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INCREASED MEMBRANE PROTEIN PHOSPHORYLATION AND ANION TRANSPORT ACTIVITY IN CHOREA-ACANTHOCYTOSIS

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ABSTRACT

Background and Objective. Chorea-acanthocytosis is a disorder characterized by neuronal degeneration and the presence of acanthocytic erythrocytes on blood smear. The abnormal function and structure of the membrane protein band 3 are considered to be of pathogenetic relevance in determining the erythrocyte defect.

Methods. In a clinically evident case of chorea-acanthocytosis, the following parameters were investigated: membrane cholesterol and fatty acid composition, sulphate influx (as a measure of the anion transport activity), membrane protein phosphorylation, membrane casein and tyrosin-kinase activities; moreover, the promoter and all exons of the EPB3 gene were screened for possible mutations by single strand conformational polymorphism (SSCP) study.

Results. The sulphate influx, the Ser/Thr phosphorylation level, and the membrane casein-kinase activity were increased in chorea-acanthocytosis compared with normal controls. In the intact vanadate-treated ^{32}P-labelled erythrocytes, Tyr-phosphorylation of the cytoplasmic domain of band 3, as well as the poly(Glu, Tyr) kinase activity in the membranes, were enhanced in the patient's sample. Apparent molecular weight and concentration of band 3 on SDS/PAGE analysis, membrane fatty acid composition and cholesterol/phospholipid molar ratio were normal and the SSCP study of EPB3 exons did not show any abnormal polymorphisms.

Interpretations and Conclusions. An abnormal degree of phosphorylation of membrane proteins, in particular of band 2 (β-subunit) and band 3, may contribute in determining both change of cell shape and increased anion transport in chorea-acanthocytosis.

Key words: Chorea-acanthocytosis, phosphorylation, anion transport, band 3, erythrocytes

Chorea-acanthocytosis (or neuroacanthocytosis) is a rare disorder, characterized by central and peripheral neuronal degeneration and the presence of acanthocytic (thorny) erythrocytes on blood smear. Although the finding of an abnormal erythrocyte shape is clinically useful in properly assessing the diagnosis of these neurological patients, the involvement of different cells such as erythrocytes and neurons remains difficult to explain.

Among the mechanisms leading to the acanthocytotic shape change, abnormalities in membrane lipid composition, either in terms of an excess of saturated fatty acids or an increased cholesterol/phospholipid ratio,[1,2] have been proposed. This latter mechanism has been connected to the acanthocyte formation in the course of severe liver disease (so-called *spur cell anemia*),[3] or associated with defective apoprotein β synthesis (a-β-lipoproteinemia).[4] Due to an elevated membrane cholesterol content, the spur cells have an exaggerated intrinsic tendency to proteolize membrane proteins, and both of these features can be transferred to normal red cells by incubating them with patient plasma affected by spur cell anemia.[5] Similarly, chorea-acanthocytic red cells show a high membrane content of saturated fatty acids[1] and an abnormally elevated degradation of membrane proteins.[6] Acanthocytes have also been described in the presence of an abnormal expression of red cell membrane antigens such as in Mac Leod syndrome.[7]

However, the current opinion about the mechanisms responsible for acanthocyte formation indicates a common component of the plasma membrane as being specifically involved in the pathogenesis of the abnormal cell shape: several data have focused on the ubiquitarious anion trans-

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porter, the membrane protein band 3.[8,9]

Although its abnormal function and structure may be of pathogenetic relevance, and despite the fact that band 3 represents one of the most important substrates of phosphorylation on the erythrocyte membrane,[10] the membrane phosphorylative process has been scarcely investigated in acanthocytosis. To the best of our knowledge, only a preliminary report, which shows that cAMP-dependent phosphorylation of erythrocyte protein 4.9 is impaired in affected patients from two families with chorea-acanthocytosis, has been made available to date.[11]

For these reasons, we studied anion transport function, red cell membrane protein phosphorylation and membrane casein and tyrosin-kinase activities in a 43-year-old woman presenting typical neurological symptoms and peripheral acanthocytosis. We also evaluated the apparent band 3 molecular weight on polyacrylamide gel and analyzed the promoter and codifying region of the erythroid band 3 (EPB3) gene. Finally, red cell membrane cholesterol and fatty acid composition were investigated in order to ascertain possible abnormalities in membrane lipid composition.

