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ALTERATIONS IN HUMAN ERYTHROCYTE SHAPE AND THE STATE OF SPECTRIN AND PHOSPHOLIPID PHOSPHORYLATION INDUCED BY CHOLESTEROL DEPLETION

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Summary

Cholesterol depletion of erythrocytes, obtained after incubation with phosphatidylcholine vesicles, induces in most of the experiments: (1) a discocyte-stomatocyte transformation as observed by scanning electron microscopy; (2) a specific decrease in spectrin phosphorylation of intact erythrocytes; (3) an increase in lipid phosphorylation. It is concluded that the effect of cholesterol on erythrocyte shape is probably mediated through its action on the activity o of membrane-bound enzymes, proteases or kinases.

In the course of a study on the effects of cholesterol on Na pump activity in erythrocytes [1], we observed by scanning electron microscopy that cholesterol depletion caused cell shape changes. Removal of 30—40% of cholesterol induced in most of the experiments a discocyte-stomatocyte transformation. The molecular mechanisms underlying the erythrocyte shape changes are at present hypothetical. It is certain that the organization of the spectrin-actin cytoskeletal proteins plays a major role in maintaining the discocyte shape [2,3]. Recently, it was shown that changes in the cell shape were associated

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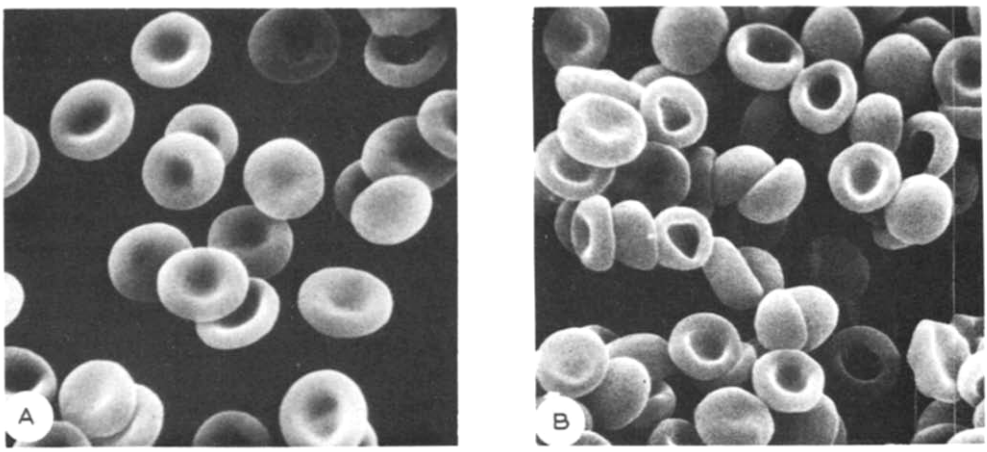

Fig. 1. Scanning electron micrographs of control (C_{II}) (A) and cholesterol-depleted erythrocytes (D) (B). Erythrocytes, collected from fresh blood, were washed four-times with 150 mM choline chloride. Unilamellar lipid vesicles were prepared by sonication and centrifugation either from egg phosphatidylcholine alone or from egg phosphatidylcholine and cholesterol in a molar 1/0.9 in the following solution (mM): NaCl, 140; KCl, 10; $MgCl_2$, 1; NaH_2PO_4/Na_2HPO_4 (20/80), 2.5 (pH 7.4); glucose, 10; sucrose, 35 [1]. Erythrocytes were incubated either with egg phosphatidylcholine vesicles (1—3 mg per ml packed cells) to decrease the cell membrane C/P, without significant changes in total phospholipids. Control cells were incubated with phosphatidylcholine/cholesterol vesicles (1—3 mg phosphatidylcholine/0.5—1.5 mg cholesterol per ml packed cells). All incubations were carried out at 37°C for 15 h at an hematocrit of about 15% with gentle agitation. At this time aliquots were taken and centrifuged. Cell pellets were washed three times to remove the vesicles and portions were taken to measure their ATP content (Boehringer Mannheim GmBH Diagnostics), membranes cholesterol and phospholipids [1] and for morphological observations. Incubation was continued for a further hour in the presence of adenine (2 mM) and inosine (10 mM). The same measurements and observations as before were done after this step. For scanning electron microscopy samples were fixed for 1 h in glutaraldehyde (1.25%), post-fixed in 1% osmium tetroxide [24] and observed under a scanning electron microscope Cameca MEB 07 at 20 kV. C_{II}: control erythrocytes (cholesterol/phospholipid molar ratio: 0.90), D: cholesterol-depleted erythrocytes (cholesterol/phospholipid molar ratio: 0.62). Both samples were fixed before the addition of adenine and inosine. ATP content was 1.02 and 0.92 $\mu mol \cdot ml^{-1}$ in C_{II} and D, respectively, at the time of the observation.

