Changes in the erythrocyte membrane cytoskeleton in microcytic hemolytic anemia – case report

Modificări ale citoscheletului membranei eritrocitare în anemia hemolitică microcitară – prezentare de caz

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Abstract

A case of microcytic anemia from the Cluj-Napoca Pediatric Hospital is presented. Light and scanning electron microscopy data indicate poikilocytosis (with a high ratio of spherocytes – but irregular-shaped cells are also present), and anisocytosis (with a high number of microcytes, many cells having diameters under 4 μm). The protein composition of the erythrocyte membranes was analysed using SDS-PAGE and the percentages of proteins in the various bands were analysed by videodensitometry. A spectrin/band 3 subunitary ratio was observed, suggesting a quantitative deficit of spectrin. The ankyrin/band 3 ratio was also reduced, under the limit of 0.2, indicating defects at the ankyrin level as well. The results of our study proved that defects in both spectrin and ankyrin from the erythrocyte membrane cytoskeleton are the main cause of microcytic anemia in this case.

Keywords: *erythrocyte, spectrin, ankyrin, microcytic anemia, SDS-PAGE*

Rezumat

Acest articol prezintă un caz de anemie microcitară înregistrat la Clinica de Pediatrie II a Spitalului Județean de Urgență Cluj. Datele de microscopie optică şi electronică arată poikilocitoză (cu un procentaj ridicat de sferocite şi prezența unor celule de formă neregulată) şi anizocitoză (cu o pondere însemnată de microcite, multe celule având diametre sub 4 μm). Compoziția proteică a membranelor celulare a fost analizată folosind SDS-PAGE, procentajul de proteine în diferite benzi fiind analizat prin videodensitometrie. S-a constatat un raport subunitar spectrină/bandă 3, sugerând un deficit cantitativ de spectrină. şi raportul ankyrină/bandă 3, este redus, sub limita de 0,2, indicând defecte şi la nivelul ankyrinei. Rezultatele obținute au dovedit că, la nivelul citoscheletului membranar eritrocitar, atât deficitul de spectrină cât şi cel de ankyrină sunt principalele cauze ale anemiei microcitare în acest caz.

Cuvinte cheie: *eritrocite, spectrină, ankirină, anemie microcitară, SDS-PAGE*

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Introduction.

Red blood cells (RBCs) are biconcave discs in the large vessels or in vivo, but they change shape in order to pass through narow capillaries. The ability of a RBC to maintain its discoid shape, deformability and elasticity in the circulation, under constant mechanical stress, is attributed to the components of its membrane (1). Actually, all these features of the RBCs are mainly provided by the structural integrity of its membrane skeleton; this skeleton is composed of a protein network (including spectrin, ankyrin and the proteins from bands 4.1, 4.2, 4.9, and 5) which, in turn, is connected to the membrane through a series of specific domains of the proteins involved (2). Defects in spectrin and other membrane-associated skeletal proteins are associated with membrane lipid loss and surface area deficiency, alteration in cation content and membrane permeability, and decreased deformability of the RBCs (3). Abnormalities in the RBC membrane are responsible for the hereditary hemolytic anemias, which include an important group of inherited disorders (4, 5). These include hereditary spherocytosis (HS), hereditary elliptocytosis (HE), hereditary pyropoikilocytosis (HPP) and the hereditary stomatocytosis (Hst) (6). HS is the most common cause of hemolytic anemia of non-immune nature being characterized by the presence of numerous spherocytes in the peripheral blood (7), in most cases having small diameters (microcytes). Although some forms of microcytic anemia are due to molecular defects concerning the heme synthesis (8), the structural modifications or absence of certain citoskeletal proteins are mainly responsible for the microcytic anemias (3, 9). In all these cases, the sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) is requested for the molecular diagnosis (10). The aim of this study was to establish the molecular diagnosis in a patient with microcytic anemia.