Materials and Methods

A 43-year-old woman, the daughter of consanguineous parents (first cousins), was admitted to the Neurological Department of Verona for a progressive neuromuscolar disturbance characterized by seizures, buccofacial dyskinesia, choreiform movements, neurogenic muscle atrophy and *pes cavus*. Several peripheral blood smears showed 10-15% of thorny erythrocytes, confirmed as acanthocytes by scanning electron microscopy. There was no anemia and reticulocyte count was in the normal range (1-2%). Plasma cholesterol and triglycerides were normal as well as circulating apo- and lipoproteins. Creatine-phosphokinase serum levels were moderately increased. Determination of blood group antigens excluded the presence of Mc Leod phenotype.

Erythrocyte membrane composition and anion transport activity

Donor and patient blood was drawn and immediately processed. Erythrocyte membrane proteins were extracted and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.[12] As positive controls we used a patient with hereditary spherocytosis (HS) due to band 3 deficiency (about 20%) and a subject displaying the band 3 Memphis variant,[13] characterized by a slower migration on SDS-PAGE, similar to that observed in a patient with acanthocytosis.[14]

Membrane cholesterol/phospholipid ratio and fatty acid composition were evaluated as previously described.[5] The anion transport activity was evaluated as described by Jerolim *et al*.[15] Red cells were washed three times in 84 mM sodium citrate, 1 mM EGTA, pH 6.5. The influx of (^{35}S)sulfate was measured using equal numbers of pathological and control red cells (determined using a cell counter) at about 10% hematocrit. The flux was measured in a medium containing 4 mM sodium sulfate, 84 mM sodium citrate, and 1 mM EGTA pH 6.5. Influx was measured after 10 min at 30°C in presence of increasing concentrations of the anion transport inhibitor di-isothiocyano-dihydrostilbene disulphonate (H_2DIDS from 0.025 mM to 4 mM).[15-17] Maximum inhibitory H_2DIDS concentration was calculated by least square linear regression analysis using Systat 3.1 software. Six controls were measured in parallel. The sulfate flux was expressed for a constant number of cells (1×10^{13} cells), in order to allow comparison between patients with variable MCV values.[16] The within- and between-run CVs were < 5%.

Phosphorylation studies

Fresh blood was centrifuged at 750 × g for 3 min. to separate the erythrocytes. To avoid leukocyte and platelet contamination, packed red cells were washed three times by centrifugation in buffer A (20 mM Tris HCl, pH 7.5; 150 mM NaCl; 10 mM KCl; 1 mM $MgCl_2$, 0.1 mg/mL streptomycin; 0.025 mg/mL chloramphenicol); the buffy coat and the upper third of the packed red cell layer were discharged.

a) *Preparation of [^{32}P]-labelled erythrocytes*
Normal and patient packed red cells (400 mL) were preincubated separately in 3.6 mL of buffer A for 4h at 35°C to deplete endogenous ATP stores, then centrifuged at 750×g for 3 min, and resuspended in 1.8 mL of the same buffer A (20% Ht) containing glucose 24 mM and adenosine 1 mM in the presence of [^{32}P]Pi (0.3 mCi) for 14 h at 35°C, essentially according to Bordin *et al*.[18]

b) *Vanadate treatment and SDS-PAGE*
Vanadate is a potent inhibitor of phosphatases; as the phosphorylation level of a biological substrate represents a balance between two opposite activities of kinases and phosphatases, vanadate pretreatment allows for a better evaluation of kinase activities. Two aliquots (1 mL) of each ^{32}P-labelled cell suspension, prepared as described above, were incubated separately for 20 min at 35°C in the presence or absence of 2 mM sodium-orthovanadate.
After incubation, each sample of ^{32}P-labelled erythrocytes was solubilized by adding 4.5 mL buffer B (Tris 50 mM, pH 8.3; containing 5mM EDTA, 380 mM Glycine and 176 mM SDS) and 1% β-mercaptoethanol (final con-

centration) followed by a 5 min treatment at 100°C.[18]

Solubilized ^{32}P-labelled erythrocytes (180 mL) were submitted to electrophoresis on 0.1% SDS/10% polyacrylamide slab gels essentially according to Laemmli.[19] After electrophoresis, the slab gels were stained with Coomassie Blue.[19] Some gels were treated in 2M NaOH solution at 55°C for 1h and fixed again. Dried gels were autoradiographed at –80°C.

c) *Preparation of erythrocyte membranes*

An aliquot (1 mL) of packed red cells was hemolyzed in 28 mL hypotonic 5 mM phosphate buffer (pH 8) containing 0.02% NaN_3 and 0.03 mM phenylmethylsulphonyl fluoride (PMSF). After centrifugation at 20,000×g for 20 min, the membranes (ghosts) were separately recovered from the hemolysate, washed twice in the same hypotonic lysis buffer and twice in 25 mM Tris HCl buffer pH 8, containing 0.03 PMSF and 0.02% NaN_3.

