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Human Red Cell Aquaporin CHIP

II. Expression during Normal Fetal Development and in a Novel Form of Congenital Dyserythropoietic Anemia

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Abstract

Channel-forming integral protein (CHIP) is the archetypal member of the Aquaporin family of water channels. Delayed CHIP expression was shown recently in perinatal rat (Smith, B. L., R. Baumgarten, S. Nielsen, D. Raben, M. L. Zeidel, and P. Agre. 1993. J. Clin. Invest. 92:2035–2041); here we delineate the human patterns. Compared with adult, second and third trimester human fetal red cells had lower CHIP/spectrin ratios (0.72±0.12, 0.94±0.22 vs. 1.18±0.11) and reduced osmotic water permeability (0.029, 0.026 vs. 0.037 cm/s); CHIP was already present in human renal tubules by the second trimester. A patient with a novel form of congenital dyserythropoietic anemia (CDA) with persistent embryonic and fetal globins and absent red cell CD44 protein was studied because of reduced CHIP-associated Colton antigens. Novel CDA red cells contained <10% of the normal level of CHIP and had remarkably low osmotic water permeability (<0.01 cm/s), but no mutation was identified in Aquaporin-1, the gene encoding CHIP. These studies demonstrate: (a) unlike rat, human CHIP expression occurs early in fetal development; (b) red cell water channels are greatly reduced in a rare phenotype; and (c) disrupted expression of red cell CHIP and CD44 suggests an approach to the molecular defect in a novel form of CDA. (J. Clin. Invest. 1994; 94:1050–1058). Key words: erythrocyte membrane • fetal blood • cell membrane permeability • water movements

Introduction

Cell membranes have long been known to exhibit limited water permeability (for review see reference 1). Recognition of very high water transport through plasma membranes of red cells, proximal renal tubules, and certain other epithelia led to the hypothesis that water-selective channels must exist. Such channels would explain the unusual biophysical behavior of these membranes: high osmotic water permeability (Pw),1 low Arrhenius activation energy (Ea), reversible inhibition by HgCl2, and lack of permeability for protons or other ions. Despite efforts by several investigators, the molecular structure of water channels eluded identification (for reviews see references 1–3).

The discovery of channel-forming integral protein (CHIP) provided the molecular structure needed to explain red cell and renal tubule water permeability (for review see reference 4). CHIP was first identified by serendipity (5), and a simple purification method permitted recognition of its unique biochemical characteristics and homology with MIP, the functionally undefined major intrinsic protein of lens (6). Determination of CHIP protein sequence led to the original cDNA isolation (7). Demonstration that CHIP is a molecular water channel was first accomplished by expression of CHIP cRNA in Xenopus oocytes (8); this conclusion was verified directly by analysis of highly purified CHIP reconstituted into proteoliposomes (9) and with red cell membranes depleted of other proteins (10). These studies led to the identification of a family of homologous water channel proteins in plants and animals which is referred to as the “Aquaporins” (11).

Water channels may be involved in numerous physiological processes such as renal water conservation, body temperature regulation, neural homeostasis, digestion, and reproduction (1–4). CHIP protein is expressed in many but not all water-permeable mammalian epithelia (5, 12–15). In situ hybridization studies revealed multiple patterns of CHIP expression during fetal development in rat (16): (a) constitutive mRNA and protein expression throughout fetal development and maturity (e.g., choroid plexus); (b) transient mRNA expression during fetal development without persistence of protein (e.g., cardiac endothelium and periosteal mesenchyme) or with persistence of protein (e.g., cornea); and (c) postnatal expression of mRNA and protein (e.g., red cells, renal proximal tubules, and descending thin limbs) (17). Expression of CHIP may involve other complexities. CHIP is already abundantly expressed by the CFU-erythroid stage of red cell differentiation (Smith, B. L., and P. Agre, unpublished observations). CHIP is among the delayed early response genes expressed by cultured fibroblasts after growth factor stimulation (18), although CHIP transcripts were found in freshly dispersed but not dedifferentiated aortic smooth muscle cells (19).

