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BIOCHEMICAL STUDIES ON ABNORMAL ERYTHROCYTE MEMBRANES

PROTEIN ABNORMALITY OF ERYTHROCYTE MEMBRANE IN BILIARY OBSTRUCTION

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SUMMARY

Biochemical studies on erythrocyte membranes from eleven obstructive jaundice patients (due to various disorders) have been undertaken. By scanning electron microscopic observation these erythrocytes were spur and target in appearance. The lipid composition showed a marked increase in both cholesterol and phosphatidylcholine. In addition to these changes, it was unexpectedly demonstrated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate that a specific membrane protein component 4.2 was reduced or absent in all cases tested. This membrane protein abnormality was identical with that of hereditary spherocytosis erythrocyte membranes. It is of particular interest to note that after surgical relief of biliary obstruction in a typical case of common duct cholelithiasis, the disc electrophoretic pattern of erythrocyte membranes became normal and both lipid composition and red cell morphology returned to normal.

INTRODUCTION

Much information has been accumulated on normal erythrocyte membranes from the biochemical as well as ultrastructural point of view and has been reviewed by some investigators [1, 2]. On the other hand, the rapid expansion of knowledge and interest of erythrocyte membranes in disorders has stimulated intensive investigation, mainly on membrane lipids. In fact, the abnormality of membrane lipid composition in various forms of liver disease has been reported [3, 4]. By comparison to the documentation of membrane lipid abnormalities in a diseased state, only a few reports of membrane protein abnormalities have appeared and were principally limited to hereditary spherocytosis [5–8]. In the present communication the proteins of erythrocyte membrane from obstructive jaundice patients were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, thereby demonstrating a marked membrane protein abnormality which is exclusively identical with that of hereditary spherocytosis. To our knowledge, this is the first report on membrane protein alteration of erythrocytes from patients with obstructive jaundice.

MATERIALS AND METHODS

Blood samples

Blood samples were obtained from eleven hospitalized patients with biliary obstruction to varying degrees due to cholelithiasis, hepatoma or pancreas head cancer. Control blood was drawn from healthy, hematologically normal subjects.

Preparation of erythrocyte membranes

Blood was freshly drawn from patients and normal subjects in acid citrate dextrose solution and used almost immediately. After serum and buffy coat were removed, packed cells were washed with normal saline three times. Isolation of erythrocyte membranes was carried out essentially according to the method by Dodge et al. [9]. Washed erythrocyte suspension was hemolyzed in 20 vols. of 20 imosM veronal/HCl buffer (pH 7.4) and ghosts were sedimented by centrifugation in Hitachi automatic preparative ultracentrifuge model 80 P at 20000 $\times g$ for 20 min. Then the ghosts were washed with same buffer three times subsequent to hemolysis. Thus the purified erythrocyte membranes were prepared for chemical analysis.

Chemical analysis

Protein concentration was measured by the method of Lowry et al. [10] by using bovine serum albumin as standard. Phospholipid phosphorus was assayed by the technique of Bartlett [11] and cholesterol was measured by enzymatic method with cholesterol oxidase and peroxidase [12]. Alkaline phosphatase was assayed according to the method of Kind and King [13], and its activity was demonstrated as a King-Armstrong unit (K.A. unit) and one unit is defined as the amount of enzyme capable of liberating 1 mg of phenol in 30 min.

Extraction and separation of erythrocyte membrane lipids

The lipid was extracted from erythrocyte membranes using isopropyl alcohol and chloroform according to the method of Broekhuyse [14] and separated by thin-layer chromatography using Silica gel H. The thin-layer chromatographic plates were developed with chloroform/methanol/acetic acid/water (60 : 30 : 8 : 4, v/v). Quantitative analysis of individual phospholipids was carried out according to the method of Rouser [15].