d) *Assay of casein-kinase and poly(glu,Tyr) kinase activies in membrane and cytosol*

The membrane-associated casein kinase activity was assayed by incubating 80 mg of white ghosts at 30°C for 5 min. in the presence of 0.65 mg/mL whole casein as an exogenous substrate in a 125 µL reaction mixture containing 100 mM Hepes- NaOH buffer (pH 7.5), 10 µM vanadate, 10 mM $MgCl_2$ and 20 mM [γ-^{32}P]ATP (6×10^6 cpm/nmol).[20]

When poly(Glu,Tyr)4:1- kinase activity was assayed, 80 mg of white ghosts were incubated in the same conditions described for the casein kinase activity, except that casein was replaced by 0.065 mg/mL poly(Glu,Tyr)4:1 as the exogenous substrate. Incubation was stopped by the addition of 2% SDS and 1% β-mercaptoethanol (final concentration) followed by a 5 min treatment at 100°C; half of the reaction mixture was then analyzed by SDS-PAGE as described above.

The ^{32}P-labelled band of casein and the ^{32}P-diffuse band of poly(Glu,Tyr) identified after NaOH treatment, performed as described above, were quantified by a Packard-Instant Imager (Electronic Autoradiography).

Studies on nucleic acids

Genomic DNA was isolated from leukocytes and purified according to the method of Goossens and Kan.[21] We amplified the promoter region and the capping site located in exon 1 as well as the following 19 exons of the EPB3 gene by using twenty-one pairs of oligonucleotide flanking primers.[22] Amplified fragment was used for the search of single strand conformational polymorphisms (SSCP). In addition, for some large polymerase chain reaction (PCR) products, further restriction enzyme digestions were performed in order to obtain smaller fragments suitable for the SSCP screening; in particular, PCR products relative to exons 4, 6 and 13 were digested utilizing Drd I, Bamh I and Pst I enzymes, respectively. SSCP was performed according to Spinardi *et al*.[23] with some modifications. Briefly, amplification products were heat-denatured in five volumes of 0.05% SDS, 15 mM EDTA, and 47% formamide. Electrophoresis was performed on a 6% polyacrylamide gel containing 5% glycerol at 350 V for 3.5 hours with circulating water; gel was stained using the Silver Stain kit (BioRad Laboratories).

Results

Red cell membrane characteristics and anion transport activity

Densitograms of red cell membrane proteins obtained on SDS/PAGE did not show significant differences between chorea-acanthocytosis and normal controls; in particular, apparent molecular weight and concentration of band 3 were normal (data not shown).

Erythrocyte membrane fatty acid composition (for brevity's sake, only the main families of fatty acids are reported), polyunsaturated/saturated ratio (P/S ratio) and membrane cholesterol/phospholipid molar ratio (chol/Pl ratio) of the patient are summarized in Table 1; as a normal control, the data of the husband living at home and consuming the same diet as the patient are included (Table 1).

The sulfate influx in erythrocytes of chorea acanthocytosis was significantly increased compared with the normal control, while no changes in the maximal inhibitory H_2DIDS concentration were evident between control and chorea-acanthocytic erythrocytes (Figure 1). Since the maximal inhibitory H_2DIDS concentration gives the concentration of band 3 in the sample used, the data can also be

Table 1. Erythrocyte membrane fatty acid composition and cholesterol/phospholipid molar ratio in patient and normal control.

Parameters	*Patient*	*Control**
SFA	47.70	47.70
MUFA	15.10	15.50
PUFA	37.00	36.70
P/S	0.78	0.77
Chol/Pl molar ratio	1.10	0.98

**for the normal control we used the husband living in the family and consuming the same diet as the patient.*

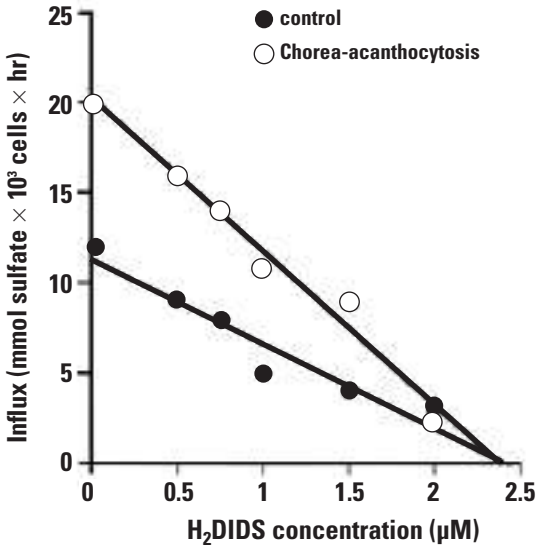

Figure 1. H_2DIDS inhibition of sulphate flux. The effect of increasing concentrations of H_2DIDS on [^{35}S] sulphate flux into erythrocytes of patient (open circles) and control (solid circles) is shown.

