4} dynes.sec/cm

Hemorheologic parameters analysed in Young Erythrocytes (YE) and Senescent Erythrocytes (SE): relaxation time (t_c), deformability index (DI), membrane elastic modulus (μ) and membrane surface viscosity (h_m)

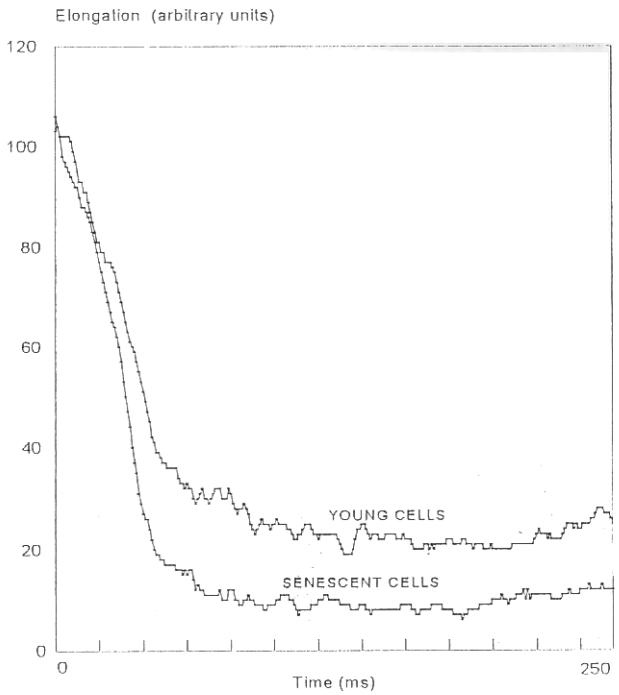

Fig. 2.– Relaxation curves obtained with young and senescent red blood cells.

TABLE 2.– *Antigenic expression of ABO system*

	Titre	Score	AG%
RBC "A"			
Young	160.0 ± 32.0	50.5 ± 3.1	65.6 ± 5.1
Senescent	40.0 ± 8.0	34.7 ± 4.9	44.2 ± 5.5
RBC "B"			
Young	160.0 ± 32.0	58.5 ± 3.5	75.5 ± 5.5
Senescent	40.0 ± 8.0	39.0 ± 1.0	50.8 ± 7.6
RBC "O"			
Young	72.0 ± 20.1	47.0 ± 2.9	62.2 ± 3.9
Senescent	16.0 ± 5.7	30.2 ± 4.5	40.5 ± 8.8

Determination of ABO System antigenic expression: titre, score and percentage of agglutination (AG%) in young and senescent red blood cell (RBC)

TABLE 3.– *Antigenic expression of MN system*

Erythrocyte antigens	Titre with YE	Titre with SE
M	35.2 ± 6.5	4.4 ± 1.1
N	64.0 ± 16.9	4.0 ± 1.3

Titre determination of MN antigens in young (YE) and senescent (SE) erythrocytes

TABLE 4.– *Interaction with monocytes*

	% of APC
YE	4.4 ± 1.3
SE	17.0 ± 2.6
Unsensitized	3.7 ± 0.5
Sensitized	27.5 ± 1.3

Percentage of active phagocytic cells (APC) with young (YE) and senescent (SE) erythrocytes. Negative and posi-tive controls were performed using unsensitized and IgG anti-Rh sensitized red cells.

Discussion

The analysis of the processes that take place during the aging of red blood cells is still very much hindered by the fact that it is very difficult to obtain homogeneous fractions that contain red blood cells of the same age. Density separation is the technique that has been used by the vast majority of authors[1, 3].

The principle behind the separation of the red cells by density is that there is progressive loss of membrane and water during the aging process[2, 15]. This is almost certainly a stochastic process and not all cells may increase in density at the same rate. Thus the densest cell fraction will include cells that are of different ages chronologically, but exhibit rheologic properties characteristic of old cells[6].

In this paper we found modifications in rheologic properties of density fraccionated senescent erythrocytes indicating a decrease in the deformability of these cells. Ektacytometry is a method which detects differences in whole cell deformability and it is claimed that it gives information about alterations in surface area, to volume (S/V) ratio and intracellular viscosity. In a study by Waugh et al[15] a lost of surface area, volume and deformability was found after ektacytometry of the most 0.8% dense fraction (Stractan-separation)[16, 4]. Deforma-bility is determined by membrane mechanical properties, the viscosity of the cytoplasm and the S/V ratio. The relative importance of these factors is not completely clear. All studies on changes in deformbility of red blood cells have been performed with the use of fractions separated on the basis of differences in cell density[17].

It has been suggested that red cell deformability decreases during the life of red blood cells and that this deformability reduction plays a role in the destruction of these cells. This declining deformability makes erythrocytes susceptible to sequestration in the spleen and other organs.

Extrapolation of these deformability data to our observations suggest that the observed decrease in surface are may be of sufficient magnitude to contribute to sequestration and removal of aged cells from circulation.

A specific recognition system has been developed that permits the removal of senescent and damaged cells and stores intact mature cells. Results of the experiments of Kay[7] demonstrate that the senescent cell is antigenically related to protein band 3, an integral membrane erythrocyte protein. In our experiments, as can be seen in Table 2, we found a decrease in the reactivity of ABH antigens, which are in protein band 3. This reuslt may be related to modifications observed by others in the said protein leading to the formation of a senescence antigen[17].

The determination of erythrocyte lifespan is a complex process affected by many cellular parameters. Among the factors that have been proposed to play an important role in erythrocyte senescence, the accumulation on the erythrocyte of autologous IgG has received much attention, because it provides a direct mechanism for removal of senescent erythrocytes via phagocytes. Modification of protein band 3, either by proteolytic cleavage or aggregation, has been suggested to lead to the formation or exposure of an antigenic site resulting in the accumulation of cell surface IgG. Other potential senescent antigens have also been proposed. Regardless of the identity of the senescent cell antigen, the mechanism responsible for its appearance has not been well defined.

There is a difference in sialic acid content between young and senescent erythrocytes[1]. This has suggested the possibility that in vivo senescence involves desialylation of red blood cells with their subsequent sequestration from circulation[3]. The loss of MN antigens expression, shown in Table 3, may be related to the desialylation observed in senescent erythrocytes since sialic acid and MN antigens are carried by the same protein glycophorin A.

After a lifespan of about 120 days, red blood cells are sequestered by the reticuloendothelial system and eliminated by mononuclear phagocytes. Many experi-ments regarding the mechanism of red blood cell senescence and elimination have been carried out, but several questions remain unanswered specially concerning the specific differentiation of aged cells from the younger ones. In this paper we demonstrate an increased rate of erythrophagocytosis by monocytes of senescent erythrocytes compared to young ones.

The significant modifications in the biomechanic properties of senescent red blood cell membrane (μ and η) and antigenic expression, resulting from modification in membrane proteins, might lead to the removal of aged cells from circulation predominantly by phagocytosis.

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