Biochimica et Biophysica Acta, 643 (1981) 269—275
Elsevier/North-Holland Biomedical Press

BBA 79210

ERYTHROCYTE MEMBRANE ELASTICITY DURING IN VIVO AGEING

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(Received November 21st, 1980)

Key words: Elasticity, Ageing membrane, Deformability; Microelastimetry, (Erythrocyte, Human, Rabbit)

Summary

Changes in the ability of senescent erythrocytes to pass through the microcirculation may cause them to be trapped in the spleen and removed from the blood. To help understand this process we have measured erythrocyte membrane elasticity, to see whether it changes during in vivo ageing. Human and rabbit red cells were fractionated by isopycnic sedimentation to obtain samples of aged and young cells. These were subjected to micropipette analysis in order to determine their membrane shear elastic modulus. We found that the membrane rigidity did not significantly alter as red cells aged. Previously we have also demonstrated that the changed size and shape of aged cells is unlikely to explain their removal from the circulation (Nash, G.B. and Wyard, S.J. (1981) Biorheology, in the press). Thus we conclude that the lifespan of erythrocytes is not determined by factors related to membrane flexibility or cell shape but may depend on changes in their viscous properties (as suggested by Williams, A.R. and Morris, D.R. (1980), Scand. J. Haematol. 24, 57—62).

Introduction

It has long been known that the in vivo lifespan of the erythrocyte is limited [1], but the process by which the senescent cells are recognised and removed is not fully understood. Whilst ageing, the physical, biochemical and physiological properties of red cells change (see Wintrobe [2] for review) and it has frequently been suggested that cellular deformability decreases [3—5]. This would make it difficult for aged cells to pass through the microcirculation and might cause them to be trapped in the spleen, thus facilitating their destruction [6,7]. In fact it has been shown that older red cells require greater pressure to force them through narrow pipettes (diameter less than 3 μm) [8] and also that the

rate at which they enter similar micropipettes is slower [9].

The ability of the red cell to become greatly deformed is the result of its large surface area/volume ratio, the fluidity of its contents and also of the flexibility of the cell membrane [10]. In order to see which of these factors might change during in vivo ageing, we measured membrane shear elastic modulus for human and rabbit erythrocytes which had been fractionated according to their age. The fractions were separated by isopycnic sedimentation, based on the widely reported variation in cell density with age [11—13]. The membrane elasticity was tested using a micropipette technique, whereby a short 'tongue' of membrane was aspirated from the flattened region of an otherwise undeformed red cell [14]. By measuring the length of the tongue as a function of pressure, the shear elastic modulus at constant area could be determined [15, 16]. This work represents the second part of a study in which the geometry of aged cells was also investigated [17]. In the light of our results, we discuss the mechanism by which red cell deformability might change during in vivo ageing.

Materials and Methods

The methods for obtaining, handling and fractionating blood were as previously described [17]. Briefly, for rabbit blood, the least dense and most dense cells were separated from the majority (approx. 90%) of the sample using a simple two-step density gradient. Human blood was fractionated using a spontaneously formed, shallow, continuous density gradient. The basic medium for the density separation was Percoll (Pharmacia Ltd., Hounslow, U.K.), a poly-(vinyl pyrrolidone)-coated silica colloid. Additionally, in the present study only, 10-ml samples of heparinised, whole human blood were fractionated by centrifugation at $2000 \times g$ for 40 min in graduated tubes [18]. The plasma was removed and 250 μl of blood cells were sampled from the uppermost 500 μl of the packed cell mass. The majority of the packed cells were then removed and, finally, 250 μl of cells was collected from amongs the lowest 500 μl. These samples were subsequently resuspended in equal volumes of plasma. In this way samples were obtained from amongst the most dense 10% and least dense 10% of cells. For all the above methods, the least dense cells are referred to as the upper sample, the most dense cells as the lower sample and the remainder as the middle sample.

Measurements on separated cells

Erythrocyte membrane shear modulus was measured by a method similar to that described by Evans and Lacelle [14]. The micropipette apparatus was as previously described [17], except that pipette diameter was between 1.0 and 1.5 μm in the present study. The tips were broken at the desired position using the method of Merril and Ainsworth [19]. Using a suction pressure of between 1.5 and 2.5 mm H_2O (depending on pipette diameter), red cells suspended in a glass chamber mounted on a microscope stage were attached to the micropipette tip at their flattened regions. They were then photographed at each pressure as it was increased in steps of 0.5 or 1.0 mm H_2O. The lengths of the aspirated membrane 'tongues' were later measured from the projected film negatives. On average four measurements were made for each cell and between

six and ten cells were tested in each sample. The suspension in the chamber was replaced about every 15 min. The same pipette was used for the separated fractions from any one donor.