with the phosphorylation of spectrin [4—6], or with the phosphorylation and redistribution of membrane lipids [7]. It was suggested that these factors were involved in the changes in shape [2,3,7]. Accordingly in the present study we investigated whether the morphological transformation induced by cholesterol depletion was accompanied by alterations in membrane protein composition and in phosphorylation of proteins and phospholipids.

Erythrocytes were incubated with egg phosphatidylcholine sonicated vesicles to reduce the cholesterol/phospholipid molar ratio of the membrane by about 35% [1]. Control incubations were carried out in the absence of vesicles (C_I) or with phosphatidylcholine-cholesterol vesicles (C_{II}) that did not change the cholesterol/phospholipid molar ratio. In about 70% of the experiments cholesterol-depleted erythrocytes were stomatocytes (D, Fig. 1B) and control cells (C_I not shown and C_{II}, Fig. 1A) were discocytes, as observed by scanning electron microscopy. In the other experiments cholesterol-depleted as well as control cells incubated with vesicles (C_{II}) presented all morphological aspects ranging from discocytes to echinocytes III [8]. Defatted human serum albumin

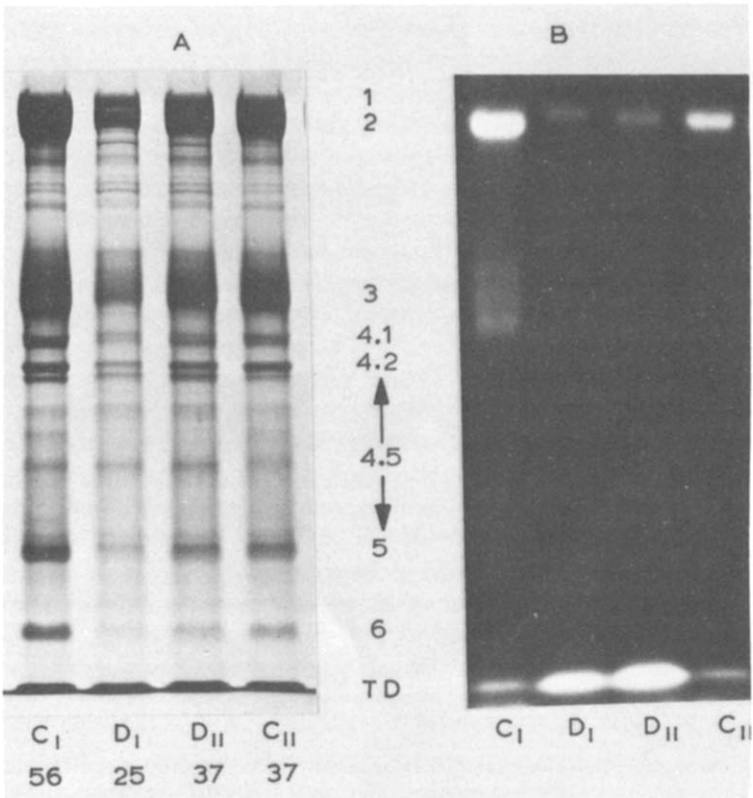

Fig. 2. Membrane protein composition and ^{32}P labeling of control (C_I, C_{II}) and cholesterol-depleted erythrocytes (D_I, D_{II}). A. Coomassie blue-stained SDS-polyacrylamide gel electrophoresis of membrane proteins. B. Radioautograms of SDS slab gels. Control and cholesterol-depleted erythrocytes were incubated in the presence of $^{32}P_i$ (1 mCi · ml^{-1} packed cells) at a 10% hematocrit, for 5 h at 37° C under agitation in the following solution (mM): NaCl, 120; KCl, 10; $MgCl_2$, 1; glucose 10; bovine serum albumin, 1 mg · ml^{-1}; $NaHCO_3$, 20, and in contact with a stream of 5% CO_2, 95% O_2 [10]. During the incubation, aliquots were taken for assay of specific activity of [γ-^{32}P]ATP [10] and ATP content. At the end, erythrocytes were washed four times and membranes were prepared by the method of Dodge et al. [25]. ATP specific activities were not significantly different in cholesterol-depleted cells and in controls and reached a plateau by 4 h. ATP contents in cholesterol-depleted cells decreased to 80—60% of their initial values by 5 h and remained constant in controls. Erythrocyte membranes were dissolved in SDS (3%) and β-mercaptoethanol (5%) for protein analysis on a 7.5% acrylamide separating gel according to Laemmli [26]. Protein concentrations were determined by the method of Lowry et al. [27]. The amounts of membrane protein (μg) applied are indicated below the gels. The gels were fixed, stained and destained according to Fairbanks et al. [28], then scanned and exposed to Kodak NS film for 90 h. Samples wells are labeled: C_I, incubated control (cholesterol/phospholipid molar ratio: 0.96); D_I and D_{II}, cholesterol-depleted (cholesterol/phospholipid molar ratio: 0.62 and 0.64, respectively); C_{II}, control incubated with phosphatidylcholine/cholesterol vesicles (cholesterol/phospholipid molar ratio: 0.97). The protein bands were numbered according to Steck [29]; T.D., tracking dye.

