DIAGNOSIS OF HEMOLYTIC ANEMIAS DUE TO DEFECTS IN MEMBRANE CYTOSKELETON BY POLYACRYLAMIDE GEL ELECTROPHORESIS: A CASE STUDY-ABSENCE OF ANKIRIN

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Summary

Detection of structural defects or absence of cytoskeletal proteins are known to be involved in pathogenesis of microcytic anemias. Venous blood samples were collected in heparinised tubes. The protein composition of the erythrocyte membranes was analysed by densitometry after their separation on SDS-PAGE. We studied 17 cases of congenital microcytic anemia from the Cluj-Napoca Pediatric Hospital. In 16 cases quantitative, or qualitative changes of spectrin were observed, or even the both. In one case the absence of ankyrin was observed. This study suggests that defects of the membrane cytoskeleton are cause of microcytic congenital anemias.

Key words: microcytic congenital anemias, SDS-PAGE, ankyrin, spectrin, cytoskeleton

Introduction

The erythrocyte biconcave shape is provided by the structural integrity of its membrane skeleton, composed of a protein network (including spectrin, ankyrin and the proteins from bands 4.1, 4.2, 4.9, and 5) that, in turn, is connected to the membrane through a series of well defined amino acid sequences derived from the polypeptide chains of the proteins involved (Ranaekers et al., 2004). Even some forms of microcytic anemia are due to molecular defects concerning the heme synthesis (Iolascon et al., 2009), the structural modifications or absence of certain cytoskeletal proteins are mainly responsible for the microcytic anemias (Lanciotti et al., 1997; Eber et al., 2004; Rocha et al., 2005; Bennett et al., 2008; Perotta et al., 2008).

Material and methods

The blood was collected by venipuncture in heparinised tubes. The erythrocytes were isolated by centrifugations and washed three times in medium S: 150 mM NaCl, 5.5 mM glucose, 5 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], pH=7.4. The erythrocyte membranes were prepared using the method described by Benga et al. (2000, 2002). Protein concentration was determined by the procedure of Lowry et al. (1951), using a Specord S 600 spectrofotometer (Analytic Jena AG, Jena, Germany), assisted by a Win ASPECT Spectroanalytical software (Analytic Jena AG, Jena, Germany). One volume of membrane proteins was added to 3 volumes of a solution containing 1.3% sodium dodecylsulfate (SDS), 10% sucrose, 53.3 mM dithioerythritol, 1.3 mM EDTA, 20 mM Tris-HCl (pH 6.8) and 0.007 mg bromphenol blue. The mixture was heated for 5 min in a 95°C bath. Membrane peptides were separated using the SDS polyacrylamide system described by Laemmli (1970). The slab gel consisted of a running gel of 10% acrylamide and 5% stacking gel. The acrylamide-to-bisacrylamide ratio was maintained at 36.5:1 in both gels. Samples of 20 μl/20 μg protein were applied and the electrophoresis was carried out at 200 V in a Mini Protean II system (BioRad Richmond, CA, U.S.A), until the dye reached the bottom of the running gel (about 1 hr) in the running
buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). The gels were fixed for 1 hr in 45% (vol/vol) methanol/10% (vol/vol) acetic acid and then stained 15 min in the solution containing 0.07% (wt/vol) Coomassie brilliant blue R-250. Destaining was performed with 10% (vol/vol) acetic acid. The percent concentration of the separated fractions was determined by densitometry, using a GS-700 Imaging Densitometer (BioRad, Hercules, CA, U.S.A.), assisted by a computer with adequate software – 1-D Analyst® II (BioRad Laboratories, Richmond, CA, U.S.A). The protein fractions separated were noted according to the nomenclature proposed by Fairbanks et al. (1971).

In order to measure the RBCs diameter, the blood samples were suspended in 0.5% Bovine Serum Albumin (BSA). The RBCs morphological aspects were examined in phase contrast microscopy, with a Nikon Eclipse 80i light microscope (Nikon Corporation, Tokyo, Japan), using an Olympus Color View 1 CCD camera (Olympus Soft Imaging Solutions GMBH, Münster, Germany). Morphological measurements were made with the CellID Olympus computer software (Olympus Soft Imaging Solutions GMBH, Münster, Germany), and the statistical analysis was performed using the Microsoft Office Excel software (Microsoft Corporation, Redmond, USA).

