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POLYMERIZATION OF MEMBRANE COMPONENTS IN AGING RED BLOOD CELLS

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Summary: The addition of malonyldialdehyde to red blood cells in vitro causes the formation of fluorescent chromolipids characteristic of those produced during the peroxidation of endogenous membrane phospholipids. Additionally, gel electrophoresis reveals that this agent also causes a decrease in bands 1 and 2 of spectrin as well as an increase in high molecular weight protein polymers. These same changes are observed in membranes of older cell populations fractionated from freshly drawn, untreated blood. The results obtained suggest that polymerization of membrane components, subsequent to the peroxidation of membrane lipids, may contribute to the altered biochemical and mechanical properties of aging cells and to their eventual sequestration.

RBC survive in the circulation for only finite periods. Partly as a consequence of their altered mechanical properties during aging, they may ultimately be sequestered in the reticuloendothelial system (1,2). As these cells age, the activities of various enzymes, e.g., G-6-PD and AcChE, are diminished (3,4). Indeed, these enzymes are often used as discriminators in the identification of RBC populations with respect to age. However, the molecular events which contribute to changes in the mechanical properties of aged cells are not well understood. It is of special interest that the peroxidation of endogenous membrane phospholipids has been reported to be associated with aging as well as with tissue injury in vivo (5). The accumulation of fluorescent chromolipids in old animals has also been described (6). These pigments are presumed to have been formed as a result of the breakdown of peroxidized fatty acids to yield MDA. This substance has the capacity to cross link, through Schiff's base formation, the amino groups of both phospholipids and proteins to produce the fluorescent chromolipids (5,7). Although the accumulation of such polymers in RBC which have been exposed to autoxidizable agents with the capacity to

Abbreviations: RBC: red blood cells, G-6-PD: glucose-6-phosphate dehydrogenase, AcChE: acetylcholineesterase, MDA: malonyldialdehyde, DTE: dithioerythrotol.

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initiate membrane lipid peroxidation has been demonstrated (8-10), it has not been known whether their levels also increase in normal RBC during in vivo aging. Here we present evidence for the increased presence of these fluorescent pigments in the membranes of older RBC. We also show that the membranes of aged RBC contain decreased amounts of spectrin and increased amounts of high molecular weight protein polymers. These findings suggest a basis for some of the altered biochemical and mechanical properties of normal, but aging, RBC.

Methods: Male Sprague-Dawley rats (150-160 g) were obtained from Simonson Labs, Gilroy, Ca. Blood was collected by cardiac puncture, under light ether anesthesia, into heparinized (10 u/ml) syringes, after overnight fasting of the animals. Pooled blood from several animals was centrifuged at 600 x g for 10 min at 21° C. Plasma and buffy coats were removed from sedimented cells which were then washed three times with 0.154 M NaCl solutions, and finally centrifuged at 30,000 x g for 60 min at 21° C. Three fractions of cells (ca one ml each) were collected with a Pasteur pipette from the top, middle, and the bottom of the tubes. Activities of AcChE and G-6-PD were determined by the methods of Ellman, et al.(11) and Beutler (12) respectively, in each of the fractions, as indicies of RBC age. The values obtained (expressed as a percent of those determined for the whole population of unfractionated cells) were 128 ±11%, 85 ±6%, and 56 ±8% for the top, middle, and bottom fractions respectively in the case of AcChE and 113 ±5%, 95 ±7%, and 66 ±6% for G-6-PD in the same fractions. These values are in keeping with the enrichment of the top fractions with younger cells and the bottom fractions with older ones, after prolonged centrifugation.

MDA was prepared by acid hydrolysis of malonaldehyde bis-(dimethyl acetal) (Aldrich Chemical Company). To 25 µl of the latter compound were added 0.965 ml of 0.154 N NaCl and 10 µl of 0.1 N HCl. Since four moles of HCHO may be formed with each mole of MDA, the treatment of cells with HCHO was utilized as a control in the initial experiments. HCHO, up to 50 µM, had no measurable effect on any of the observed parameters. MDA was added to washed, but unfractionated, cells resuspended to a 5% hematocrit in 0.128 M potassium phosphate buffer, pH 7.4. Cell suspensions (3.0 ml) were then incubated for 2 h at 37° C in a shaking water bath.

Fluorescent chromolipids were determined after extracting cells with isopropanol:chloroform (3:2) by the method of Rose and Oklander (13). Fluorescence measurements were made with a Farrand Spectrofluorometer standardized with quinine sulfate (14) and the values obtained expressed in terms of phospholipid phosphorus assayed by the method of Bartlett (15).

RBC ghosts were prepared from MDA-treated cells and from fractionated cells after hemolysis in 10 mM Tris-HCl, pH 7.9 as described by Kumar, et al. (16). Protein was determined by the method of Lowry, et al. (17).

The protein profiles of the hemoglobin-free ghosts were analyzed after polyacrylamide gel electrophoresis in 1% SDS by the method of Fairbanks, et al. (18). Proteins were incubated with DTE before loading the gels. The current was set at 6 mA per tube and the usual running time was 3 h. Each gel was loaded with 80 µg of membrane protein. After staining with Coomassie blue, electrophoretograms were obtained on a Gilford spectrophotometer equipped with a gel scanner. The quantitative values of the various protein classes are expressed as a percentage of the total membrane proteins.

