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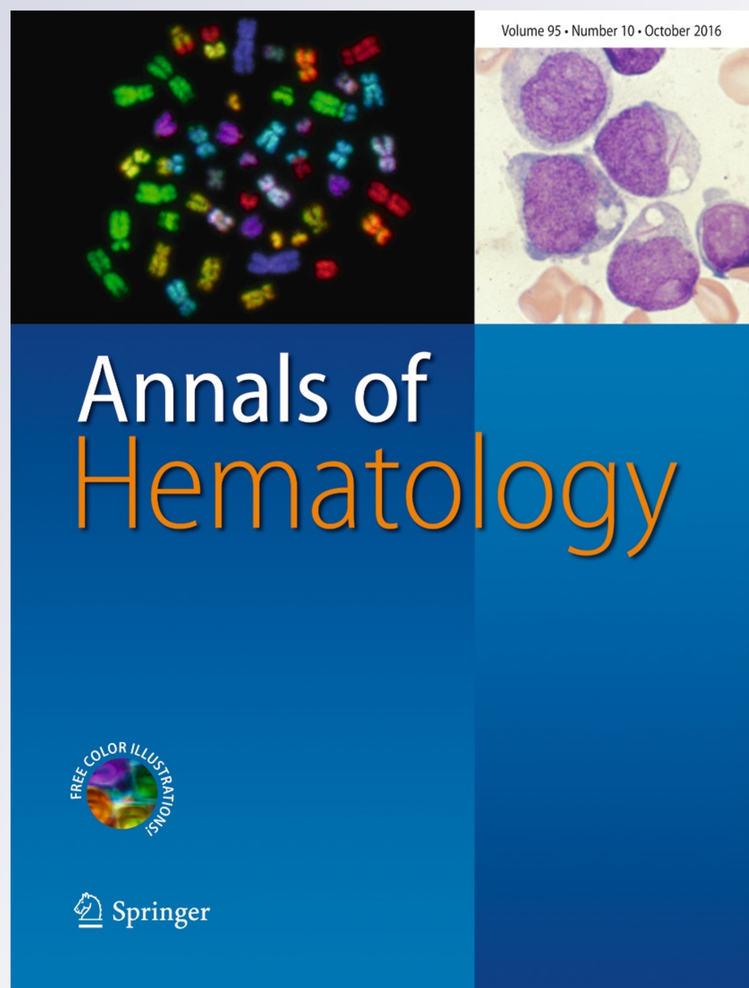

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ORIGINAL ARTICLE

Red blood cell aquaporin-1 expression is decreased in hereditary spherocytosis

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Abstract Aquaporin-1 (AQP1) is the membrane water channel responsible for changes in erythrocyte volume in response to the tonicity of the medium. As the aberrant distribution of proteins in hereditary spherocytosis (HS) generates deficiencies of proteins other than those codified by the mutated gene, we postulated that AQP1 expression might be impaired in spherocytes. AQP1 expression was evaluated through flow cytometry in 5 normal controls, 1 autoimmune hemolytic anemia, 10 HS (2 mild, 3 moderate, 2 severe, and 3 splenectomized), and 3 silent carriers. The effect of AQP1 inhibitors was evaluated through water flow-based tests: osmotic fragility and hypertonic cryohemolysis. Serum osmolality was measured in 20 normal controls and 13 HS. The effect of erythropoietin (Epo) on AQP1 expression was determined in cultures of erythroleukemia UT-7 cells, dependent on Epo to survive. Independent of erythrocyte size, HS patients showed a lower content of AQP1 in erythrocyte membranes which correlated with the severity of the disease. Accordingly, red blood cells from HS subjects were less sensitive to cryohemolysis than normal erythrocytes after inhibition of the AQP1 water channel. A lower serum osmolality in HS with respect to normal controls suggests alterations during reticulocyte remodeling. The decreased AQP1 expression could contribute to explain variable degrees of anemia in hereditary spherocytosis. The finding of AQP1 expression induced by Epo in a model of erythroid cells may be interpreted as a mechanism to restore the balance of red cell water fluxes.