This study was presented at the annual meeting of the American Federation for Clinical Research in Baltimore, MD on 1 May 1994 and was published in abstract form (1994. Clin. Res. 42:238a).

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Received for publication 7 April 1994 and in revised form 24 May 1994.

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0021-9738/94/09/1050/09 $2.00
Volume 94, September 1994, 1050–1058

1. Abbreviations used in this paper: CDA, congenital dyserythropoietic anemia; CHIP, channel-forming integral protein; Ea, Arrhenius activation energy; Pw, coefficient of osmotic water permeability.
Although fetal rat is a rewarding developmental model, fundamental differences between the red cells and kidneys of human and rats are known to exist. For example, fetal hemoglobin is expressed in the red cells of unborn humans but not in fetal rats (20). Human kidneys are known to concentrate effectively at birth (21), whereas neonatal rat kidneys concentrate poorly (22). Also, excellent murine models are known for some but not all human diseases. Therefore, the expression of CHIP was investigated during normal human fetal development and in a congenital disease state.

**Methods**

**Clinical samples**

**Fetal blood and tissue samples.** Human blood samples anticoagulated with buffered sodium citrate were obtained through the Perinatology Service at Johns Hopkins Hospital with approval of the institutional clinical investigation board. Samples were stored on ice and processed within 48 h. These blood samples consisted of surplus tubes of cord blood from term or premature deliveries or postdelivery samples from abortuses. Institutional restrictions permitted identification only by gestational age. All samples were analyzed for reticulocyte count by the methylene blue staining and Wintrobe indices by clinical chemistry lab at Johns Hopkins Hospital. Human kidneys were procured through the International Institute for the Advancement of Medicine (Philadelphia, PA).

**Patient with congenital dyserythropoietic anemia (CDA).** Blood samples from patient S.F. and her family members were drawn at the Righshospital in October of 1993, more than 1 yr since her last transfusion. The blood was anticoagulated with citrate phosphate dextrose adenine and analyzed within 72 h.

**Patient S.F.** was born in 1982 to normal, unrelated Danish parents, and her only sibling is also normal. As a neonate she was noted to be hyperbilirubinemic with marked Coombs negative anemia. She received 10 blood transfusions during her first year of life, but this need has declined steadily. Her bone marrow revealed a normal female karyotype, but microscopic evaluation was consistent with a novel form of CDA. Myeloid and megakaryocytic progenitors appeared normal, but intense erythroid hyperplasia with basophilic stippling was noted. Electron microscopic analysis revealed abundant erythroblasts with unusual nuclear condensation and karyorrhexis, cytoplasmic inclusions which may represent smooth endoplasmic reticulum in both erythroblasts and circulating red cells (23). In addition to the ineffective hematopoiesis, there was evidence of increased hemolysis: chromatin-51 red cell survival of 12 d (normal 21–31 d), undetectable haptoglobin level, and total bilirubin of 3 mg/dl (normal < 1 mg/dl). The patient has normal platelet counts, white blood counts, and differential. Without transfusions, she now maintains a hemoglobin of 6.5–9 g/dl (normal 11–15), a mean corpuscular volume of 92 fl (normal 80–97), and reticulocyte counts of 2–16% (normal < 1%). Her peripheral blood smear revealed moderately anisocytic and poikilocytic red cells with occasional nucleated red cells. The serum ferritin was 882 μg/liter (normal 10–120 μg/liter). Extensive red cell glycolytic enzyme studies revealed normal or slightly elevated levels. Normal results were obtained from the direct antiglobulin test, osmotic fragility test, sucrase lysis, and Ham test against seven normal fresh sera. Globin chain biosynthetic studies revealed the ratio of non-α/α as 0.63, and the child was found to have measurable embryonic hemoglobins, with both ε and ξ chains, and 30–40% fetal hemoglobin, with both γ and δ chains (normal < 1%). Extensive molecular genetic studies were performed which revealed normal ζ-α and γ-δ clusters (24). Although nucleotide sequence variants were identified 5' to the cap site in both the γ and β promoters, these also were found in her parents and her sister and were concluded to be normal. Since the other family members are clinically normal. Extensive red cell antigen typing recently demonstrated the unique phenotype In(a–b–), Co(a–b–), and lack of the high incidence antigen AnWj with reduced LW reactivity (25). Despite numerous transfusions, the patient never developed Colton antibodies. Although routine agglutination studies with anti-Coa were negative, adsorption–elution revealed that her cells had weak Coa reactivity. Testing for > 40 other blood group antigens revealed normal strength reactions including Lu(a—b+) and I antigens. Her father was In(a–b+), Co(a+–b–); her mother was In(a+b+), Co(a+b–); her sister was In(a–b–), Co(a+b–); all three were LW positive, and AnWj positive. In addition, her parents and sister had normal routine hematology laboratory tests.