The exact position of the pipette tip is difficult to observe as it may be partly obscured by the attached cell. Additionally, the dimensions measured are of the same order as the diffraction limit of the resolution of the microscope. Thus, the change in tongue length can be more accurately measured than its absolute dimensions [20,21]. Accordingly we expressed data as change in tongue length (dL) as a function of change in suction pressure (dP). However, use of the exact theory of Evans [15,16] requires knowledge of the variation of the absolute tongue length. Thus an approximation was used. In this study the values for the ratio L/R (tongue length divided by pipette internal radius) varied approximately between 2 and 4. In this range the ratio is predicted to be nearly directly proportional to the product $P \cdot R$ [15,16]. Hence, a straight line was fitted to the theoretical curve relating these two quantities in the region $2 < L/R < 4$, using a least-squares fit (see Results). From the data for each cell a value for the quotient $(dL/R)/(dP \cdot R)$ was calculated by a least-squres method. This could then be related to the slope of the above linear approximation by a constant factor which included the shear elastic modulus u. The values for u calculated in this manner included the multiplicative factor of 0.5 by which the original theory has since been amended [21].

Measurements were made on cells picked at random from those in suspension. However, bias might occur as only those cells with a relatively flat region are suitable for measurement by this method and shape might consistently differ between fractionated samples. Thus, diameter was measured for the cells attached to the pipette tip and compared to values for cells settled on the bottom of the chamber, to see if any selectivity had occurred. The morphology of the cells in the different samples was also noted.

A Coulter Counter Model ZF with Channelyser (Coulter Electronics Ltd., Harpenden, U.K.) was used to measure cell volume and to count the cells in each fraction. Sample dilution and instrument calibration were as previously described [17]. In addition, reticulocytes were counted microscopically, after staining concentrated cell suspensions with brilliant cressyl blue.

Results

Table I summarises the data for the different fractionation methods. Volume and diameter were smallest for the most dense cells. Reticulocytes were concentrated in the uppermost samples. Thus, as it is known that red cells become denser and smaller in volume as they age [11,12], we conclude that both the rabbit and human cells were successfully fractionated according to age. In general, the differences between the fractions were more marked for rabbit erythrocytes. For human blood little variation occurred between the separated samples when whole blood was centrifuged without the use of gradients. Although separation was improved if step gradients similar to those for rabbit blood were used (data not shown), the best results were obtained using the shallow, spontaneously formed gradients. Overall, the fractionated cells appeared morphologically normal and did not have altered volume compared to

TABLE I

PROPERTIES OF CELLS IN SEPARATED FRACTIONS

Data represent the means of the values obtained from all the experiments in each group. The number of experiments was as follows, (a) One donor, four experiments. (b) Three donors, two experiments on each. (c) Three rabbits, two or more experiments on each. Figures in brackets after Coulter volume are the volumes expressed relative to those for the lower (least dense) cell samples. For freshly suspended, unfractionated cells the Coulter volumes were: (a) 97 μm^3, (b) 99 μm^3, (c) 73 μm^3. In each experiment two orthogonal diameters were measured for 36 cells from each sample. Values for diameters are followed by the average coefficient of variation for the experiments (shown in brackets).

Sample	Fraction	Coulter volume (μm^3)	Diameter (μm)	Reticulocyte count (%)	Cell count (% of total)
Human blood					
(a) Separated without gradient	Upper	101 (1.05)	8.25 (±5%)	—	≈10
	Middle	98 (1.02)	—	—	≈80
	Lower	96 (1.0)	8.1 (±5%)	—	≈10
(b) Separated on continuous gradient	Upper	104 (1.15)	8.33 (±5%)	1.5	13.5
	Middle	98 (1.08)	8.1 (±5%)	0.3	78.5
	Lower	90 (1.0)	7.85 (±5%)	0	8.0
Rabbit blood					
(c) Separated on step gradient	Upper	83 (1.20)	7.5 (±6%)	13	6.0
	Middle	73 (1.06)	6.9 (±8%)	0.9	89.0
	Lower	68 (1.0)	6.6 (±7%)	0	5.0

freshly suspended erythrocytes. Nevertheless, the densest cells tended to include relatively large numbers of cup-shaped cells (stomatocytes), especially for rabbit blood which is known to exhibit anisocytosis [22].

