Biochimica et Biophysica Acta, 685 (1982) 207–210
Elsevier Biomedical Press

BBA Report

BBA 70011

FREEZE-FRACTURE CHARACTERIZATION OF 'YOUNG' AND 'OLD' HUMAN ERYTHROCYTES

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(Received August 4th, 1981)

Key words: Erythrocyte membrane; Freeze-fracture; Aging

While 'young' and 'old' erythrocytes separated by density show differences in various biochemical properties, the membane ultrastructure as demonstrated by freeze-fracture remains unchanged. This implies that although superficial membrane components may be affected by aging, and whole segments of membrane may be lost, the structural relationship between integral protein and lipid in the remaining membrane is not altered.

Erythrocytes become denser as they age [1–3] and undergo progressive biochemical changes including loss of membrane protein, sialic acid, lipid, and activity of such membrane-associated enzymes as acetylcholinesterase [4–6]. Whether these changes are due to the preferential loss of specific membrane components or the non-specific loss of whole membrane fragments is not known.

We have brought the technique of freeze-fracture electron microscopy to bear on this problem. The intramembranous particles seen in freeze-fracture replicas of erythrocyte membranes are thought to correspond to transmembrane proteins set in the membrane's lipid bilayer [7–9]. If the progressive biochemical changes which occur in aging erythrocytes involve preferential loss of specific lipids or proteins in the erythrocyte membrane, they could be expected to be associated with alterations in the freeze-fracture appearance of the membrane. We sought to determine whether such alterations do in fact occur.

Fresh heparinized blood was separated into fractions of differing density (and therefore age) by the method of Murphy [10]: after two 20-min centrifugations at $2000 \times g$ and 4°C to remove the buffy coat, the cells were placed in 13×98 mm polypropylene tubes and centrifuged in serum for 60 min at $39000 \times g$ and 30°C. Fractions were then carefully aspirated from the tubes and resuspended in serum. Subsequent analyses were done on the top 10–15% and the bottom 10–15% of the cells in each tube.

The density separation was verified by measuring the mean corpuscular hemoglobin concentration in each fraction using a Coulter Model S cell counter. The cell density distribution was determined by matching the samples against phthalate ester standards of varying density [1]. Reticulocytes were counted on thin smears stained with new methylene blue. Acetylcholinesterase was assayed by the method of Ellman [11] on whole blood with $2 \cdot 10^{-5}$ M quinidine sulfate to inhibit non-specific esterase activity. Red cell ghosts were prepared according to the method of Dodge et al. [12] and used for the assay of protein [13] and sialic acid [14]. The results were expressed per cell, based on the pre-hemolysis red cell count.

Freeze-fracture electron microscopy for red cell intramembranous particle count was performed as previously described [15,16]. Whole red cells were fixed (except as noted below) in buffered 3% glutaraldehyde, infiltrated with 30% glycerol, frozen in Freon cooled with liquid nitrogen, and fractured and replicated in a Balzers BAF-301

freeze-fracture apparatus. The thickness of the platinum coat was controlled with a quartz monitor. The replicas were then cleaned, mounted on grids and photographed in a Zeiss EM-10 microscope. The particles were counted in quarter square micrometer areas on 100000 × or 200000 × prints; 20 membrane fracture faces (ten P faces and ten E faces) were counted for each sample. Mean particle diameters were determined by measuring 50 particles per sample on 400000 × prints. Particle distribution was evaluated by measuring particle-free areas more than 20 nm wide on five prints per sample.

The fractions produced by our density separation technique differed consistently in mean corpuscular Hb concentration, reticulocyte count, and mean cell specific gravity (Table I). The overlap in cell density between the dense and light cell fractions averaged only 3% (Fig. 1). We found an average of about 20% less total membrane protein, 10% less sialic acid, and 30% less acetylcholinesterase activity per cell in the older, denser fractions (Table I).

These results are consistent with those found by others using similar techniques [5,6], and demonstrate that the cell fractions were well separated by density and age. The average difference in mean corpuscular Hb concentration between our fractions is not as great as reported previously [5,10], perhaps because our use of an automated cell counter produced values unaltered by differences in packing density, which tend to artificially exaggerate differences in mean corpuscular Hb concentration calculated from spun hematocrits.

