

BIOMARKERS

Changes in Protein Profile of Erythrocyte Membrane in Bronchial Asthma

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Objectives. Erythrocyte membrane proteins reflect the prototype of multifunctional proteins of various erythroid and non-erythroid cells, which demonstrate various cellular functions. The protein profile of cells changes in various diseases. Therefore, the objective of this study was to understand the changes in protein profile of erythrocyte membranes in bronchial asthma. **Methods.** The study included 20 patients of bronchial asthma and 20 healthy subjects. Erythrocytes were isolated from peripheral blood, membranes were prepared followed by the determination of protein contents, and protein profile was assessed using SDS-PAGE. **Results.** In bronchial asthma, the protein contents of erythrocyte membranes in asthmatic patients were significantly higher ($p < .005$) than in healthy controls. Analysis of protein profile showed absence of the proteins, namely, band 4.2 and adducin subunit-II, and appearance of protein bands of molecular weights corresponding to galectin-3, glyceraldehyde 3-phosphate dehydrogenase, β -actin, dematin, band 4.1, and adducin (subunit-I) in asthmatic patients when compared with healthy controls. **Conclusions.** In asthma, there are quantitative and qualitative changes in proteins of erythrocyte membranes. The absence of band 4.2 protein may cause impairment of the erythrocyte membrane integrity, and presence of galectin-3 may lead to the activation of various inflammatory cells. The altered protein profile may possibly lead to altered response of the inflammatory cells to the asthmogenic stimuli, which may be responsible for pathophysiology and manifestation of the symptoms of bronchial asthma.

Keywords blood, bronchial asthma, erythrocytes, membrane, protein profile

INTRODUCTION

The plasma membrane performs a very important function of isolating the cell contents from the exterior besides regulating the intracellular ionic concentrations and interacting with the external environment via the cell surface receptors (1). Its lipid bilayer is believed to function as a neutral two-dimensional solvent, having little influence on the functions of the membrane proteins. Of the total cellular proteins, 30% have been reported to be the transmembrane proteins (1). These proteins are involved in a variety of functions such as transduction of signals, transport of metabolites, cellular contraction, movement, and growth (2). In the membrane, the proteins are distributed in lipid rafts of the lipid bilayer. The lipid rafts are rich in sphingomyelin and cholesterol and constitute the functional domains (3). The membrane proteins are included in or excluded out of these lipid rafts to variable extents and thus build up a dynamic membrane structure. Proteins, which possess affinity to rafts, include glycosylphosphatidylinositol-anchored proteins (4, 5), doubly acylated proteins such as Src-family kinases or the α -subunits of heterotrimeric G proteins (6), cholesterol-linked and palmitoylated proteins such as Hedgehog (7), and transmembrane proteins, particularly the palmitoylated ones (4). One type of protein may associate with different rafts with different kinetics or partition coefficients. Thus, a monomeric transmembrane protein may have lesser affinity for one type of lipid raft,

while the same protein after cross-linking together or oligomerization may have higher affinity for the same rafts (8).

The proteins of erythrocyte membranes serve as prototype for the multifunctional proteins in various erythroid and non-erythroid cells. They perform specific functions in different cell types and tissues (9). They are responsible for their normal morphology and functions. Any alteration in their profile may lead to the development of pathophysiology and the disease.

Erythrocytes traverse through the vascular system for which the mechanical strength and flexibility is provided by the actin cytoskeleton network, which is organized by the lipid rafts (1). The stress causes morphofunctional changes in them (10). For example, the oxidative stress causes changes in band 3 protein, which changes rheological functions of the erythrocyte membranes (11). Similarly, the stress by ethanol and formate radicals makes the spectrin (bands 1 and 2) and ankyrin (band 3) more sensitive to radiations (12). In severe megaloblastic anemia, the erythrocyte membranes lack spectrin (bands 1 and 2) and band 3 protein (13). In diabetes, there are quantitative and qualitative changes in spectrin (bands 1 and 2) in erythrocyte membranes (14). In patients with autoimmune haemolysis, the bands 3, 4.1, and 4.2 proteins decrease, while in HIV patients, band 4.2 protein decreases (15). In patients with essential hypertension, the bands 4.5 and 6 proteins increase in erythrocyte membranes (16). In non-small cell lung carcinoma, the expression of glycoproteins, sialoglycoproteins, and band 3 proteins changes in erythrocyte membranes (17). In cystic fibrosis patients, changes in chloride channel in erythrocyte membrane are also documented (18). However, we are not aware of any