Polyacrylamide disc gel electrophoresis of erythrocyte membrane proteins

Sodium dodecyl sulfate-acrylamide gel electrophoresis was carried out essentially according to the procedure of Fairbanks [16] in 0.1 % sodium dodecyl sulfate/0.1 M Tris/acetate buffer at pH 7.4 using 5.5 % acrylamide gel, in discs of 7 mm diameter and 8 cm length. For solubilization of membrane proteins, erythrocyte membranes were suspended in 1 % sodium dodecyl sulfate in 10 % sucrose containing 10 mM Tris · HCl (pH 8.0), 1 mM EDTA (pH 8.0), 40 mM dithiothreitol and 10 μg/ml Pyronin Y (tracking dye), and were incubated at 37 °C for 20 min. 25–35 μg protein was applied to each column and electrophoresis was carried out for 3 h at 4 mA/tube. The gel was fixed in 25 % isopropyl alcohol/10 % acetic acid and stained in Coomassie Brilliant Blue. For better visualization of stained gels, gels were scanned with the gel scanner of the Gilford spectrophotometer at 0.5 inch per min using a slit width of 0.05 mm at 550 nm.

Scanning electron microscopy

The samples were fixed for 1 h with 1 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After dehydration in acetone, dried erythrocytes were placed on grid, shadowed with carbon-gold, and examined by a scanning electron microscope (JSM-U3) [8].

RESULTS

Fig. 1 shows the representative disc electrophoretic patterns of erythrocyte membrane proteins from normal and four selected patients (2 cholelithiases, hepatoma and pancreas head cancer) out of eleven patients with apparent jaundice. As compared with protein pattern of normal erythrocyte membranes, a significant change is clearly demonstrated in the protein band 4.2. Although there are some variations in the total amount of protein loaded on each gel, band 4.2 became markedly faint or completely absent in erythrocyte membranes in all patients. Other cases (not shown here) showed the same trend. Furthermore, it is well known that the red cells of patient with obstructive jaundice due to various liver diseases show abnormal shapes which can be observed by scanning electron microscopy. Erythrocyte morphology of a typical case of biliary obstruction (case A in Fig. 1) is demonstrated in Fig. 2A. Most erythrocytes are "targeted" in appearance, possessing