Keywords Aquaporin-1 · Spherocytosis · Serum osmolality · Erythropoietin

Introduction

The extensive remodeling of the red blood cell membrane throughout erythropoiesis plays a major role in determining the expression of membrane proteins. Two main levels of regulation have been described for this process: the selective distribution occurring during enucleation of the erythroblast [1], and the subsequent remodeling during reticulocyte maturation [2]. Aquaporin-1 (AQP1) is the membrane water channel responsible for fast volume changes in red cells in response to the tonicity of the medium and its expression in the immature red cell suffers changes depending on medium tonicity. In mouse reticulocytes, AQP1 is located in the plasma membrane and the endosomal compartment, whereas in the mature erythrocyte it only remains expressed in the plasma membrane. AQP1 ubiquitination or phosphorylation mechanisms probably determine the inclusion of the AQP1 pool to the membrane or its release to plasma via the exosomal pathway. Evidence confirming the exosomal release has been

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reported by Blanc et al. in animal models and in vitro assays [3]. When reticulocytes are suspended in a hypertonic medium, AQP1 release is blocked while its expression increases in the cell membrane [3]. Membrane remodeling during reticulocyte maturation is a crucial step, since it leads to modifications in rheological and morphological membrane properties [2]. Likewise, throughout the enucleation process, proteins are selectively distributed between the extruded nucleus and the reticulocyte membrane. Salomao et al. found that an aberrant sorting of membrane proteins, leading to deficiencies in proteins other than those codified by the mutated gene, occurs in *nb/nb* mice (murine model of hereditary spherocytosis) as well as in protein 4.1-knockout mice (murine model of hereditary ellyptocytosis) [1]. This finding raises the possibility that combined protein deficiencies in hereditary spherocytosis (HS) patients may not only be due to the continued loss of the erythrocyte membrane but be already determined in the nascent reticulocyte.

Several confirmatory tests to diagnose HS, such as osmotic fragility (OF) [4], flow cytometric osmotic fragility [5], and cryohemolysis (CH) [6] are based on the different behavior of spherocytes and normal erythrocytes when exposed to osmotic changes after brief incubation. On the assumption that the short time required to attain results by these tests indicates that the passage of water through the membrane is a prevalent mechanism, we decided to determine AQP1 expression in the red blood cells of HS patients. Furthermore, as the expression levels of AQP1 in the red cell membrane could be influenced by the tonicity of the medium [3], we compared serum osmolality between patients and normal controls.

Other authors observed that erythropoietin (Epo) treatment appears to be effective in the management of anemia in most HS infants suggesting that it could serve as a valuable alternative to packed RBC transfusions [7]. Although little is known about a possible role of Epo on aquaporin regulation, some evidence suggests that increased AQP4 and AQP1 expression induced by Epo may mediate its neuro- and renoprotection, respectively [8, 9]. Besides, an increase in AQP1 levels in newly formed erythrocytes was found after Epo administration to healthy adults [10]. Based on these reports, it was interesting to investigate whether one of the possible mechanisms of Epo protection in HS erythrocytes could be related to water regulation mediated by AQP1. The UT-7 cell line, dependent on Epo to grow, is a useful model of erythroid progenitors to study the ability of Epo to induce AQP1 expression.

Materials and methods

Patients

The study protocol was approved by the Ethic and Research Committee. Informed consent was obtained from patients or parents (in the case of children) before entering the protocol. The study was performed according to the Helsinki international ethical standards on human experimentation.

A total of 28 samples from normal controls and 25 samples from HS patients were included. According to clinical severity [11, 12], anemia was defined as severe (hemoglobin <8 g/dL), moderate (hemoglobin = 8–10 g/dL), and mild (hemoglobin >10 g/dL). Splenectomized patients were considered a separate group. Distribution of HS patients was mild 10, moderate 5, severe 3, and splenectomized 7. Three healthy individuals showing a defect in any of the membrane proteins by SDS-PAGE but otherwise asymptomatic and with negative results for other diagnostic tests were considered as HS silent carriers. A patient diagnosed with CLL (chronic lymphatic leukemia), who developed an autoimmune hemolytic anemia (Hct 28 %, Hb 8.1 g/dL, DAT ++++, spherocytes + (~4–5 per field 100x), Cryohemolysis 1.22 % and normal EMA test) was also included in the study to compare the results with those of the HS patients.

Cell line

Human UT-7 cell line, kindly provided by Dr. Patrick Mayeux (Cochin Hospital, Paris, France), shows growth dependence on Epo. These cells were grown on Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 U/mL Epo (Hemax, Biosidus, Argentina) [13].

Methods

Osmotic fragility test

Osmotic fragility test (OF) was performed using the usual standard method by Parppart et al. [4].