Results: The addition of authentic MDA to washed RBC in vitro causes the formation of fluorescent material extractable with isopropanol:chloroform

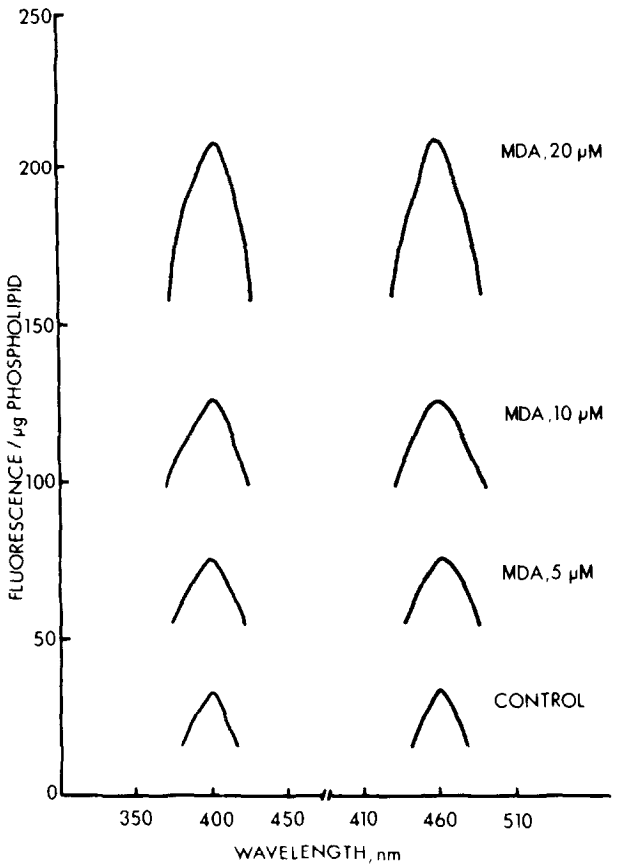

Fig. 1. The effects of MDA-treatment on lipid fluorescence in erythrocytes. Ten fluorescence units are equivalent to 0.01 ng quinine sulphate in 0.1 N HCl.

(Fig.1). The extracted pigment exhibited maximal excitation between 390 and 400 nm and maximal emission at 460 nm. The increase in pigment content was proportional to the amount of MDA (between 5 and 20 µM) added to the cells. The fluorescent pigment was associated exclusively with the plasma membrane of the RBC and was not demonstrable in samples of freshly prepared, pure phospholipids. It should be noted in Fig. 1 that untreated (control) cells also contained fluorescent chromolipid. Fig. 2 shows that the chromolipid extracted from untreated cells does not have a uniform distribution in the RBC population. Cells with the highest density, the bottom fraction after centrifugation, contained about 50% more pigment than those of the top fraction. This finding is particularly striking since there is only a partial

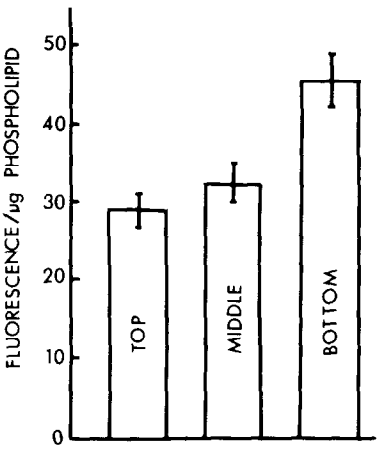

Fig. 2. The distribution of fluorescent pigments in erythrocytes separated on the basis of age. Top: top fraction (youngest cells), Middle: middle fraction, Bottom: bottom fraction (oldest cells). Fluorescence units are as in Fig. 1.

enrichment of the bottom fraction with older cells as identified by enzyme assays (see Methods). Even those cells from the middle fraction consistently exhibited a small (but not statistically significant) increase in fluorescence compared with the top fraction enriched with the youngest cells.

In addition to causing the formation of fluorescent pigment after addition to RBC in vitro, MDA also caused a decrease in the protein of bands 1 and 2 and a concomitant increase in high molecular weight proteins (Fig 3). These changes were proportional to the amount of MDA added to the cells (between 1 and 10 µM). The protein pattern of untreated and unincubated cells is shown in the gel marked A. After incubation for 2 h, RBC exhibited an increase in material which moved behind band 2 (gel B). HCHO (50 µM) did not produce any alterations in the protein pfofiles (gel C). MDA at a concentration of 0.1 µM was also without effect on the membrane proteins (gel D). However, of the total membrane protein, spectrin (bands 1 and 2) in the gels shown in Fig. 3 accounted for 28%, 25%, and 23%, when the cells were incubated with 1, 5, and 10 µM MDA respectively (gels E, F, and G). Incubated control cells contained 31% spectrin (gel B). The treated cells contained 5%, 9%, and 13% of high molecular weight material as compared to control values of 3%. These shifts were consistantly observed and other classes of membrane proteins appeared to be unchanged.