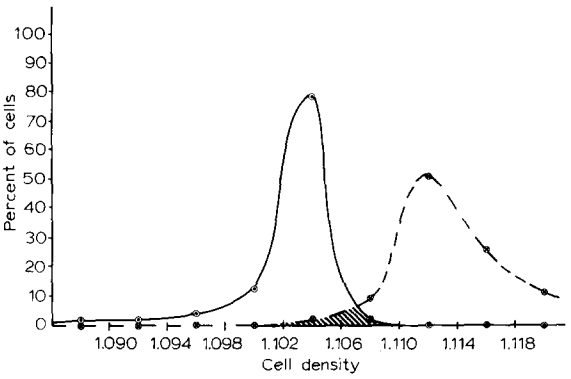

Fig. 1. Typical erythrocyte density separation. The cell density distributions for the younger, lighter cell fraction (solid line) and older, denser fraction (broken line) were determined by measuring the percent of the packed cell volume which sedimented above and below phthalate ester standards of varying specific gravity [1].

In contrast to the biochemical findings, freeze-fracture analysis showed no significant difference in the number of intramembranous particles per μm^2 in both P faces and E faces of density-separated red cells from each of six separate donors (Table II and Fig. 2). There was also no difference in the size or distribution of intramembranous particles in these samples. One pair of samples processed without fixation similarly showed no difference in intramembranous particle density.

This remarkable uniformity of freeze-fracture

TABLE I

RED CELL PROPERTIES IN LIGHT AND DENSE FRACTIONS

MCHC, mean corpuscular Hb concentration.

Red cell property	Value in light fraction [a]	Value in dense fraction [a]	Significance P (by paired two-tailed t-test)
MCHC (g/dl)	34.0 ±0.2	35.8 ±0.2	<0.001
Reticulocytes (%)	1.5 ±0.3	0.1 ±0.1	<0.01
Mean cell density	1.101 ±0.001	1.110 ±0.001	<0.01
Ghost protein (pg/cell)	0.58 ±0.04	0.47 ±0.03	<0.05
Sialic acid molecules/cell ($\times 10^{7}$)	2.8 ±0.2	2.5 ±0.2	<0.01
Acetylcholinesterase (absorbance change/min per cell) ($\times 10^{11}$)	2.2 ±0.1	1.5 ±0.1	<0.01

[a] Values given are the mean of at least four separation runs ± S.E. of the mean.

TABLE II

IMP, intramembranous particle.

	Value in light fraction [a]	Value in dense fraction [a]	Significance P (by paired two-tailed t-test)
P face IMP density (μm^{-2})	3801 ±154	3856 ±166	>0.1
E face IMP density (μm^{-2})	297 ± 25	286 ± 12	>0.1
P face IMP diameter (nm)	8.5± 0.4	8.3± 0.5	>0.1
E face IMP diameter (nm)	8.5± 0.5	8.4± 0.6	>0.1
P face particle-free area (%)	8.1± 1.5	8.3± 1.0	>0.1

[a] Values given are the mean of samples from at least five separate donors ± S.E.