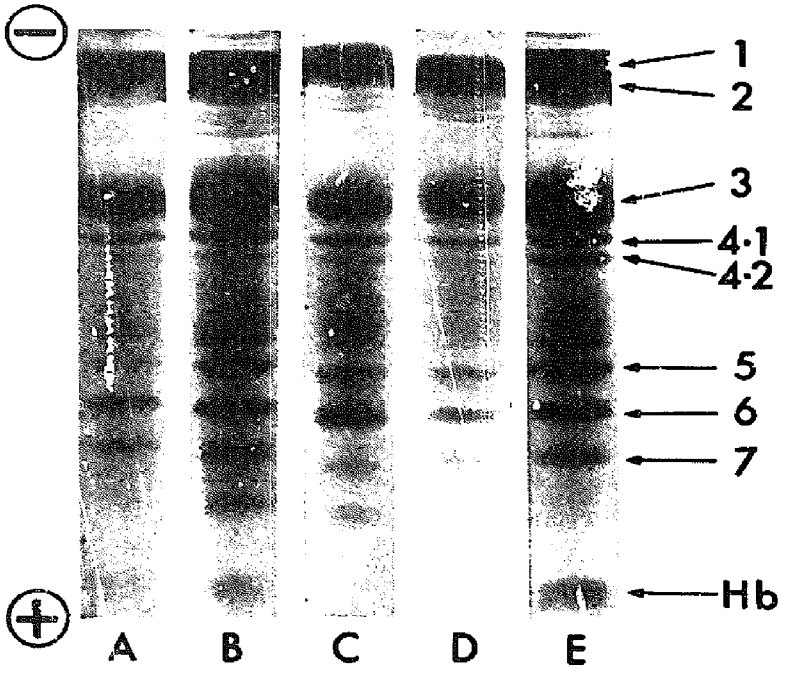

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of erythrocyte membrane. A; cholelithiasis, B; cholelithiasis, C; hepatoma, D; pancreas head cancer, E; control. Ghosts were dissolved in 1 % sodium dodecyl sulfate as described in Materials and Methods and electrophoresed in 5.6 % polyacrylamide gels in 0.1 % sodium dodecyl sulfate/0.1 M Tris/acetate, pH 7.4 [16]. Electrophoresis was carried out for 3 h with a constant current 4 mA per gel. Gels were stained with Coomassie Brilliant Blue. The nomenclature of individual protein bands was adapted from that of Fairbanks [16].