Although no longer requiring transfusions, the patient suffers from recurrent gall stones and has a slightly enlarged spleen. Her growth and development have been normal, and she has normal vision and normal pulmonary studies. The patient has no evidence of renal dysfunction; her electrolytes, urea nitrogen, and creatinine are normal. After overnight fluid restriction, her urine osmolality was 755 mmol/kg.

**Materials**

Polyclonal, affinity-purified rabbit antibodies to human red cell CHIP or spectrin were described previously (6, 13, 17). FITC goat anti–rabbit IgG was from Boehringer Mannheim Corp. (Indianapolis, IN), and Texas red goat anti–rabbit IgG was from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Electrophoresis reagents were from Bio-Rad (Melville, NY), and enhanced chemiluminescence reagents were from Amersham Corp. (Arlington Heights, IL). Other reagents and supplies were from Sigma (St. Louis, MO), Eastman Kodak Co. (Rochester, NY), or J. T. Baker, Inc. (Phillipsburg, NJ).

**Methods**

**Membrane preparations.** Red cells were washed in 7.5 mM sodium phosphate (pH 7.4), 150 mM NaCl (PBS), and membranes were prepared by hypotonic lysis with 7.5 mM sodium phosphate (pH 7.4), 1 mM Na3EDTA, 4 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml diisopropyl fluorophosphate as described (26).

Electrophoresis and immunoblot methods. SDS-PAGE was performed using the buffer system of Laemmli (27) and 0.1 X 9 X 6 cm 10 or 12% acrylamide slabs. Immunoblotting was performed with enhanced chemiluminescence as described (13). To quantitate the abundance of CHIP relative to spectrin, 10-μl vol of serially diluted membranes were electrophoresed into SDS-PAGE gels. The approximate membrane protein applications were: 1/5 = 8 μg, 1/10 = 4 μg, 1/20 = 2 μg, 1/40 = 1 μg, and 1/80 = 0.5 μg. Identical slabs were processed in parallel by anti-CHIP immunoblot or Coomassie staining. CHIP signals (area, mm2) at the 26-kD level of the immunoblots were quantitated at each dilution by densitometric scanning of the autofluorograph with an UltraScan XL laser densitometer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Spectrin was quantitated by elution of dye from gels (absorbance at 550 nm). Linear regressions of the measured values at four dilutions were performed, and the calculated reciprocal dilution corresponding to 50% of maximum CHIP area (or spectrin absorbance) were compared as the ratios (CHIP/spectrin) in arbitrary units.