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study so far that reported the changes in protein profile of erythrocyte membranes of the asthmatic patients.

Since the protein profile of erythrocyte membranes changes in various diseases including conditions of oxidative stress, which is one of the important factors for development of inflammation, we hypothesized that there may be changes in the total protein contents and the protein profile of erythrocyte membranes of asthmatic patients and report the findings in the present communication.

MATERIALS AND METHODS

Study Design

A total number of 40 subjects (20 asthmatics and 20 healthy volunteers) of either sex (age range; 18–60 years) were enrolled in the study. Blood was drawn by venipuncture. The erythrocyte membranes were prepared and their total protein contents and protein profile were determined. The values were expressed as mean $\pm$ SEM. $P < .05$ was considered significant.

Chemicals and Biochemicals

Acrylamide, bis-acrylamide, bovine serum albumin (BSA), bromophenol blue, Coomassie Brilliant blue R250, Folin–Ciocalteu's phenol reagent, glycine, glycerol, histopaque (specific gravity 1.077), 2-mercaptoethanol, sodium dodecyl sulfate (SDS), and trizma were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetic acid, ethanol, methanol, sodium carbonate, sodium chloride (NaCl), sodium dihydrogen ortho phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), sodium potassium tartrate, sodium sulphite, and so on, were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, India). Heparin was purchased from Biological Evans Ltd. (Hyderabad, India).

Subjects

Patients. Asthmatics (20 patients) of either sex (age range 18–60 years) were enrolled in the study. They were selected from the outpatient department of Viswanathan Chest Hospital, Vallabhnbhai Patel Chest Institute, University of Delhi, Delhi, India. The diagnosis was made on the basis of detailed history, physical examination, and pulmonary function test as per the NHLBI guidelines (19). Accordingly, the patients showing reversible airway obstruction, that is, increase in forced expiratory volume in 1 second (FEV_1) by $\geq 12\%$ and 200 ml after inhalation of 200 μg of salbutamol and mild persistent asthmatics were included in the study. Patients showing any sign or symptoms of any other pulmonary or systemic disorder, pregnant and lactating females, and receiving any oral steroid were excluded. Bronchodilator, if any, was withdrawn 24 hours before collecting the blood sample. Their demographic details are given in Table 1. The study was conducted after obtaining the approval from the ethics committee of the institute.

TABLE 1.—History of disease.

Age (years) (Mean $\pm$ SD)	31.6 $\pm$ 6.67 (range 19–43) ($n = 20$)
Sex	
Male (n)	11
Female (n)	09
Familial history of asthma/atopy	
Present (n)	8
Absent (n)	12
Self-recorded history of allergies to	
Cold (n)	7
Exercise (n)	2
Smoke (n)	9
Dust (n)	13
Pollens (n)	5
Fumes (n)	2
Diet	
Vegetarian	13
Non-vegetarian	7
$\text{FEV}_1\%$ (Predicted) (Mean $\pm$ SD)	75.1 $\pm$ 4.23 (range 68–80) ($n = 20$)
Percent change in FEV_1 after β_2 agonist use (Mean $\pm$ SD)	13.65 $\pm$ 1.90 (range 12–20) ($n = 20$)

Healthy Controls. Healthy and nonsmoker volunteers (20 subjects) of either sex (age range 18–60 years), with no personal or family history of any respiratory or systemic disease, constituted the control group.