Fig. 1 shows the slope $(dL/dP) \cdot u/R^2$ plotted as a function of the ratio L/R for both the exact theory of Evans [15,16] and for the approximation used to analyse our experimental data (in the region $2 < L/R < 4$). Some typical data

TABLE II

DATA FOR FRACTIONATED CELLS SUBJECTED TO MICROPIPETTE ANALYSIS

The number of experiments in each group was as in Table I. Values given for elastic shear modulus represent the mean ± S.D. for (n) cells. Values for the diameter of aspirated cells represent the mean and are followed by the average coefficient of variation (±x%) for (n) cells.

Sample	Fraction	Elastic shear modulus (u) (dyn/cm) ($\times 10^3$)	Diameter of aspirated cells (μm)
Human blood			
(a) Separated without gradient	Upper	4.7 ± 0.9 (35)	8.7 (±5%) (27)
	Lower	5.1 ± 1.4 (36)	8.5 (±4%) (33)
(b) Separated on continuous gradient	Upper	5.8 ± 0.7 (48)	8.7 (±4%) (47)
	Lower	6.0 ± 1.1 (49)	8.2 (±5%) (49)
Rabbit blood			
(c) Separated on step gradient	Upper	3.8 ± 0.6 (56)	8.0 (±4%) (57)
	Lower	3.4 ± 0.6 (57)	7.4 (±5%) (54)

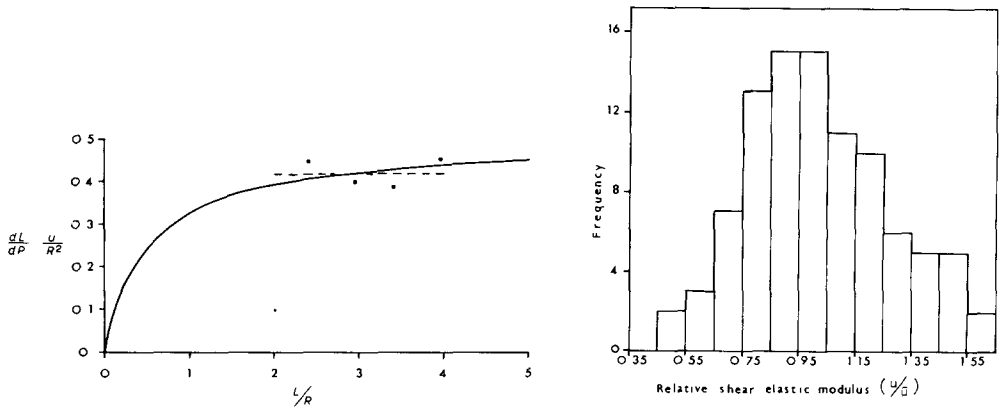

Fig. 1. Rate of change of membrane 'tongue' length, $(dL/dP) \cdot u/R^2$, plotted as a function of the ratio of the 'tongue' length to the pipette radius (L/R). Calculated using the theory of Evans [15,16] (———). In the region $2 < L/R < 4$ the slope was approximate to be constant (- - - - -), so that the shear modulus u could be determined without knowledge of the absolute tongue length. Data points (■) are shown for four measurements made on a single human erythrocyte ($u = 4.8 \cdot 10^{-3}$ dyn/cm, $R = 0.6$ μm).

Fig. 2. Frequency distribution of values for the membrane shear elastic modulus for erythrocytes obtained by repeated sampling from a single donor. The modulus is expressed relative to the mean value $\bar{u}$, which was 5.5 ± 1.0 (S.D.) · 10^{-3} dyn/cm ($n = 94$).

points from measurements on a single human red cell are also shown. Data fitted the modified theory well. The change in aspirated tongue length was approximately proportional to the increase in suction pressure. Values for the shear modulus obtained for a single donor are reproducible, even when measurements are repeated using pipettes of different diameters.