Case report

A 12-year-old girl (35 kg weight, 150 cm height), diagnosed with congenital hemolytic anemia in early infancy, was admitted in October 2009 to the Cluj-Napoca Pediatric Hospital with bleeding gums, epistaxis and intense jaundice. Previously, a viral etiology of hepatocytolytic syndrome has been excluded.

At admission the patient showed intense jaundice, facial acne, dry teguments, pale mucous membranes, and laterocervical adenopathy. The heart rate was 96/min and a low blood pressure (95/60 mmHg) was recorded. The measurement of *splenic* length obtained *at ultrasound* examination (14.1 cm) suggested a higher volume of this organ.

The blood group test resulted in O Rh-positive; erythrocyte sedimentation rate (ESR) was 12 mm/h, and the hematocrit, 30.2%. Hematological examination revealed: RBCs count 3.42 $x10^{12}$/L, mean corpuscular volume (MCV) 91 fL, hemoglobin (Hb) 9.8 g/dL, mean corpuscular hemoglobin (MCH) 29.5 pg, mean corpuscular hemoglobin concentration (MCHC) 32.5 g/dL, reticulocytes count 72.5‰, white blood cells (WBC) count 11,400 $x10^6$/L (among which lymphocytes 38%, neutrophils 40%, eosinophils 4%, basophils 0.0%, and monocytes 18%), and platelet count 422$x10^9$/L.

Liver function tests revealed: total bilirubin 12.59 mg/dL (of which 1.18 mg/dL was direct bilirubin), alkaline phosphatase 258 U/L, aspartate *transaminase* (ASAT) 62 U/L, alanine *transaminase* (ALAT) 26 U/L, cholinesterase 6681 U/L, γ-glutamyl transferase (γGT) 16 U/L, total protein 7.3 g/dL, albumin 4.8/dL; other biochemical tests: fibrinogen 259 mg/dL, iron 160 µg/dL, ferritin 37 µg/L.

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 RBCs morphological aspects were studied both in phase contrast microscopy, and scanning electron microscopy (SEM).

Light microscopy. The separated and washed RBCs were suspended in 0.5% bovine serum albumin in medium S, and studied 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 CellD Olympus computer software (Olympus Soft Imaging Solutions GMBH, Münster, Germany). We measured the diameter of the RBCs, and the mean volume of RBCs was calculated using values of the hematocrit and number of RBCs/mm^3. In order to obtained the mean value of the hematocrit capillary tubes, a Hawksley micro-hematocrit centrifuge and a Hawksley reader (Hawksley & Sons Ltd., Lancing, England) were used; the number of RBCs/mm^3 was obtained by counting the cells on a Thoma slide (Carl Zeiss, Jena, Germany). The statistical analysis (mean diameter and standard deviation) and the calculations of the number of RBCs/mm^3, and of the mean RBCs volume were performed using the Microsoft Office Excel software (Microsoft Corporation, Redmond, USA).

Electron microscopy. The separated RBCs were prepared for SEM examination according to the usual protocols (11). RBCs were prefixated for 1.5 hours in 1% (v/v) glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4, at 0-4ºC), washed three times with 0.1 M phosphate buffer, post-fixated for 2 hours in 1% (w/v) osmium tetroxide (in 0.1 M phosphate buffer, pH 7.4, at room's temperature), and dehydrated in an ethanol series (5 solutions) of 50–100% (v/v), 15 minutes each. RBC samples placed on glass cover slips were fastened on aluminum stubs used as holders, and then placed in a Polaron E–5100 plasma-magnetron sputter coater (Polaron Equipment Ltd., Watford, Hertfordshire, UK) and maintained over several hours at low vacuum (0.5 torr) for desiccation. The samples were coated with gold in a high-purity argon gas at low pressure (0.05 torr), for 1 minute at 2 kV and 20 mA. The examination of RBCs was performed in a JEOL JSM–25 Scanning Microscope (Jeol Ltd. Tokyo, Japan), at 30 KV acceleration voltage. The images were captured with a Deben Pixie–3000 image processor (Deben UK Ltd., Debenham, Suffolk, UK).