Cryohemolysis

Cryohemolysis (CH) test was performed according to the method described by Streichman et al. [6], slightly modified [14].

Serum osmolality

It was measured in triplicate using a pressure vapor osmometer (WESCOR VAPRO 5600) on serum samples kept frozen at −20 °C immediately after withdrawing.

Inhibition assays

Inhibition assays were performed on heparinized blood samples pre-incubated for 1 h at room temperature with and without the addition of $HgCl_2$ (final concentration 40 μM) and

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$CuCl_2$ (final concentration 400 μM). Blood samples were subsequently used to perform OF and CH tests.

AQP1 expression by flow cytometry

(A) Blood samples were fixed by incubation (10 min) with cold 0.05 % glutaraldehyde in phosphate-buffered saline (PBS) and spun down. Red blood cells were washed with 0.1 % bovine serum albumin (BSA) in PBS, incubated (5 min, room temperature) with 0.1 % Triton in BSA-PBS, spun down, and suspended in the washing solution ($1x10^6$ cells/mL). The cell suspension was incubated (1 h, room temperature) with anti-AQP1 antibody (Millipore). After washing, cells were suspended in 0.1 % BSA-PBS and incubated (30 min, in the darkness) with FITC-conjugated secondary antibody. Then, the cells were washed and suspended in 1 % formaldehyde in 0.1 % BSA-PBS for acquisition by flow cytometry. Results are expressed as percentage of the normal control simultaneously processed. (B) Before the onset of experiments, UT-7 cells were Epo-deprived for 18 h. Then, 10^6 cells per treatment were fixed (15 min, 4 °C) with Cytofix/Cytoperm (BD Biosciences), washed with PermWash buffer (PWB, BD Biosciences) and incubated with anti-AQP1 antibody in PWB with 10 % FBS (1 h on ice). After washing off the primary antibody, the cell suspensions were incubated with the appropriate Alexa Fluor 488-conjugated secondary antibody (Life Technologies) in PWB (1 h on ice in the dark). Cells were then washed with PWB and suspended in PBS until acquisition of events in a FACSort flow cytometer (Becton Dickinson). Data were analyzed with the WinMDI 2.9 software.

Statistical analysis

Statistical analysis was performed using GraphPad Software, Inc. Results of laboratory tests were evaluated by nonparametric methods (Mann-Whitney test, Kruskal-Wallis test). Spearman's rank correlation coefficient was used to evaluate relationships between sets of data. Results are presented as mean ± SEM and statistical significance defined as $P < 0.05$.

Results

AQP1 expression was evaluated in 5 normal controls, 10 patients with HS (2 mild, 3 moderate, 2 severe, and 3 splenectomized), 3 silent carriers, and 1 autoimmune hemolytic anemia (AIHA) with spherocytes in the blood smear (Table 1). HS and silent carriers presented single or combined deficiencies of ankyrin, spectrin, protein 4.1, and protein 4.2 detected by polyacrylamide gel electrophoresis [14]. The selection of patients represents the variability we found in the study of protein deficiencies in Argentina [14].

AQP1 expression was significantly lower in HS patients and silent carriers than in the simultaneously processed normal control, while no AQP1 decrease was detected in the AIHA sample (Fig. 1a). To investigate whether the decreased fluorescence observed in patient red blood cells was a result of the reduction in AQP1 density or in spherocyte surface area, we comparatively analyzed AQP1 expression in control and HS erythrocyte populations of similar size. In 5 different assays, patients' red cell AQP1 was lower than in the simultaneously analyzed control, independent of cell size. In contrast, similar AQP1 levels between the control and AIHA samples were observed despite the presence of many spherocytes in the blood smear of the latter, thus suggesting that the decreased expression of AQP1 in spherocytes is not related to their smaller size (Fig. 1b). The decrease in AQP1 expression was greater as HS was more severe (Fig. 1c). Moreover, a significant correlation between AQP1 expression and hemoglobin levels was also found (Fig. 1d).