Collection of Blood and Isolation of Erythrocytes. Peripheral blood (10 ml) was collected from the human subjects by venipuncture, directly in tubes under aseptic conditions, in EDTA (5 mg/ml). Lymphocytes were separated by the method of Boyum (20). Briefly, the blood was diluted 1:1, with physiological saline (0.15 M NaCl solution), layered on histopaque (specific gravity 1.077), and centrifuged at 1800 rpm ($600 \times g$) (Rota 4R-V/FM Plasto Crafts, Plasto Crafts Industries (P) Ltd., Andheri (E), Mumbai – 400 059, India) for 10 minutes. The interface containing lymphocytes along with plasma was removed. The packed red cells were washed twice with physiological saline and used for preparation of the membrane.

Preparation of Erythrocyte Membranes. The erythrocyte membranes were prepared by the method as described by Hanahan and Ekholm (21). To 1.0 ml of packed RBCs, 40 ml of 5P8 (5 mM sodium phosphate, pH 8.0) was added. The contents were mixed thoroughly, kept at 4°C for 1 hour to lyse the cells and then centrifuged in a Sorvall RC-5B (Kendro Laboratory Products, Texas, USA) refrigerated centrifuge (rotor-SS-34) for 30 minutes at 10,000 rpm ($7000 \times g$). The supernatant was discarded. The pellet consisting of erythrocyte membrane was washed twice similarly and finally suspended in 5 mM sodium phosphate buffer.

Protein Estimation. Protein contents of the membrane preparation were determined by the method of Lowry et al. (22). An aliquot (250 μl) was mixed with an equal volume of 10% chilled trichloro acetic acid (TCA), kept at 4°C for 15 minutes, and then centrifuged at 2000 rpm ($700 \times g$) for 5 minutes in a REMI (R8C) centrifuge

(Remi Sales & Engineering Ltd, Remi House, Goregaon (East), Mumbai, India). The TCA was removed by inverting the tube carefully and the pellet dissolved in 125 μ l of 2N NaOH. After dissolving the pellet, the volume was made up to 1.5 ml with distilled water. Then, aliquots (250 μ l) of the sample were taken in duplicates and their volume made up to 500 μ l. This was followed by addition of 2 ml of freshly prepared alkaline copper reagent (prepared by mixing 1.0 ml of 2% sodium potassium tartarate and 1.0 ml of 1% copper sulphate to 98.0 ml of 2% sodium carbonate solution in 0.1N NaOH) to each tube. The contents of the tubes were mixed gently on a vortex mixer and kept in dark at 37°C for incubation for 15 minutes. Then, 0.2 ml of 1N Folin–Ciocalteu's reagent was added to each tube with immediate mixing. The tubes were kept in dark at 37°C for 45 minutes. The optical density was measured at 690 nm. BSA solution (1 mg/ml in 0.1N NaOH) was used as standard. A reagent blank and a set of standards (20–140 μ g) were simultaneously run. Standard curve was plotted to calculate the protein contents of the samples.

Protein Profiling by SDS-PAGE. The proteins were resolved using SDS-PAGE on 4% stacking and 10% resolving polyacrylamide gel (using BIO-RAD mini Protean II apparatus, Bio-Rad Laboratories, Inc. 2000 Alfred Nobel Drive, Hercules, Ca 94547, USA). The electrophoresis was done at constant voltage, initially at 80 V for 20 minutes and then at 120 V for 90 minutes. The standard protein markers were simultaneously electrophoresed. The gels were stained with Coomassie Brilliant blue R-250 to visualize the protein bands. The intensity of the protein bands was measured using Quantity One software (BIO-RAD).

Statistical Analysis. "Prism" software was used for analyzing the data for calculating mean $\pm$ SEM and for applying Student's *t*-test. *P* < .05 was considered significant.