expressed as the ratio between sulfate flux and maximal inhibitory H_2DIDS concentration. This ratio gives a measure of the relative transport activity per molecule of band 3,[15] and it does not allow for changes in cell shape, size or density of membrane proteins in the red cell membrane. The ratio for the patient's red cells was increased (8.23) compared to the normal controls (4.57±0.18; n=6), suggesting an increased anion transport activity per molecule of band 3.

Phosphorylation studies

Figure 2 shows that ^{32}P-labelled erythrocytes from the patient present a higher membrane protein phosphorylation level, principally of band 2 (spectrin β-subunit) and band 3 (lane b), with a 40% increase compared with control erythrocytes (lane a).

After alkali-treatment of the gels, the alkali-labile ^{32}P-labelling of Ser-residues disappears, thus making the Tyr-phosphorylation of the band 3 protein evident.[24] In these conditions, only after a phosphatase blockade by vanadate incubation of ^{32}P-labelled erythrocytes (lane c', d') is it possibile to recognize the alkali-stable ^{32}P-labelling of Tyr-residues. This stresses a 40% increase of band Tyr-phosphorylation in the acanthocytic sample (lane d') compared to the control (lane c'), while without vanadate incubation, neither sample shows any ^{32}P-labelled proteins (lanes a', b'). Such enhanced levels in Ser/Thr- and Tyr-phosphorylation may be explained by the concomitant increase in kinase activity found in the corresponding patient membranes compared to the control (Table 2).

Studies on nucleic acids

SSCP screening of the promoter and all the exons of the EPB3 alleles revealed no abnormality within the reliability range of SSCP analysis;[25] in particular, exon 19, which codifies for the last transmembrane segment of band 3 and is directly involved in the anion transport mechanism, appeared normal. Moreover, MSPAII analysis of amplified B3 exon 19 supported the absence of the P868L mutation (not shown).[26]

Discussion

In the case reported here, the abnormal erythrocyte shape on peripheral blood was ascribed to a form of neuroachanthocytosis, because of the prominent neurological manifestations with no evi-

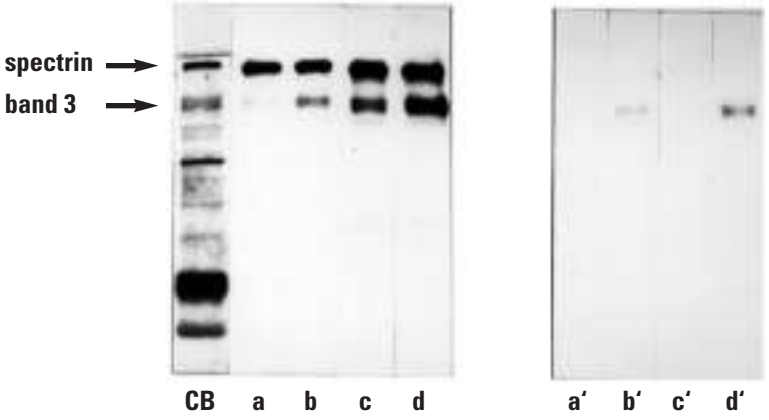

Figure 2. Phosphorylation pattern of erythrocytes from the normal control (lanes a, b, a', b') and the propositus (lanes c, d, c', d'). Intact erythrocytes were incubated with [^{32}P]Pi alone (lanes a, c, a', c') or in the presence of 2 mM vanadate (lanes b, d, b', d') as described in the *Materials and Methods* section. Whole ^{32}P-labelled erythrocytes were solubilized and analyzed by SDS-PAGE as described in the *Materials and Methods* section. Lanes a'-d' show the alkali-treated gels corresponding to the alkali-untreated gels (lanes a-d). Lane CB shows the Coomassie Blue stained gel of membrane proteins of solubilized whole erythrocytes. Autoradiograms were exposed for 3 days (lanes a-d) and 6 days (lanes a'-d').

Table 2. Casein kinase and Poly(Glu,Tyr) kinase activities displayed by membranes from normal and acanthocytotic erythrocytes.