SDS-PAGE. The erythrocyte membranes were prepared using the method described by Benga et al (12). Protein concentration was determined by the procedure of Lowry et al (13), using a Specord S 600 spectrophotometer (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% SDS, 10% sucrose, 53.3 mM dithioerythritol, 1.3 mM EDTA, 20mM Tris-HCl (pH 6.8) and 0.007 mg bromphenol blue. The mixture was heated for 5 min in a 95ºC bath. Membrane peptides were separated using the SDS polyacrylamide system described by Laemmli (14). 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% (v/v) methanol/10% (v/v) acetic acid and then stained 15 min in the solution containing 0.07% (w/v) Coomassie brilliant blue R-250. Destaining was performed with 10% (v/v) 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).

Results

On the images of light (*Figure 1*) and electron microscopy (*Figure 2*) we observed a wide range of shapes for different RBCs: normal discocytes, spherocytes, ovalocytes and elliptocytes, irregular-shaped cells, and rare teardrop-shaped cells, bite cells and echinocytes. Poikilocytosis is accompanied by anisocytosis indicated by the presence of microcytes, normocytes, and macrocyites. The mean value of the RBCs diameter was 6.019±1.54 µm (n=480). A high number of microcytes has been observed: 575‰ of RBCs have diameters under 6 µm (245.6‰ with diameters between 5 and 6 µm, 158.7‰ with diameters between 4.5 and 5 µm, 106.2‰, with diameters between 4 and 4.5 µm, and 64.5‰ have the diameter's value under 4 µm). Macrocytes are present in a lower amount: only 15‰ of RBCs have diameters over 9 µm. At a hematocrit of 29% (RBCs suspended in medium S) we obtained a value of 3,462,562.5 RBCs/mm^3, and the calculated mean value of the RBCs volume was 83.82 µm^3.

RBC membrane proteins from a control subject and a patient with microcytic anemia from the Cluj-Napoca Pediatric Hospital were separated by denaturated electrophoresis on a 10% polyacrylamide gel. The separated bands were designed after the Laemmli's system (14): 1 (α-spectrin), 2 (β-spectrin), 2.1 and 2.3 (ankyrin), 3 (band 3), 4.1., 4.2, 4.5, 5 (actin), 6, 7, and 8. (Fig.3). After the densitometric analysis of the electrophoreograms of control RBCs membrane proteins we found a normal distribution of the bands. In the case of the investigated patient, we found the following percentage for the bands of interest: 18.09% spectrin, 3.77% ankyrin, and

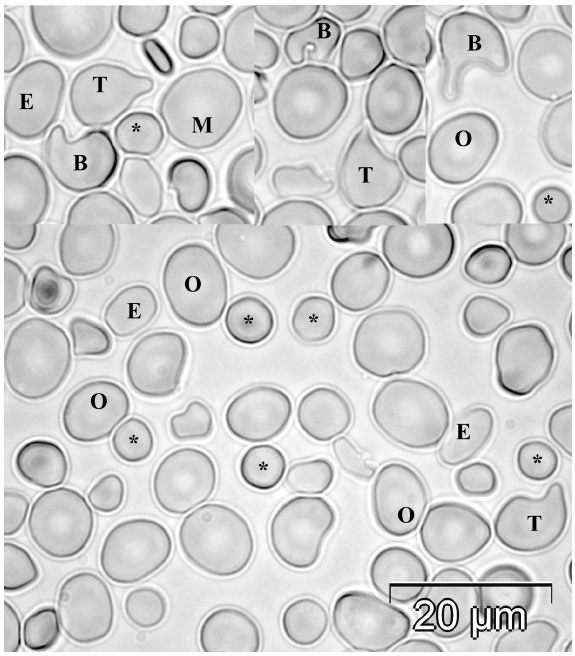

Figure 1. Photomicrograph of red blood cells from patient D.I. A high number of spherocytes (*) is observed, and also irregular-shaped cells (ovalocytes – O, eliptocytes – E, tear drop-like cells – T, bite cells – B) can be observed mainly in upper pannels (poikilocytosis). Cells of different sizes are present (anisocytosis), many cells having diameters under 4 µm (microcytes, *), and also macrocytes (M). Original magnification 100×.