A significant correlation between AQP1 expression and percentage of cryohemolysis was observed for the whole population studied (Rho Spearman = −0.6501, $P = 0.0161$). We hypothesized that different levels of AQP1 expression should affect channel inhibition differently in normal erythrocytes and in spherocytes. Therefore, we performed the traditional diagnostic tests CH and OF in the presence of Hg^{2+} or Cu^{2+} ions to observe the effects of these AQP1 inhibitors on the passage of water when red cells were suspended in hypertonic (CH test) or hypotonic medium (OF test). The ion concentrations we used in AQP1 inhibition assays were in accordance to those reported in the literature since Hg^{2+} concentrations greater than 50 μM might interfere with cation channels [15]. The inhibition of AQP1 increased the CH of red cells, such effect being significantly lower in HS patients than in normal controls expressing more AQP1 (Fig. 2). In simultaneously performed assays, the inhibitory effect of Hg^{2+} ions on CH results showed a direct correlation with the level of AQP1 expression ($n = 4$; $r^2 = 0.865$) while the presence of Hg^{2+} or Cu^{2+} ions did not affect the hemolysis of erythrocytes in hypotonic solutions when the OF test was performed either at room temperature or at 0 °C (data not shown).

Taking into account that AQP1 expression seems to be influenced by medium tonicity during reticulocyte membrane remodeling, we were interested in comparing serum osmolarity in samples from patients with those from normal controls. We found that HS patients (5 mild, 3 moderate, 2 severe and 3 splenectomized) showed significantly decreased serum osmolality in comparison to normal controls (Fig. 3).

To investigate whether Epo could affect AQP1 expression in erythroid progenitors, we employed the human erythroleukemia UT-7 cells, which is an Epo-responsive cell line known as a well-established model for inducible erythroid differentiation. The cells were arrested for 18 h

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Table 1 Data of the patients with hereditary spherocytosis in whom AQP1 expression was evaluated

Patient	Age	Protein deficiency	Abnormal test results	Severity
1	14 y	Protein 4.1	None	Silent carrier
2	48 y	Protein 4.1; protein 4.2	None	Silent carrier
3	35 y	Protein 4.1; spectrin	None	Silent carrier
4	32 y	Protein 4.2	FC	Mild
5	26 y	NA	FC, CH, AH, OF	Mild
6	18 y	NA	FC, CH, AH, OF	Moderate
7	3 m	NA	FC, CH	Moderate
8	18 m	NA	FC, CH, OF	Moderate
9	8 m	NA	FC, CH, AH, OF	Severe
10	5 m	NA	FC, CH, AH, OF	Severe
11	11 y	Ankyrin	FC, CH, AH, OF	Splenectomized
12	37 y	Protein 4.1; protein 4.2	FC, CH, AH, OF	Splenectomized
13	37 y	Spectrin; protein 4.1	FC, CH, AH, OF	Splenectomized

FC eosin-5'maleimide flow cytometry test, *CH* cryohemolysis test, *AH* autohemolysis test, *OF* osmotic fragility test, *NA* not available