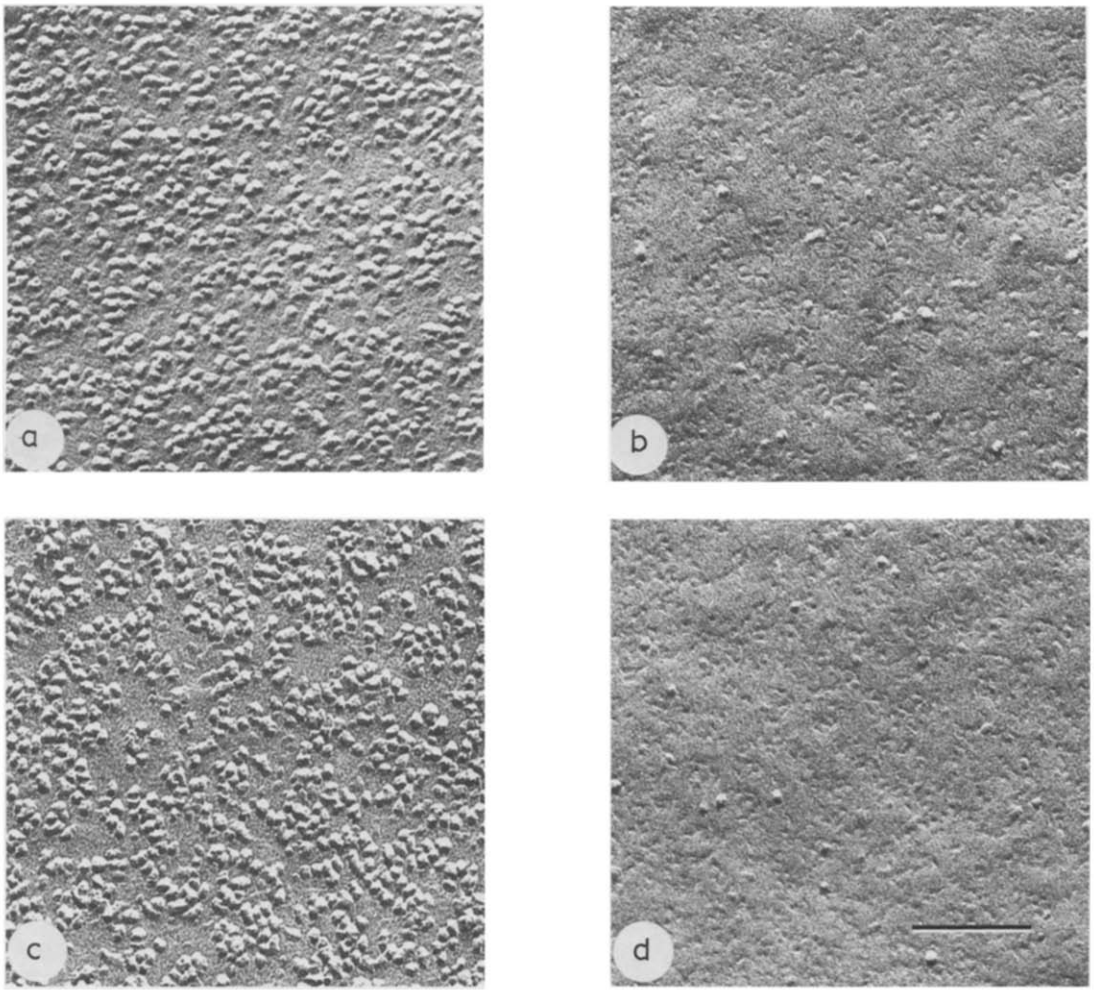

Fig. 2. Uniformity of intramembranous particles in density-separated erythrocytes. (a) Light fraction P face. (b) Light fraction E face. (c) Dense fraction P face. (d) Dense fraction E face. Final magnification 160000×. Bar=0.1 μm.

appearance suggests that the lipids and proteins which make up the hydrophobic core of the erythrocyte membrane are unaltered by aging. Thus the biochemical changes that occur are probably not the result of selective removal of specific integral membrane components. It is more likely that what membrane is lost with aging is removed whole, with preservation of structural relationships between integral protein and lipid in the membrane that remains.

This conclusion is supported by studies which have shown the surface charge density of erythrocytes to remain constant with aging [17], since surface charge is largely due to sialic acid carried on the integral membrane protein glycophorin [18,19]. These results are also consistent with studies of erythrocytes aged in vitro by ATP depletion [20], which show the release of vesicles with similar integral membrane protein content to intact membrane. Our findings suggest that in vivo aging may occur by a similar mechanism.

Since intramembranous particle density remains constant in aging erythrocytes, one may determine in a general way which biochemical properties are associated with intramembranous particles by ascertaining how the properties vary with aging. Those properties which are closely associated with intramembranous particles should, like the intramembranous particles themselves, be relatively resistant to the effects of age.

The authors thank Dr. David Goodman for his review of the manuscript. This work was supported by a center grant from the Muscular Dystrophy Association and by N.I.H. grant NS08075.

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