RESULTS

Protein Contents of the Erythrocyte Membrane

The total protein contents of the erythrocyte membranes of healthy controls were 0.94 ± 0.23 mg/ 10^9 RBCs and that of asthmatics were 2.313 ± 1.273 mg/ 10^9 RBCs. The values ranged between 0.746 and 1.36, and 1.06 and 4.45/ 10^9 RBCs in healthy subjects and asthmatic patients, respectively. Thus, in asthma, the total protein contents were observed to be higher, which were greater than two-fold (245%) of the control group (*p* < .005).

Protein Profile of the Erythrocyte Membrane

Protein profile of healthy controls and asthmatics were interesting, which showed both qualitative and quantitative differences (Figure 1) (Table 2). The nomenclature of the protein bands was made on the basis of the available literature (1, 2, 23, 24). In healthy subjects, we observed nine protein bands. Their molecular weights ranged between 190 and 52 kDa, which were 190, 160, 118, 100, 96, 80, 58, 55, and 52 kDa. In asthmatics, we

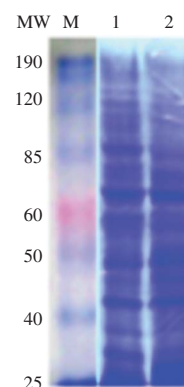


FIGURE 1.—SDS-PAGE for protein profiling of erythrocyte membrane protein. Lane M: Marker lane; Lane 1—Asthmatic Patients; Lane 2—Healthy Subjects.

observed 13 protein bands between 190 and 32 kDa. Interestingly, in asthmatics the proteins of molecular weight of 80 kDa (band 4.2) and 100 kDa (adducin subunit-II) were not substantially present but there was the presence of another six bands of proteins of 32 kDa (galectin-3 or galactose-specific lectin 3), 37 kDa (glyceraldehyde 3-phosphate dehydrogenase), 44 kDa actin cytoplasmic 1 (β -actin), 47 kDa (dematin), 88 kDa (band 4.1 protein), and 105 kDa (adducin subunit-I) (Table 1), which were absent in healthy controls.

DISCUSSION

In this study, we observed that the total protein contents of erythrocyte membranes of asthmatics were significantly higher than that of the healthy controls. This might have occurred due to increased expression and synthesis of some of the proteins or probably decrease in proteolysis, apparently due to decreased protease activity (25).

The protein profile of asthmatics also changed compared with the healthy controls, which showed the absence of protein bands of adducin (subunit-II) and band 4.2 protein, and presence of additional bands, namely, adducin (subunit-I), band 4.1 protein, dematin (erythrocyte membrane protein band 4.9), actin cytoplasmic 1 (β -actin), glyceraldehyde 3-phosphate dehydrogenase, and galectin-3 (galactose-specific lectin 3).

The altered membrane protein profile of asthmatics could be related to the increased protein contents of the erythrocyte membranes. The band 4.2 protein maintains the erythrocyte membrane integrity, and its deficiency is known to be associated with abnormalities in erythrocyte shape and membrane stability (26). Its absence in the asthmatics suggests the instability of the membrane structure of the erythrocytes, which may lead to the possibility of impaired signaling in the cells due to unstable orientation of proteins of the lipid raft in them. Another protein, the band 4.1 protein, plays a crucial role in stabilizing spectrin cross-links between actin filaments (24). In this study, this protein appeared as an additional protein band in asthmatics compared with healthy controls. This protein

TABLE 2.—Protein profile of asthmatics and healthy controls by SDS-PAGE.

Molecular weight (kDa)	Name of the protein	Healthy controls	Asthmatics
190	Unknown	+	+
160.41	Unknown	+	3+
118.42	Unknown	3+	4+
105	Adducin (subunit-I) ²⁴	Absent	2+
100	Adducin (subunit-II) ²⁴	4+	Absent
95.78	“Band 3” or anion exchanger protein ²⁵	5+	5+
88	Band 4.1 protein ²	Absent	5+
80	Band 4.2 protein ²	3+	Absent
58.0	T-complex protein 1, delta subunit ¹	5+	4+
55	55 kDa erythrocyte membrane protein (P55) ¹	3+	2+
52.24	Dematin 52 kDa subunit ¹	3+	3+
47	Dematin (erythrocyte membrane protein band 4.9) ¹	Absent	+
44.1	Actin, cytoplasmic 1 (β -actin) ¹	Absent	2+
37.0	Glyceraldehyde 3-phosphate dehydrogenase ¹	Absent	2+
32.4	Galectin-3 (galactose-specific lectin 3) ¹	Absent	2+