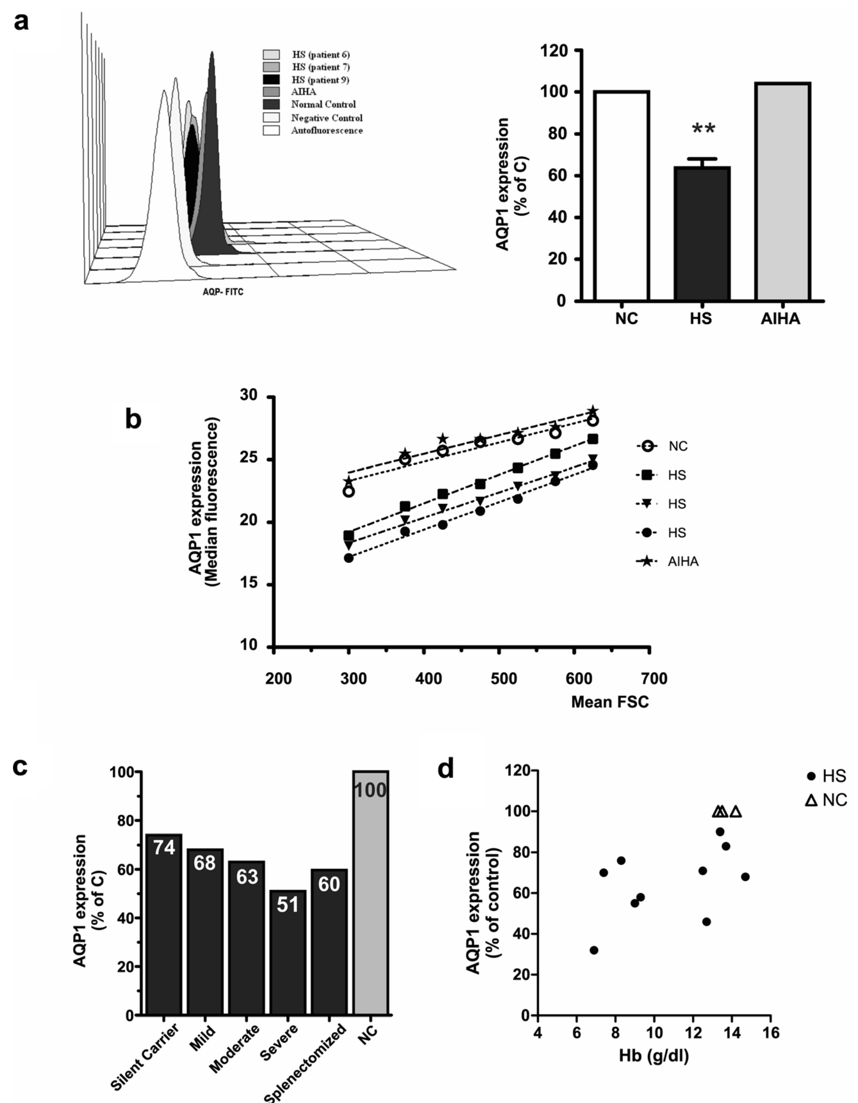

Fig. 1 Aquaporin-1 expression. **a** Superimposed flow cytometric histograms corresponding to a representative experiment and percentage of AQP1 expression in HS patients (n = 13) and the AIHA patient with respect to normal controls (NC, $n = 5$); **P < 0.005 vs. NC. **b** AQP1 expression with respect to cell size, calculated by flow cytometry at different values of the FSC parameter. The figure shown is representative of other 5 assays comparing erythrocyte AQP1 levels between patients and normal controls simultaneously analyzed. **c** Mean AQP1 expression in the subgroups with different severity of the disease (3 silent carriers; 2 mild; 3 moderate; 2 severe and 3 splenectomized) expressed as percentage of controls ($n = 5$); Kruskal-Wallis test, $P < 0.05$. **d** Significant correlation between erythrocyte AQP1 expression and blood hemoglobin concentration. HS patients ($n = 10$; splenectomized patients are not included) and normal controls ($n = 5$), (Spearman Rho = 0.5913, $P < 0.05$)

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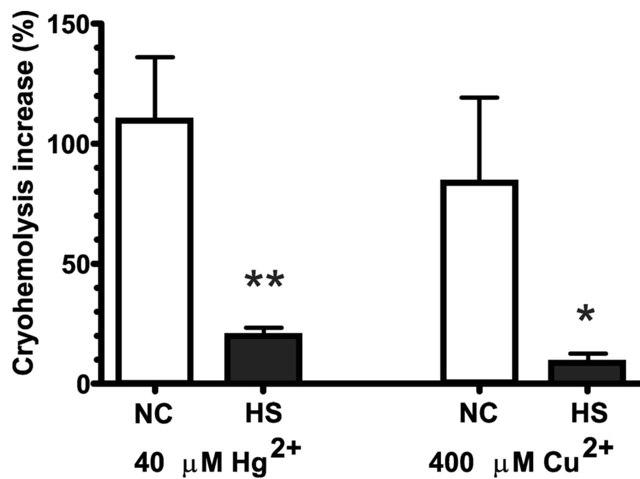

Fig. 2 AQP1 and Cryohemolysis. Heparinized blood samples preincubated with or without $HgCl_2$ (40 μM final concentration) or $CuCl_2$ (400 μM final concentration) were subsequently used to perform CH test. In the presence of Hg^{2+}, red cell hemolysis increments were of 109 ± 26.2 % for normal controls (n = 8) and 20 ± 3.5 % for patients (n = 9) and in the presence of Cu^{2+} the values were 83 ± 35.7 % for normal controls (n = 3) and 9 ± 3.8 % for patients (n = 4). Significant differences: **P < 0.0001 Hg^{2+} vs. NC; *P < 0.05 Cu^{2+} vs. NC