**Immunofluorescence microscopy.** Methods were adapted from our previous report (17). Red cell membranes were suspended to 10 vol in PBS containing 3% (vol/vol) paraformaldehyde, incubated 15 min at 20°C, and settled for 10 min upon poly-L-lysine coated coverslips. The coverslips were washed 3 X with PBS, incubated 30 min with 50 mM NH4Cl in PBS, and again washed 3 X in PBS. The membranes were permeabilized by 15 min of incubation in 0.1% (vol/vol) Triton X-100 in PBS, and then incubated for 20 min and washed 2 X with 10% (wt/vol) PBS in PBS. The coverslips were then sequentially covered with the following agents in 50 μL PBS/PBS: (a) anti-CHIP, 0.1 μg, 45 min; (b) FITC-conjugated goat anti–rabbit IgG, 2.5 μg, 20 min; (c) antiserum, 0.2 μg, 45 min; and (d) Texas red–conjugated goat anti–rabbit IgG, 0.4 μg, 20 min; four PBS/PBS washes followed each step. Coverslips were mounted with 0.1 M 1,4-diazobicyclo[2.2.2]octane in glycerol for microscopy (Nikon Microphot, Melville, NY) and photography with filters for FITC or Texas red and Kodak Tri-X Pan 400 film.

**Immunohistochemical staining of kidneys.** Tissue blocks were pre-
pared from human fetal kidneys (12–24 wk gestation), postnatal (1 mo), and adult (34 yr of age) by fixation for 4 h at 4°C in 4:1 methanol-acetic acid (Carnoy’s fixative) or freshly prepared 4% (vol/vol) paraformaldehyde, 5% (wt/vol) sucrose in PBS. Blocks were embedded in paraffin, and 6-μm sections were cut. After rehydration of sections in a graded series of ethanols, endogenous peroxidase was blocked by 0.3% (vol/vol) hydrogen peroxide for 30 min, and nonspecific staining was blocked with 10% (vol/vol) normal goat serum. Then, sections were incubated with affinity-purified anti-CHIP (0.1 μg/ml) in PBS with 2% (wt/vol) bovine serum albumin for 45 min at room temperature. Localizations were detected with streptavidin-biotin-peroxidase colored by aminoethylcarbazole following the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). Specificity of the immunoreactions was confirmed as described (13): (a) omission of anti-CHIP; (b) substitution of nonimmune rabbit IgG for anti-CHIP; and (c) preincubation of anti-CHIP with pure CHIP protein. Sections were mounted and viewed under a Nikon FXA-Microphot with Nomarsky optics.

Measurement of red cell osmotic water permeability. Methods were adapted from our previous reports (9, 17, 28). P_f was measured by abruptly doubling the extracellular osmolality around intact red cells with an equal volume of PBS/sucrose by stopped flow fluorimetry, dead time of 0.9 ms (SF.17MV: Applied Photophysics, Leatherhead, United Kingdom). Red cell volumes were measured by light scattering. The excitation wavelength, 600±1.5 nm, was generated with a 150 W mercury-xenon arc lamp and monochromator, f/3.4 grating (Applied Photophysics), and emission wavelength >515 nm was measured through a cut-on filter (Oriel Corp., Stratford, CT). The red cells acted as perfect osmometers over the osmolalities tested. The relative volume (absolute/initial) was inversely related to relative signal (absolute/initial), and data averaged from up to 16 determinations were fit to single exponential curves (9, 17, 28) with parameters to determine P_f by applying linear conversion from relative fluorescence to relative volume and iteratively solving with MathCAD software (MathSoft, Cambridge, MA):

\[
dV(t)/dt = (P_f) \times (SAV) \times (MVW) \times \left( C_{os}/V(t) - C_{sw} \right).
\]

V(t) is relative red cell volume as a function of time; P_f is in cm/s; SAV is the vesicle surface area to volume ratio; MVW is the molar volume of water (18 ml/mol); and C_{os} and C_{sw} are the initial concentrations of total solute inside and outside the cell. Red cell radii were calculated from cell volume. In some experiments, 1 mM HgCl_2 was added to the red cells for at least 5 min before analysis.