	Casein	*Poly(Glu, Tyr)*
Controls (n=3)	19,675±1050	8580±790
Acanthocytosis	24,000	9150

Membranes were prepared from control and patient erythrocytes as described in the Materials and Methods section. The protein kinase activities are expressed as cpm incorporated in 5 min. at 30°C into whole casein and Poly(Glu, Tyr)4:1 by membranes of 100 ml packed erythrocytes.

lipoprotein abnormalities. The evaluation of erythrocyte membrane lipids (i.e. cholesterol/phospholipid molar ratio and fatty acids) excluded a spur cell anemia-like form and the determination of blood group antigens excluded the presence of a McLeod phenotype.

An abnormally increased functional activity of anion transporter band 3 was observed on the erythrocytes of the propositus. In the literature, there is controversy about the occurrence of acanthocytotic shape change and increased anion transport activity: in one case of chorea-acanthocytosis and in two patients with neurological symptoms but no acanthocytes, reduced anion transport activity has been reported.[27] However, other studies agree with our results: neuroacanthocytosis was shown to display increased anion transport activity associated with an alteration of band 3 C-terminal 150 amino acids.[14] In acanthocytosis (without neurological symptoms) an unusual form of erythrocyte band 3 was described, presenting slower migration on SDS/PAGE, restriction of rotational diffusion in the membrane, decreased number of high-affinity ankyrin-binding sites and markedly increased anion transport activity.[28]

Further studies on the erythrocytes of one subject carrying this abnormal band 3 demonstrated the presence of a single amino acid change in the protein (Pro-868→Leu) and its altered capability of binding H_2DIDS.[26]

All together, these data suggest that abnormalities such as a single amino acid mutation in critical sequences of the band 3 are also associated with modifications of the anion transport activity and with acanthocytotic shape change of the erythrocytes. In our case of neuroacanthocytosis, SSCP screening of the promoter and all the exons of the EPB3 alleles revealed no abnormality within the reliability range of SSCP analysis; furthermore, by means of enzymatic digestion, the presence of the mutation described by Bruce *et al*.[26] was ruled out as well. However, despite the fact that SSCP analysis is an accurate method widely accepted for a rapid screening of mutations, it does not allow for a 100% exclusion of the presence of single mutations in the EPB3 gene. Therefore, firm conclusions about this point shoud be avoided.

Band 3 is the most abundant protein of the erythrocyte membrane. The protein consists of two major domains:[9] the COOH-terminal 56KD membrane domain mediates the anion exchange across the membrane, whereas the NH_2-terminal 45KD cytoplasmic domain has the main function of anchoring the cytoskeleton to the membrane via interactions with ankyrin, protein 4.2 and protein 4.1.[9,29] However, contiguous membrane-spanning segments of the two domains may interact, so that changes in structure and Ser/Thr-phosphorylation of the NH_2-terminal 45 KDa cytoplasmic domain may influence anion transport function.[9,30] The Tyr-phosphorylation of membrane proteins almost exclusively involves the cytoplasmic domain of band 3, as evidenced by incubating white ghosts in the presence of [γ^{32}P]ATP,[31,32] and intact red cells in the presence of [^{32}P]Pi and vanadate.[18,33]

This band 3 Tyr-phosphorylation is known to produce, *in vitro*, conformational changes in the cytoplasmatic domain that are capable of negatively modulating the binding with cytoskeleton proteins (for example ankirin), hemoglobin and glycolytic enzymes[34] and, *in vivo*, it is known to be involved in regulating red cell metabolism[29] and shape.[18] The present report shows that chorea-acanthocytosis red cells incubated with [^{32}P]Pi present an abnormally increased Ser/Thr-phosphorylation level of membrane proteins (mainly band 2 and band 3), correlated with enhanced anion transport activity, thus supporting the idea of a connection between these two phenomena.[30] In addition, the increased phosphorylation level of band 3 Tyr-residues, previously suggested as being correlated with acanthocytotic shape change in pervanadate and vanadate-treated normal erythrocytes,[30] might be a reasonable explanation for the presence of thorny erythrocytes in our patient.

In consideration of these data, it seems plausible to hypothesize that an abnormal phosphorylation (mainly Tyr-phosphorylation) may be involved in weakening the interactions between integral protein band 3 and cytoskeletal proteins (ankyrin, band 4.2) in acanthocytosis. In any event, the observed increase of anion transport activity and the increase of the ^{32}P-labelling of Ser/Thr- and Tyr-residues on band 3 indicates the NH_2 cytoplasmatic domain of band 3 as a possible crucial structure involved in the pathogenetic mechanisms of acanthocytosis.

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