The results for the measurements of membrane elastic modulus are summarised in Table II. It can be seen that rabbit erythrocytes have more flexible membranes than human cells. However, for no single donor was there a significant difference ($P < 0.01$, Student's t-test) between the elastic moduli for the least dense and most dense cells. When data for the three rabbits are grouped together, then a small but statistically significant decrease ($P < 0.01$) in shear modulus for aged cells is found. However the size of the change (approx. 10%) is only of the order of the accuracy of the method [21]. Moreover, if blood is repeatedly sampled from a single donor, then the distribution of values of u about the mean can be determined for freshly suspended cells. Fig. 2 shows a frequency histogram for u from such a set of experiments on a human donor. The distribution is essentially unimodal without a marked shoulder or skew. In addition, the values obtained for the fresh blood, unfractionated and without anticoagulant, were similar to those for the cells which had undergone the separation procedure.

On average, the diameter of the aspirated cells in a given fraction was greater than the average for all the cells in that suspension, implying some selectivity. However this applied equally for the older and younger cell fractions. Thus the larger of the dense and less dense cells had been tested.

Discussion

Micropipette analysis was used to measure red cell membrane shear elastic modulus. The theory of Evans [15,16] allows normalisation of results when pipettes of different size are used. However, in this study an approximation was applied as absolute tongue length could not be reliably determined. We found that the change in length was approximately proportional to the increase in suction pressure. From computations of the rate of change of this length with pressure, values for the shear modulus were calculated. By this method reproducible results were obtained for human and rabbit erythrocytes. Data for human shear moduli were similar to those obtained by Waugh and Evans [21] at comparable temperatures (20—25°C). To our knowledge no previous results exist for rabbit cells.

We found that there was no significant difference in the modulus between cells from the least dense (youngest) and most dense (oldest) samples from any one donor. Additionally, the shape of the distribution of values of u for a single donor was neither bimodal nor positively skewed as might be expected if the modulus increased with age. Because there was some variation in cell shape between the fractions, and as cells with a relatively flattened region are required for micropipette measurements, cell diameter was measured to see if bias had occurred in selecting cells for analysis. It was found that the aspirated cells tended to be larger than the average cells in suspension. But as this was true for all samples we conclude that relatively old and young cells were indeed compared.

Previous studies have demonstrated that red cell deformability decreases during in vivo ageing [8,9]. It has also been shown that the ratio of membrane protein/lipid content increases and that the lipid fluidity decreases [23]. In addition it has been suggested that membrane protein polymerisation occurs during ageing [24]. However, we have found no evidence for an increase in membrane rigidity. Thus, although aged cells require greater pressure to force them to completely enter micropipettes [8], this does not necessarily mean that their membranes are less elastic. These observed differences between relatively young and old cells can only be interpreted empiricaly. They could be the result of changes in other cell properties which affect erythrocyte deformability (e.g., the state of the internal matrix). Additionally, changes in the lipid region of the membrane need not necessarily result in alteration of its elastic properties, as these are largely imparted by the underlying protein network [14,25]. On the other hand, it is possible that the membrane viscosity would be altered. In fact Shiga et al. [9] have demonstrated that aged cells deform less quickly. They linked this to a change in the 'Young's modulus' of the membrane but their theoretical analysis neglected viscous effects which are important when measuring membrane relaxation phenomena [26,27].

Thus, during in vivo ageing, red cell membrane elasticity does not significantly alter. In previous work [17] we have also shown that although red cell volume and surface area decrease with time, their ratio remains nearly constant. Thus, the minimum cylindrical diameter through which they can pass is not less for aged cells. On this basis aged erythrocytes should be able to pass through the microcirculation. However, we have not tested their viscous properties. If

the internal, cytoplasmic viscosity and/or the membrane viscosity increased then the rate at which erythrocytes could deform would decrease. Indeed, Williams and Morris [28] have shown that aged cells contain an increasingly viscous cytoplasm. They suggested that this would cause the residence time in the spleen to increase, aiding in removal of senescent cells by phagocytosis. Also, if the internal concentration of haemoglobin continued to increase then a rigid gel might form. The cells would then be unable to negotiate the microcirculation. From these results it appears that the lifespan of erythrocytes may be determined by changes in their viscous properties rather than by changes in the flexibility of the cell membrane or by factors related to cell size and shape. These changes might be the result of the decreases in enzymatic and metabolic activities which have been shown to occur during ageing [29,30].

Acknowledgements

This work was supported by MRC Project Grant No. G978/659/N. We gratefully acknowledge the help and advice given by Dr. M. Adinolfi and the aid of Miss S. Dodds, Mrs. S. Bayer and Miss N. Dobson in obtaining blood samples.

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