Results

Expression of Aquaporin CHIP during human fetal development. Human fetal and adult red cells contain very similar patterns of membrane proteins when evaluated by Coomassie stained SDS-PAGE (Fig. 1, top). Unlike fetal rat (17), CHIP was present in human fetal red cells, although the fetal and adult immunoblot patterns were not identical (Fig. 1, middle). Oligosaccharides attached to human red cell membrane proteins are known to exhibit the fetalm pattern (i antigen, a linear glycan chain comprised of repeating galactose-N-acetylgalcosamine disaccharide units) and the adult pattern (I antigen, a branched structure with multiple linear glycan chains). As expected, the adult red cell membranes contained the diffuse 40–60-kD glyCHIP, presumably branched polyolactosaminoglycans (I antigen), whereas the fetal samples contained a smaller, focused band of ~40–45-kD, presumably unbranched polyolactosaminoglycans (i antigen).

To quantitate its abundance, dilutional immunoblots of membranes prepared from human fetal and adult red cells were visualized with anti-CHIP. Samples from second trimester fetuses (17–28 wk of gestation) exhibited approximately half the signal at the 28-kD band, (Fig. 1, middle, CHIP28). Human fetal red cells are larger than adult and contain more reticulocytes; the mean cellular volume (femtoliters) and reticulocyte count (percentage) of the second trimester samples (127±10 fl; 11.3±3.1%) and third trimester samples (107±9 fl; 4.3±3.7%) were above the adult samples (80–95 fl; <1%). Therefore, the abundance of CHIP was quantitated relative to spectrin, an index of membrane surface area (29). Autoradiographs of dilutional anti-CHIP immunoblots were scanned by densitometry, and the CHIP28 signal was plotted against dilution. Spectrin was quantitated by elution of dye from identical gel slabs stained with Coomassie blue. The abundance of CHIP relative to spectrin was expressed in arbitrary units (Fig. 2). Second trimester fetal samples had CHIP/spectrin ratios (0.72±0.12) which were consistently below that of normal adult red cells (1.18±0.11), a difference which was statistically significant (P < 0.001 by t test). Third trimester fetal samples exhibited a greater range of CHIP/spectrin ratios with a mean which was intermediate between second trimester fetal and adult (0.94±0.22, P < 0.01 compared with adult). Unlike rats which have a new population of red cells released into the circulation after birth (17), all
human fetal red cells were stained by anti-CHIP, consistent with the existence of a single population of red cells (Fig. 3 A).

The expression of CHIP in human fetal kidneys was investigated by immunohistochemical staining of tissue sections. Rat kidneys expressed minimal CHIP until 3 d before birth (17). CHIP was detectable in human kidneys as early as 14 wk of gestation (Fig. 4 A). By 17 wk, human fetal kidney already exhibited strong anti-CHIP immunostaining in developing proximal tubules and also in newly forming thin limbs in the outer cortex (Fig. 4 B). By 24 wk, CHIP immunostaining was prevalent in thin limbs of Henle’s loop in medulla (not shown) resembling the pattern in postnatal kidney (Fig. 4 C) and adult kidney (not shown). No staining was seen without primary antibody, with nonimmune IgG, or with anti-CHIP previously incubated with an excess of purified CHIP (Fig. 4 D). These species differences in histochemical staining correlate with recognized species differences in developmental physiology: human kidneys can concentrate urine at birth (21), but rats only achieve this at the time of weaning (22).

Osmotic water permeability of human fetal red cells. The functional consequence of a partial reduction in CHIP was studied by water flux measurements of fetal and adult red cells. Red cells were abruptly exposed to a doubling of extracellular osmolality, and light scattering was monitored with a stopped flow apparatus at 37°C. Measurements of adult red cells revealed coefficients of osmotic water permeability ($P_f = 0.037$ cm/s) which became reduced (0.009 cm/s) after incubation in 1 mM HgCl₂, a known inhibitor of CHIP water channels (9). Second and third trimester samples exhibited somewhat lower $P_f$s but were also reduced by HgCl₂ (Table I). Arrhenius activation energies were calculated from measurements performed over a range of temperatures without HgCl₂, and fetal values ($E_a = 6.7$ and $6.0$ kcal/mol) were above adult values ($4.6$ kcal/mol), consistent with greater channel-mediated water permeability in the latter.