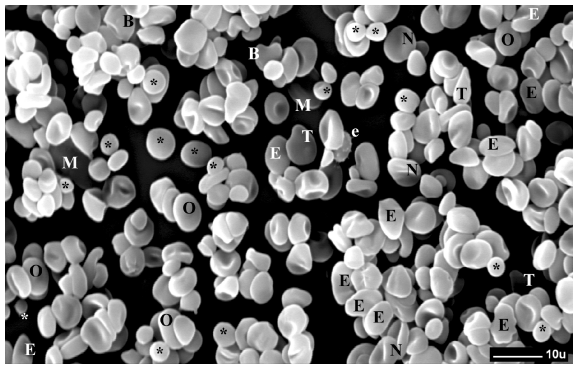

Figure 2. Scanning electron micrograph of blood cells from patient D.I. A high number of spherocytes (*) is observed, and also normocytes (N) and irregular-shaped cells (ovalocytes – O, eliptocytes – E, echinocytes – e, tear drop-like cells – T, bite cells – B). Cells of different sizes are present (anisocytosis), many cells having diameters under 4 µm (microcytes*), and also macrocytes (M), and normocytes (N). Original magnification 1000×.

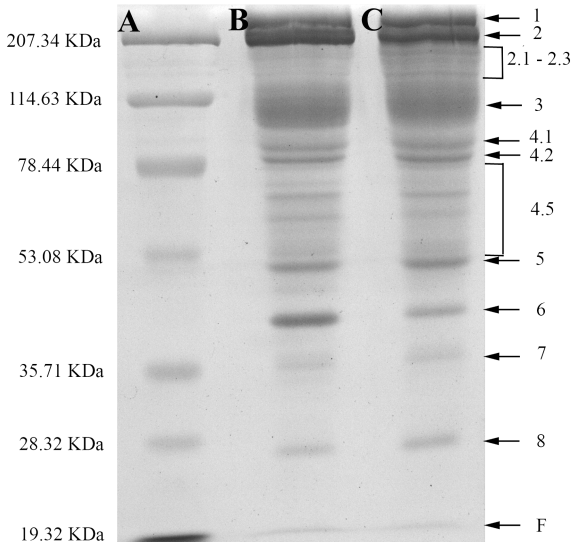

Figure 3. Electrophoretograms of red blood cells proteins from a control subject (lane B), and from patient (lane C: band 1: α spectrin, band 2: β spectrin, band 2.1-2.3: ankyrin). In lane C bands 1 and 2 2.1-2.3 are less intense. Markers are on lane A: Myosin (207.34 kDa), β-galactosidase (114.63 kDa), Bovine serum albumin (78.44 kDa), Ovalbumin (53.08 kDa), Carbonic anhydrase (35.71 kDa), Soybean trypsin inhibitor (28.32 kDa), Lysozyme (19.32 kDa).

22.45% band 3. It should however be noted that the spectrin/band 3 ratio was 1.14 in the control subject and 0.805 in the patient. Also the ratio ankyrin/band 3 was 0.2 in the healthy control and 0.16 in the investigated patient.