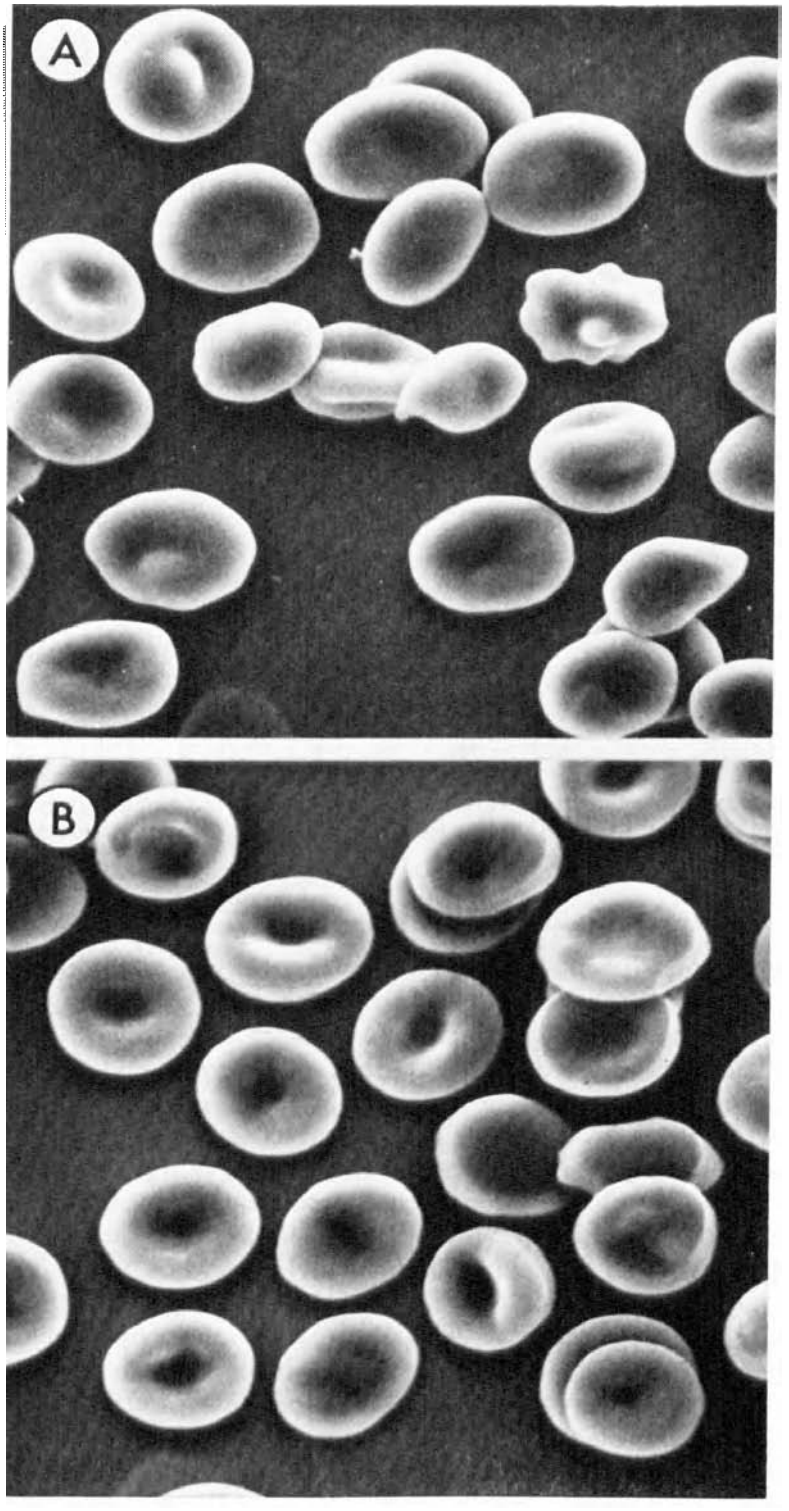

Fig. 2. Scanning electron micrographs of erythrocytes of cholelithiasis (case A) before (A) and after (B) surgical relief of biliary obstruction. Magnification, × 3000.

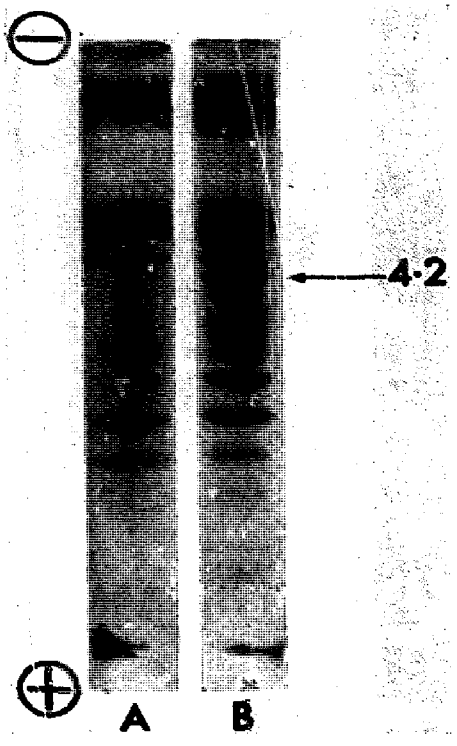

Fig. 3. Comparison of electrophoretic erythrocyte membrane protein pattern before (A) and after (B) surgical relief of biliary obstruction (case A). Electrophoresis was carried out in 5.6 % polyacrylamide gels in 0.1 % sodium dodecyl sulfate as described in Fig. 1.

either a central hemispherical projection or a more elongated ridge extending across the central depressions. Some "spur" shaped red cells are also seen. After surgical relief of biliary obstruction due to cholelithiasis, band 4.2 which was almost completely absent before operation reappeared just like that of normal erythrocyte membranes (Fig. 3A, B). This protein alteration can be easily visualized by the densitometer tracing of the stained gels shown in Fig. 4A, B. The membrane lipid composition of the patient showed that there was a striking elevation of phosphatidylcholine (31.3 % → 41.8 %) and cholesterol levels (cholesterol/phospholipid ratio; 0.94 → 1.39). Associated with higher values of phosphatidylcholine was a decrease in sphingomyelin (26.1 % → 21.3 %) and phosphatidylserine plus phosphatidylinositol percentages (11.3 % → 6.7 %) (Table I). These high levels of phosphatidylcholine and cholesterol in red cells agree well with the results of Cooper et al. [4]. However, after surgical operation such altered lipid composition was also restored to normal. With normalization of both protein and lipid composition of erythrocyte membrane after the surgery, morphologic abnormalities were no longer noted (Fig. 2B). In analogy to the morphological and biochemical parameters, the clinical-chemical values also returned to normal; icterus index 160 → 14, alkaline phosphatase 22 K.A. units → 4 K.A. units and total serum bilirubin 16.4 mg/dl → 1.3 mg/dl.