Novel CDA with deficiency of Colton antigens. Although the expression of CHIP in human fetal development occurs earlier and more gradually than in fetal rat, all studies suggest that CHIP expression will be reduced or absent during human embryonic or during very early human fetal development. The possibility that this could be of pathological significance in certain human disorders was suggested by the recent demonstration that Colton blood group antigens were a surface epitope

![Figure 2](image2.png)

**Figure 2.** Concentration of Aquaporin CHIP relative to spectrin in fetal and adult red cell membranes. Dilutional immunoblot analysis of CHIP expression from fetuses of defined gestational ages or normal adults were prepared and incubated with anti-CHIP. Identical SDS-PAGE gels were stained with Coomassie. The abundance of CHIP relative to spectrin was assessed (see Methods) and is expressed in arbitrary units.

![Figure 3](image3.png)

**Figure 3.** Anti-CHIP and antispectrin immunofluorescence of fixed, permeabilized red cell membranes. (A) Red cell membranes from a 17-wk-old fetus and a normal adult. The same microscope slides were stained with anti-CHIP (visualized with FITC goat anti–rabbit IgG) and then with antispectrin (visualized with Texas red goat anti–rabbit IgG). Optical filters were used for differential anti-CHIP or antispectrin immunofluorescence (see Methods). Note that all cells in both fetal and adult slides were stained with both antibodies, and, except for areas where cells were folded, the intensity of staining was consistent in both slides. (B) Red cell membranes from a patient with a novel form of CDA and a normal adult. Note that a few cells in the novel CDA slide exhibit strong anti-CHIP immunofluorescence, whereas most are weak. In contrast, all cells in the adult slides were stained with both antibodies, and, except for areas where cells were folded, the intensity of staining was consistent.
of red cell CHIP (30) and the recent discovery of a patient suffering from a novel form of CDA with persistent embryonic globins (ε- and ζ-chains) with marked deficiency of CD44 and Colton blood group antigens (see Methods and references 23–25).

Similar to fetal membranes, the red cell membranes of the novel CDA patient showed the appearance of some additional bands on Coomassie-stained SDS-PAGE gels, but were otherwise similar to adult red cell membranes (Fig. 5 A, top). Anti-CHIP immunoblotting revealed a marked reduction in the amount of CHIP in membranes from the CDA patient, but no reduction in membranes from her family members (Fig. 5 A, bottom). No evidence of proteolytic degradation was noted with antibodies to the amino terminus, which is known to resist proteolysis (not shown). When examined by dilutinal immunoblot, the CDA membranes were found to contain < 10% of the normal amount of signal (Fig. 5 B). The electrophoretic mobilities of the glyCHIP subunits in the normal and CDA membranes appeared similar when signal intensities were adjusted by varying the radiographic exposure time (Fig. 5 C), consistent with an adult polyglycosaminoglycan structure (1 antigen) on the CDA glyCHIP. Examination of the peripheral blood by anti-CHIP immunofluorescence revealed a markedly heterogeneous pattern in the novel CDA red cells, with a few strongly positive cells, frequent faintly stained cells, and some unstained cells (Fig. 3 B), whereas the red cells from her family members (not shown) were equivalent to the control.