Discussion

Proteins in RBCs cytoskeleton are responsible for the normal size and shape of RBCs. Spectrin is the most abundant among these proteins. The basic spectrin unit comprises two subunits, α and β, which are encoded by two different genes located on chromosome 1q22-q23 and 14q23-q24.2 respectively (15). These two subunits share a common structural organization, which consists of a succession of approximately 106 aminoacid repeats (16). The two subunits of spectrin associate in an antiparallel fashion to form a heterodimer. It has been suggested that this association of the two subunits is initiated at specific sites within the two proteins, termed "nucleation sites", which involve repeats 19-22 on α subunit and repeats 1-4 on β subunit (17). Spectrin heterodimers associate at their head region to form tetramers. This head-to-head connection involves the most N-terminal region of the α subunit on one heterodimer and the 17th repeated segment of the β subunit of the other heterodimer which is located toward the C-terminal end of the molecule. Together, these two regions form a typical spectrin triple helical structure, which stabilizes the spectrin tetramer (15). The spectrin network is attached to band 3 by ankyrin, an anchor-like protein. Ankyrin is encoded by genes located on chromosome 8p11.2 and is composed of three structural domains (15). The 89 kDa N-terminal domain consists of 24 repeated-sequence motifs and contains two binding sites for band 3 (18). The 62 kDa domain contains the binding site for spectrin (19) and the C-terminal 55 kDa domain which regulate the binding of ankyrin to band 3 and spectrin (20).

The presence of spherical erythrocytes in blood is due to defects in cytoskeleton proteins; the cause of the molecular defects is represented either by problems of the individual proteins or a combined deficiency.

Thus, in many cases were reported a) defects in spectrin – reduced concentration of spectrin, normal amount of non-functional spectrin, or the presence of spectrin with mutations at the level of amino acids (21, 22); b) defects in ankyrin – which are the most frequent and typical for hereditary spherocytosis (23), and which may range from a reduced synthesis or the synthesis of mutant ankyrin forms to the total absence of ankyrin (24); c) changes in band 3; d) changes in band 4.2 (24), and e) very rare the absence of Rh complex, which binds to ankyrin, for a stabile expression in erythrocyte (25). In other cases deficiencies in both spectrin and ankyrin may be associated and responsible for the hereditary spherocytosis.

We reported here a situation in which the spherocytosis is caused by deffects in both spectrin and ankyrin. We also found many RBCs with abnormal shapes, proving the involvement of the two proteins in this pathology. It is known that defects mainly in spectrin may result in RBCs with abnormal shapes. On another hand, we must take into account that the ankyrin defficiency revealed in our study may be responsible for a reduced concentration of spectrin in RBCs membrane, as previously mentioned by Coetzer et al (26), who also reported a normal synthesis of spectrin. Many cells have a very small diameter as consequence of cytoskeletal problems followed by disruption of RBCs (27) and hemolysis; this aspect is also confirmed by the biochemical data (high values of total and indirect bilirubin).

Mutations associated with isolated spectrin deficiency are defects of both α-spectrin and β-spectrin (SPTA1 and SPTB, respectively) genes (28). In general, hereditary spherocytosis caused by α-spectrin and β-spectrin mutations is associated with recessive and dominant inheritance, respectively. In healthy erythroid cells, production of α-spectrin chains is three-fold to four-fold greater than β-spectrin production. Thus, a mutation of one β-spectrin allele is sufficient to cause spherocytosis whereas both α-spectrin alleles have to be affected for the disease to arise. β-spectrin defects account for about 15-30% of cases of hereditary spherocytosis in northern European populations. With rare exceptions, mutations of the β-spectrin gene are isolated and might be associated with monoallelic expression, suggesting that null mutations are common. Several de-novo mutations of β-spectrin have been described. α-spectrin defects account for about 5% of patients with hereditary spherocytosis and are only clinically apparent in the homozygous or compound heterozygous state. These patients have a severe degree of disease.

It is also known that patients with ankyrin defects display a prominent spherocytosis without other morphological abnormalities. In this situation, the low amount of ankyrin present in RBCs (due to a deficiency either in its synthesis, or its accumulation) is followed by a decrease in ankyrin assembling in RBCs cytoskeleton membrane. Spherical red cells also result from a loss of membrane surface area and the consequent increased sphericity and reduced deformability (28).

Conclusion

Our results prove the involvement of defects both in spectrin and ankyrin from the RBCs membrane cytoskeleton as the main cause of microcytic anemia in this case.

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