Results

RBC membrane proteins were separated by denatured electrophoresis on a 10% polyacrylamide gel in case of 17 patients with hemolytic anemia from the Cluj-Napoca Pediatric Hospital and a control subject. The densitometric analysis of the electrophoreograms of RBCs membrane proteins from all cases was performed. In 16 patients we observed quantitative modifications in spectrin, while in one case the absence of ankyrin (band 2.1-2.3) was noted (Fig.1). For the diameters of RBCs (n=12), we obtained a mean value of 6.24 µm (STDEV 0.68).

![Figure 1](image1.png)

Figure 1. Lane 1: patient without band 21-23 (ankyrin) red blood cells membranes (RBC). Lane 2: normal RBCs membranes. Lane 3: molecular weight markers (BioRad) 200 Da: myosin; 116.2 Da: E. coli β-galactosidase; 97.4 Da: rabbit muscle phosphorylase b; 66.2 Da: BSA; 45 Da: hen egg white ovalbumin; 31 Da: bovine carboxy anhydrase; 21.5 Da: soybean trypsin inhibitor.

![Figure 2](image2.png)

Figure 2. Phase contrast image of a blood sample of the patient diagnosed with congenital microcytic anemia.
Discussion

RBCs spherocytic aspect is caused by the absence of ankyrin, which binds the membrane cytoskeleton to band 3 – a major protein in the plasma membrane (Bennett et al., 1979, 1980). Many studies reported ankyrin implication in modifying RBCs morphologic aspect, showing that ankyrin deficit is one of the most common erythrocyte membrane anomalies in patients with microcytic anemia. Sometimes it can be associated with a spectryn deficit as well (Pekrun et al., 1993; Savvides et al., 1993; Miraglia del Giudice et al., 1994; Saad et al., 1994; Lanciotti et al., 1997; Premetis et al., 1999; Lee et al., 2000; Ricard et al., 2000; Sanchez-Lopez et al., 2003; Boguslawska et al., 2004).

Ankyrin binds the cytoskeleton to the cellular membrane via β-spectryn – a protein that presents a specific binding site for ankyrin (Bennett et al., 1979, 1980). Gallagher et al. (1998) showed that the absence of ankyrin generates the appearance of spherical RBCs. Mutations at ankyrin level or a diminished concentration of ankyrin in the cytoskeleton determine a dysfunctional attachment of the cytoskeleton to the cellular membrane, generating the appearance of spherocytes. As spectryn assembling is influenced by ankyrin (Bennett et al., 2008), a decrease in ankyrin synthesis determines a concomitant decrease of both spectryn and ankyryn concentrations.

Davis et al. (1990), reported that spheric RBCs can appear if the ankyrin binding site to the spectryn is missing or presenting defects. On the other hand, modifications in RBC’s morphology can be due to structural modifications of ankyrin induced by a decreased interaction between band 4.2 protein (pallidin) and band 3 protein. In hereditary spherocytosis, band 3 defects stand along side ankyrin defects as the most frequent causes of this disease. Lack of certain amino acid sequences determines the abolishing (Glu 721 – Ser 764) or diminishing (Cys 347 – Asn 567) of ankyrin binding affinity to band 3 protein (Davis et al., 1991). Since 1989 Davis pointed out the influence of 174-186 sequence in ankyrin – band 3 interactions (Davis, et al. 1989). Absence or a diminished concentration of ankyrin, or the presence of a mutant ankyrin, determines a dysfunctional attachment of the cytoskeleton to the cellular membrane via band 3 protein, generating the appearance of spherocytes (spherocytosis). Also mutations in α or β spectrin produce spherocytosis (Becker et al., 1993; Hassoun et al., 1997; Miraglia del Giudice et al., 1998).

Concomitant deficiencies in both spectrin and ankyrin is not that unexpected, as a diminishing in ankyrin synthesis or assembling generates lower rates in spectrin assembling if the ankyrin binding site is absent or deficient.

In our study, the absence of ankyrin, observed on the SDS-PAGE (Fig.1) is the main reason for the abnormal morphological SDS-PAGE of the RBCs (Fig.2) and provides a useful tool for the diagnosis of microcytic anemia.

Conclusions

This study suggests that defects of the membrane cytoskeleton are the cause of microcytic congenital anemias. The majority of cases revealed changes of spectrin, quantitative, qualitative or both.

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