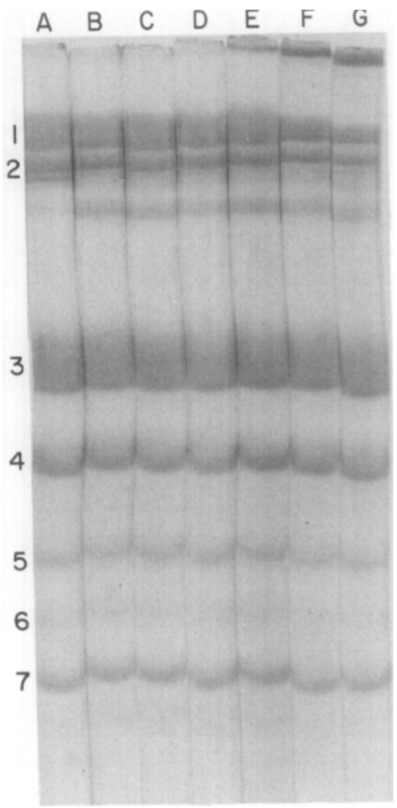

Fig. 3. The effect of varying concentrations of MDA on erythrocyte membrane proteins separated (after DTE) by SDS gel electophoresis. A: unincubated control, B: incubated control, C: 50 μM HCHO, D: 0.1 μM MDA, E: 1.0 μM MDA, F: 5.0 μM MDA, G: 10.0 μM MDA.

SDS gel electrophoresis patterns of membranes prepared from fresh, untreated RBC obtained from the top, middle, and bottom layers of tubes after centrifugation were also obtained. Quantitative data for these gels is shown in Table 1. The bottom fractions enriched with older cells contained a lightly staining band wich corresponds to the high molecular weight material found in MDA-treated cells. The amount of protein in bands 1 and 2 of these gels also exhibited a small (ca 20%), but significant, decrease ($P<.01$). The other protein classes were unchanged in these older cells.

Discussion: During aging RBC become less deformable and as a consequence unable to pass the splenic circulation (1,2). Such sequestration removes them from the circulation. The changes which result in this altered flow behavior include a

Table 1. Membrane proteins in RBC separated into age groups by centrifugation.

RBC FRACTION	TOP (YOUNGEST)	MIDDLE (INTERMEDIATE)	BOTTOM (OLDEST)
PROTEIN BAND		% TOTAL PROTEINS	
HMWP*	3.6 ±0.3	4.3 ±0.6	5.4 ±0.8**
1 + 2	34 ±2	32 ±1**	27 ±1***
3	24 ±1	24 ±1	25 ±1

Values are means ±SD of three different experiments.
* High molecular weight proteins.
** Significantlt different as compared to top fraction ($P<0.05$).
*** Significantly different as compared to top fraction ($P<0.01$).

decrease in surface/volume ratios. Thus more rigid RBC may be formed as a result of either membrane loss (19) or an increase in cellular volume (20). In the latter case, it has been observed that the ATP content of cells is decreased with aging (21). This as well as other changes in the activities of RBC enzymes with time may contribute to an increase in cell volume and hence rigidity (22). It is also possible that an increase in the intrinsic membrane rigidity and a decrease in the fluidity of membrane components may contribute to the altered mechanical behavior of cells (23). Doberstov, et al (24) have recently shown that lipid peroxidation results in an increased phospholipid bilayer rigidity. In this paper, we show that normal cells separated into fractions based on age by centrifugation (25) contain, with increasing age, increasing amounts of chromolipids characteristic of those known to be formed after the peroxidation of membrane phospholipids. Additionally, older cell populations contain lesser amounts of bands 1 and 2 proteins (spectrin) and increasing amounts of high molecular weight substances. Since calcium was absent during the preparation of membrane fractions it is unlikely that these changes are the result of the activity of transglutaminase (26). Moreover, the changes are mimicked by the addition of MDA in vitro. It seems reasonable to assume that the presence of polymers caused by cross-linking of membrane components subsequent to lipid

peroxidation may be a feature which contributes to the altered physical as well as biochemical properties of aging cells.

These speculations do not exclude the possibility that polypeptide aggregates may be formed in RBC membranes by other mechanisms. For example, Johnson, et al (27) have recently reported the presence of spectrin-containing polypeptides in the membranes of G-6-PD mutants with chronic hemolytic disease. Unlike the experiments described in this paper, in that instance, the aggregates were dissociated by DTE. Their presence is in keeping with the well-known sensitivity of G-6-PD deficient RBC to oxidant substances (28,29). These substances have the capacity to react with O_2 to form reactive species such as O_2^-, $\cdot OH$, and H_2O_2. In a similar fashion, it is possible that the autoxidation of hemoglobin (30) may generate species with the capacity to initiate the free radical reactions which lead to the formation of MDA from peroxidized lipids or the capacity to oxidize essential sulfhydryl groups. In either event, normal, but aging cells (with low G-6-PD), and mutant G-6-PD cells may share a diminished ability to maintain effective reducing systems in the presence of oxidizing species. This failure may ultimately be reflected in the accumulation of polymerized membrane components.

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