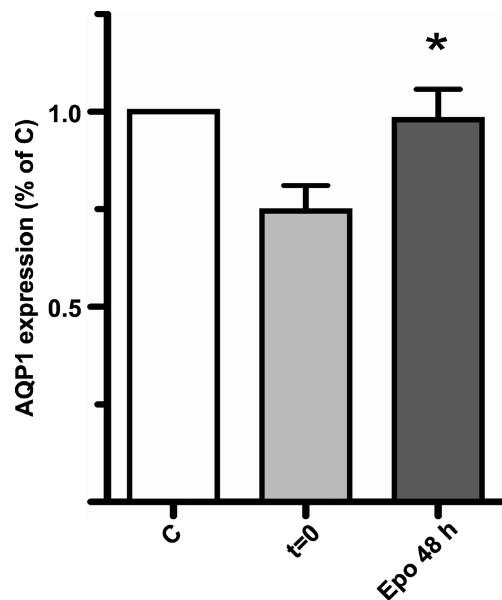

Fig. 4 Epo effect on AQP1 expression. UT-7 cells maintained with 2 U/mL Epo (control) were Epo-deprived for 18 h (t = 0) and then incubated in the presence of 2 U/mL Epo for additional 48 h (Epo 48 h). AQP1 expression was determined by flow cytometry. Each *bar* represents percentage of control simultaneously analyzed (mean ± SEM). Significant increase in AQP1 expression was induced by Epo, *P < 0.05 vs. t = 0, n = 4

without growth factor and then cultured in the presence of 2 U/mL Epo for additional 48 h to further analyze AQP1 levels by flow cytometry (Fig. 4). Initially, we found AQP1 expression in growing cells maintained with 2 U/mL Epo. After Epo deprivation, the cells showed decreased AQP1 levels. However, further stimulation with the growth factor induced a significant increase in AQP1 expression comparable with that of control cells.

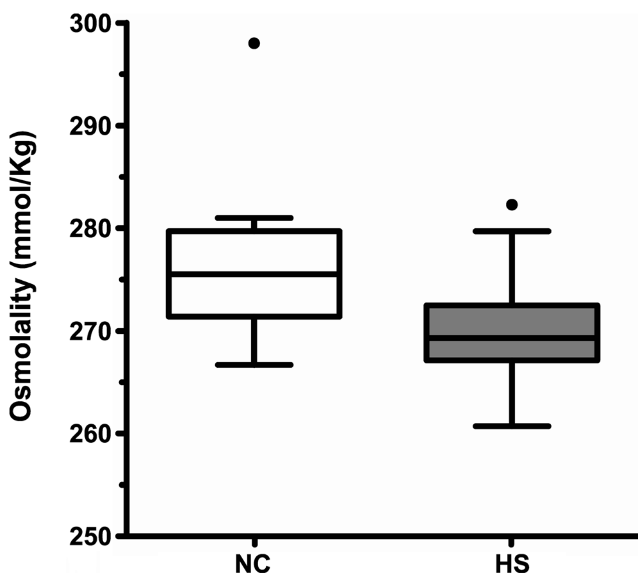

Fig. 3 Serum osmolality. Each measurement, made in triplicate samples from HS (n = 13) and normal controls (n = 20), is expressed as mean ± SEM. Significant difference between both groups P < 0.01

Discussion

The increased "osmotic fragility" of HS red blood cells suggested the hypothesis that water channels, such as aquaporins, might be involved in the physiopathology of hereditary spherocytosis.

This study for the first time demonstrates a decreased AQP1 expression in HS erythrocytes which correlates with altered results of the CH test. On the other hand, the Epo-induced AQP1 expression in erythroleukemia cells allows us to suggest a possible role of Epo in the process of cell water regulation.

The first report concerning AQP1 decrease associated to a disease of the red blood cells was published by Agre et al. in a patient with congenital dyserythropoietic anemia [16]. A later study on the band 3-based macrocomplex showed decreased AQP1 expression in a patient with homozygous band 3 deficiency, as well as in band $3^{-/-}$ knockout mice [17]. A recent study reported that mice with a mutated GATA1 gene suffered from severe hemolytic anemia with the presence of spherocytes associated to decreased expression of band 3, α-spectrin, and AQP1 [18]. Our results showed decreased AQP1 expression in the whole HS group studied; furthermore, this decrease correlated directly with the clinical severity. This deficiency could contribute to explain variable degrees of anemia regardless of the protein deficiency involved since the patients belonged to different families and presented single or combined deficiencies of several membrane proteins associated to HS. Worthy of note, the 3 splenectomized HS patients included in this work

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presented normal hematological parameters (as usually occurs after surgery) despite a decrease in red blood cell AQP1 expression, which raises the possibility of a marginal contribution of AQP1 deficiency to hemolysis in this disease. Ensuing research is therefore needed to clarify this point.