Notes: Gradings: + to 5+ indicates presence and intensity of the protein band as measured by Quantity One software from BIO-RAD. ODU, optical density unit:

0–2000 ODU: +
 2001–4000 ODU: 2+
 4001–6000 ODU: 3+
 6001–8000 ODU: 4+
 8001 ODU onward: 5+

may be playing an important role in maintaining the stability of the membrane, which otherwise would be compromised due to absence of band 4.2 protein in asthmatics. Further, we observed that in asthmatics, dematin (erythrocyte membrane protein band 4.9) and actin cytoplasmic 1 (β -actin) appeared as additional bands. Actin cytoplasmic 1 (β -actin) is involved in disassembly of the membrane skeleton proteins, whereas dematin (erythrocyte membrane protein band 4.9) is involved in bundling actin protein (25). Presence of both these proteins in asthmatics represents an unusual pattern in comparison to the healthy controls, where these proteins are absent. The presence of these proteins in asthmatics may influence the flexibility of the erythrocyte membrane thereby affecting the movement of erythrocytes in the vascular system. In asthmatics, the other additional protein bands observed by us were that of glyceraldehyde 3-phosphate dehydrogenase and galectin-3 (galactose-specific lectin 3). Glyceraldehyde 3-phosphate dehydrogenase catalyzes the sixth step of glycolysis, whereas galectin-3 (galactose-specific lectin 3) plays a role in activating various types of cells, including monocytes/macrophages, mast cells, neutrophils, and lymphocytes (27). The role of increase in glyceraldehyde 3-phosphate dehydrogenase in asthmatics suggests an increase in glycolytic pathway to provide more energy possibly due to higher energy demand of the asthma patients. Since galectin-3 (galactose-specific lectin 3) activates inflammatory cells, the role of these proteins in causing inflammation may be considered to be very important in asthmatics compared with that in healthy controls.

Distinct classes of proteins are incorporated in lipid rafts in the plasma membrane. They are glycosylphosphatidylinositol-anchored proteins, doubly acylated peripheral membrane proteins, cholesterol-linked proteins, and transmembrane (often palmitoylated) proteins. The transmembrane domains of proteins intercalate with raft lipids

for maintaining the tight packing of the liquid-ordered phase (28). Thus, by accumulating signaling molecules and selectively admitting transmembrane proteins, lipid rafts have the potential to compartmentalize signals within the plasma membrane.

These changes in protein profile may presumably result in the causation of the disease or its predisposition, or might have been acquired secondarily subsequent to the development of asthma. Since we collect the blood samples from patients, it is quite likely that these changes might be occurring secondarily to the development of the disease. Since proteins play important roles in the functioning of the lipid rafts as their structural component and in the orientation of the membrane receptors, it may be speculated that the quantitative or qualitative changes in membrane proteins, as observed in the present investigation, may be proposed to lead to the impaired functioning of the proteins of the membranes, including those present in the lipid rafts of the membranes, causing abnormal cellular response, which may possibly result in the pathophysiology and perpetuation of the symptoms of asthma.

CONCLUSIONS

In asthmatics, there are quantitative and qualitative differences in the protein profile of erythrocyte membranes. These proteins may influence the structure and functions of the plasma membrane and its lipid rafts. Based on these observations, it may be proposed that the change in protein profile of the plasma membrane may affect the cellular signaling, which in turn may activate the inflammatory cells to release the inflammatory mediators in inappropriate amounts that may cause the perpetuation of inflammation and manifestation of the symptoms of bronchial asthma.

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DECLARATION OF INTERESTS

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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