(5 mg · ml^{-1}) added to the vesicles prior to their incubation with erythrocytes prevented the echinocytic transformation suggesting that lysophosphatidylcholine generated during vesicle sonication was responsible for this shape change [9]. The ATP content of stomatocytes was never decreased by more than 10% relative to control cells (see legend of Fig. 1). The increase in ATP content (30—50%) induced by a further incubation with adenine and inosine

for 1 h did not suppress the stomatocytic appearance. These results show that the discocyte-stomatocyte transformation cannot be attributed to the presence of vesicles fused or adsorbed on the membrane or to a metabolic depletion.

Cholesterol-depleted (D) and control erythrocytes (C_I and C_{II}) were further incubated in the presence of $^{32}P_i$ and analyzed for membrane protein composition and phosphorylation [10]. No major changes in membrane protein pattern appeared on the Coomassie-blue stained gel (Fig. 2A) and particularly there was no detectable loss of spectrin or actin as estimated by densitometric scanning (not shown). Only minor modifications could be detected by careful observations in the region between band 2.1 and 3 and at the level of bands 4.2 and 4.5 which need further confirmation (Fig. 2A). In agreement with previous studies [10], the greatest incorporation of ^{32}P was found in bands 2 and 3 (Fig. 2B). Cholesterol-depleted membranes derived from stomatocytic cells exhibited a significant reduction in spectrin phosphorylation but had no significant effect on phosphorylation of other protein bands (Table I, see also Fig. 2B). Interestingly, radioautograms (Fig. 2B) showed areas of increased labeling just behind the dye marker in cholesterol depleted membranes. It has been shown that this labeling corresponds to radioactive phospholipids [11,12], but it could also include low molecular weight phosphorylated proteins or protein breakdown products [13]. Membranes were thus extracted by acid-chloroform-methanol [14] and the recovery of ^{32}P in the organic phase containing the lipids was mea-

TABLE I

MEMBRANE PROTEIN AND PHOSPHOLIPID PHOSPHORYLATION OF CONTROL AND CHOLESTEROL-DEPLETED ERYTHROCYTES FOLLOWING 5 h INCUBATION WITH $^{32}P_i$ (SEE LEGEND OF FIG. 2)

After destaining, the gels were sliced between the stained bands into the following regions: band 1, band 2, band 2.1 to band 2.6, band 3, band 4.1, band 4.2, band 4.5, band 5, and band 6. The gel slices were placed in 10 ml of H_2O in scintillation vials and counted in a liquid scintillation counter by Cerenkov radiation [10]. Membrane protein phosphorylation was calculated by using the [γ-^{32}P]ATP specific activity at 4-h incubation. Only bands where significant phosphorylation accurred are presented. To determine the percentage of radioactivity in the lipid fraction, membranes (50 μl) were extracted with 3 ml HCl/chloroform/methanol (1 : 200 : 100, v/v) followed by 5 ml 0.1 N HCl [14]. The lower chloroform phase containing the lipids was evaporated, counted as above and compared with the total radioactivity of unextracted membranes. Values are means ± S.E. The number of experiments is indicated in parenthesis.