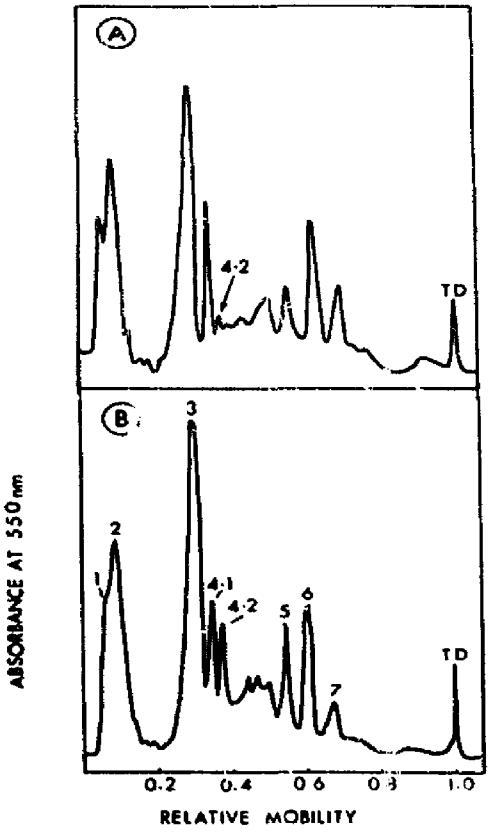

Fig. 4. Spectrophotometric scans of stained bands on sodium dodecyl sulfate polyacrylamide gels in Fig. 3A, B. Gels were scanned with a Gilford gel scanner at 550 nm. Note the reappearance of band 4.2 protein after removal of biliary obstruction.

TABLE I

RELATIVE PERCENTAGE OF INDIVIDUAL LIPIDS OF ERYTHROCYTE MEMBRANES FROM PATIENTS AND NORMAL SUBJECTS

Lipids were extracted from erythrocytes according to the method of Broekhuyse [14]. Individual phospholipids were separated on a Silica gel H thin-layer chromatographic plate and analyzed by the method of Rouser [15]. Free cholesterol was measured by an enzymatic method with cholesterol oxidase and peroxidase [12]. The numbers in parenthesis indicate the number of analyses and the patient values are the average of two analyses.

Lipids	Removal of biliary obstruction		Control (5)
	Patient before	Patient after	
Lysophosphatidylcholine	1.4	1.4	2.0±0.99
Sphingomyelin	21.3	26.1	26.3±0.48
Phosphatidylcholine	41.8	31.3	30.3±1.4
Phosphatidylserine plus phosphatidylinositol	6.7	11.3	12.2±0.87
Phosphatidylethanolamine plus phosphatidic acid	28.7	30.3	29.6±1.4
Cholesterol/phospholipid molar ratio	1.39	0.94	0.91±0.02 (3)

DISCUSSION

The disappearance or reduction of membrane protein components has been found by several investigators in hereditary spherocytosis. Gomperts et al. [5] reported that electrophoresis of acetic acid solubilized membrane protein on urea-starch and polyacrylamide gel revealed a consistent absence of one protein component. In 1974, Hayashi et al. [7] and Nozawa et al. [8] independently demonstrated the partial or complete absence of 4.2 band protein using disc gel electrophoresis in sodium dodecyl sulfate.

In the present study, an abnormal pattern of erythrocyte membrane protein was first shown in patients with obstructive jaundice. It was surprising for us to discover that this protein abnormality was observed not only in hereditary spherocytosis but also in the obstructive jaundice erythrocyte. It should be of further interest to note that in a patient with common duct cholelithiasis (case A in Fig. 1) the membrane protein change which was associated with alterations in lipid composition and morphology was reversible and disappeared by surgical relief of biliary obstruction.

Although the mechanism for the genesis of such membrane abnormality is unresolved, a possibility can be speculated that this specific membrane protein 4.2 would be solubilized from erythrocyte membranes with bile acids and lost during the preparation of ghosts, since it has been proposed by several lines of evidence from investigations of erythrocyte membrane architecture that this protein component 4.2 is localized on internal surface of erythrocyte [2, 17]. In order to clarify this mechanism, some in vivo and in vitro studies are required, these are therefore in progress in our laboratory.

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