It is likely that an abnormal AQP1 secretion occurs during the enucleation process, as has been demonstrated for deficiencies in proteins other than those codified by the mutated gene causing HS [1]. Blanc et al. reported that modulation of medium tonicity in in vitro maturation of mouse reticulocytes regulated the secretion of AQP1, showing that extracellular osmotic conditions can drive sorting of selected proteins by the exosomal pathway [3]. Here, we found significant differences in serum osmolality between patients and controls. As reticulocytes of HS patients are immersed in plasma with lower osmolality than normal, the loss of AQP1 might be increased during the subsequent reticulocyte maturation process. We suggest that these two mechanisms, abnormal secretion during the enucleation process and loss during reticulocyte maturation, could help explain the lower expression of AQP1 in erythrocytes of patients compared to normal controls. Considering that increased plasma osmolality should lead to cellular dehydration, we postulate that the decreased osmolality found in patients with HS would work as a compensatory mechanism to protect erythrocytes from a greater dehydration. Furthermore, a decrease in extracellular osmolality gives rise to fast cell swelling so that regulatory volume decrease occurs, leading to a potassium efflux and the subsequent calcium influx [19]. We postulate that this mechanism would contribute to an increased sensitivity of the Gardos channel, which has been proposed as a paradoxical but protective effect for the spherocyte [20].

The inhibitory effect exerted by certain molecules and ions on the water transport mediated by aquaporins has been studied [21]. Taking this concept into consideration, we investigated whether the presence of AQP1 inhibitors may affect usual diagnostic tests which involve fast water passage through the membrane. Although the channel allows the passage of water in both directions, we found that the presence of Hg^{2+} or Cu^{2+} ions modified the water efflux from erythrocytes when suspended in hypertonic medium, but not in hypotonic medium. The osmotic shock created by the hypertonic medium in the cryohemolysis test induces Ca^{2+} influx and the subsequent activation of the Gardos channel. As a consequence, an efflux of potassium, chloride, and osmotically obliged water occurs, leading to cell shrinkage. Treatment of red cells with $HgCl_2$ prevents such water efflux by blocking the AQP1 channel. Thus, intracellular potassium is promptly exhausted and a significant increase in lysis occurs.

Hemolysis in hypotonic medium (OF test) was not influenced by AQP1 inhibition through the used range of inhibitor concentrations. Since the test is performed at room temperature, a large inflow of water occurs by diffusion through the lipid layer. For this reason, we performed the same test at 0 °C, thus blocking the diffusional passage. In the same way, no differences were found when the inhibitor was present and the diffusional passage had been blocked by the effect of temperature. This finding is consistent with that described by Mathai et al., who determined a Pf/Pd ratio of 3.4 in Colton-null red cells showing that, in addition to AQP1 and diffusion, other mechanisms are involved in the water influx [22]. They suggested the glucose transporter Glut-1 as responsible for such a finding. In agreement with this observation, Bruce et al. found that this transporter was increased in the red blood cells of a patient with HS [17].

The effect of erythropoietin on AQP1 expression deserves a commentary. Rentsch et al. reported that AQP1 levels increased in circulating erythrocytes during a 4-week period of Epo administration to healthy adults [10]. Nevertheless, no information has been reported about modulation of the AQP1 levels by this growth factor. Since erythrocytes are anucleated cells, we decided to investigate whether the rise in AQP1 expression observed in mature cells could also be detected in immature cells of the erythroid lineage committed to erythrocyte production. This way, we employed the Epo-dependent UT-7 cell line which represents a useful model of erythroid progenitor cells. The finding of the AQP1 expression induced by Epo in these in vitro assays may explain the in vivo data reported by Rentsch et al. [10]. Moreover, it can be suggested that the Epo-induced AQP1 levels may be at least one of the mechanisms involved in the amelioration of the clinical course observed in patients with severe HS treated with erythropoietin [7].

In conclusion, we found decreased AQP1 expression in erythrocytes from patients with HS which was independent of cell size and appeared to be related to clinical severity. In agreement, spherocytes were less sensitive to cryohemolysis than normal erythrocytes after inhibition of the AQP1 water channel. The results let us suggest that the decreased AQP1 expression may be related to aberrant distribution of membrane proteins during reticulocyte remodeling due to decreased serum osmolality. The Epo-induced AQP1 expression, observed in a model of erytroid immature cells, reveals a potential treatment that may restore the balance of red cell water fluxes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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