Band no.	^{32}P incorporation in proteins (pmol $^{32}P/\mu g$ protein)		^{32}P incorporation in lipids (% of total radioactivity)	
Cholesterol/ phospholipid (molar ratio)	Control	Cholesterol-depleted	Control	Cholesterol-depleted
	0.90 ± 0.03 (9)	0.63 ± 0.01 (6)	0.85 ± 0.05 (4)	0.67 ± 0.04 (4)
1	0.37 ± 0.04	0.18 ± 0.03 *		
2	1.79 ± 0.13	1.15 ± 0.20 *		
2.1 to 2.6	0.90 ± 0.13	0.90 ± 0.14		
3	1.14 ± 0.15	0.87 ± 0.21	36.7 ± 2.5 *	55.0 ± 6.2 *
4.1	0.42 ± 0.08	0.32 ± 0.09		
4.5	0.60 ± 0.06	0.77 ± 0.15		
6	0.16 ± 0.03	0.32 ± 0.11		

* $P < 0.05$.

sured. Results gathered in Table I show that the percentage of ^{32}P incorporated in this fraction was increased after cholesterol depletion.

These preliminary results show that erythrocyte membrane cholsterol is one of the numerous factors interfering with the cell shape and with the membrane protein and lipid phosphorylation. Cholesterol depletion caused the cells to adopt stomatocytic shape, reduced spectrin phosphorylation and increased phospholipid phosphorylation. None of these effects have, to our knowledge, been described before. Only swollen or spherocytic shapes [15,16] or a lack of shape change in cholesterol-depleted erythrocytes have been briefly reported [17], an observation which contrasts with the broad and flat appearance of the cholesterol-loaded cells [15]. The results of Lange et al. [18] obtained in erythrocyte ghosts, suggest however, that cholesterol may act physiologically to constrain the membrane contour against invagination, which is in agreement with our finding on the stomatocyte formation induced by cholesterol removal. The mechanism of action of membrane cholesterol is probably complex because both membrane protein and lipid phosphorylation are altered and because agents such as lysolecithin may mask the change in shape. Eyrythrocyte shape has been reported to be dependent on the state of spectrin phosphorylation [4—6]. If the level of cholesterol controls the activity of the membrane-bound enzymes involved in the phosphorylation-dephosphorylation equilibrium, the above mentioned theory can account for the shape effect induced by cholesterol depletion. However, the control of cell shape by spectrin phosphorylation has been recently questioned because of the observation that change in cell shape induced by ATP depletion occurs before any spectrin dephosphorylation [19]. The decrease in phosphorylation that we observed could thus arise from a breakdown of spectrin dimer following an activation of one of the proteases which have been described in the erythrocyte membrane [20]. This could result in the formation of peptides containing some of the phosphorylation sites, which are clustered at the extreme end of band 2 in peptides of 2500—4600 daltons [21]. The resulting rearrangement of spectrin could lead to the stomatocyte shape. An alternative hypothesis is that this shape change results from the increased phosphorylation of phospholipids. In erythrocytes incubated with ^{32}P, significant labelling of phospholipids has been reported to be due to phosphorylation of phosphatidate [22] or phosphatidylinositol [14,23]. The former has been implicated in the stomatocyte formation induced by phospholipase C treatment [7]. If the same holds under our experimental conditions, the increased synthesis of phosphatidate by activation of diacylglycerolkinase could account for the stomatocytogenic effect.

At the present time clear evidence in favour of one of the hypotheses discussed above is not available. More work is needed to determine whether spectrin breakdown products are generated by cholesterol depletion and to identify which lipids are phosphorylated in cholesterol depleted cells. Whatever the mechanism involved it is probable that the effects of cholesterol on the erythrocyte shape are mediated through the modulation of the activity of membrane-bound enzymes.

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