*Figure 4. CHIP immunohistochemical staining of kidney sections from prenatal and postnatal human fetuses. (A) A 14-wk-old human fetal kidney shows CHIP immunostaining within a newly developing proximal tubule. Fetal glomeruli, condensates, S-bodies, and interstitial cells are unstained. (B) CHIP immunostaining is prominent at the apical membrane of newly forming proximal tubules and is detectable in a newly descending thin limb of Henle (arrow) in the outer cortex of a 17-wk-old human fetal kidney. (C) CHIP immunostaining is present over apical and basolateral membranes of proximal tubules and thin limbs of Henle (arrows) in a 1-mo-old postnatal human kidney. (D) No immunoreaction is observed on sections of normal 1-mo-old postnatal human kidney section after incubation with preabsorbed anti-CHIP. ×160 (A), and ×80 (B–D).*
The Pf of fetal red cell samples and normal adult red cell travel controls were each analyzed as described (see Methods). Highly consistent values were obtained on multiple occasions from all of the different normal adults. Statistical comparisons of fetal to adult values: P<; Hg-Pf, NS; E<, P < 0.01.

The existence of human red cells with marked deficiency of CHIP permitted direct biophysical evaluation of Pf. When abruptly exposed to a doubling of extracellular osmolarity, the CDA cells shrunk to half of their original size (Fig. 6), reaching equilibrium in ~2 s at 37°C (Pf < 0.01 cm/s; Table II). Normal adult red cells shrunk similarly, however equilibrium was reached approximately eight times faster (Pf ~ 0.04 cm/s).

Analyses of red cells from the patient’s family members (not shown) were equivalent to that of the control red cells. Treatment of red cell samples with HgCl2 blocked water permeability of control red cells but not the CDA red cells. Arrhenius plots of measurements between 8 and 39°C revealed a notable increase of the activation energy for the CDA red cells (E< > 7 kcal/mol), consistent with markedly reduced water channel activity (Table II).

**Discussion**

Our previous studies demonstrated three patterns of Aquaporin CHIP expression during fetal development in rat (16), with concurrent appearance of CHIP in erythroid and renal proximal tubules and descending thin limbs at the time of birth (17). Those observations suggested a physiologic link between the presence of CHIP in red cells and kidney. Red cells may use CHIP for rapid rehydration as the cells leave the markedly hypertonic renal medulla, and proximal renal tubules use CHIP to reabsorb water from the glomerular filtrate. It was anticipated that concurrent appearance of CHIP in erythroid and renal tissues would also be found in human fetal development. Unlike rat kidneys, however, human kidneys are functionally mature at the time of birth (21). Consistent with this, newborn human red cells and renal tubules contained nearly adult levels of CHIP.

Table 1. Osmotic Water Permeability of Second and Third Trimester Fetal and Adult Red Cells

<table>
<thead>
<tr>
<th>Red cell sample</th>
<th>No.</th>
<th>Pf ± SD (cm/s at 37°C)</th>
<th>Hg-Pf (cm/s in 1 mM HgCl2)</th>
<th>E&lt; (kcal/mol, 8–39°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second trimester (17–28 wk)</td>
<td>6</td>
<td>0.029±0.009</td>
<td>0.008±0.002</td>
<td>6.7±0.4</td>
</tr>
<tr>
<td>Third trimester (28 wk–term)</td>
<td>6</td>
<td>0.026±0.006</td>
<td>0.006±0.003</td>
<td>6.0±0.5</td>
</tr>
<tr>
<td>Normal adult</td>
<td>11</td>
<td>0.037±0.007</td>
<td>0.009±0.005</td>
<td>4.6±0.8</td>
</tr>
</tbody>
</table>

![Figure 5](image_url) Red cell membranes from an unrelated normal adult control, normal family members, and a patient with a novel form of CDA. (A) An SDS-PAGE slab was stained with Coomassie or blotted with anti-CHIP. (B) A dilutional immunoblot of red cell membranes from an unrelated control and the novel CDA patient was visualized with anti-CHIP. (C) Approximately 8 μg membrane protein from the control or the CDA patient was analyzed by anti-CHIP SDS-PAGE immunoblot, and the difference in CHIP signal intensities was corrected by varying the radiographic exposure times. (Because of rapidly decaying signal, differences in exposure times only approximate the relative differences in abundance.)

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expression, and the developmental program was already advanced even at the middle of the second trimester. We were unable to obtain blood samples from fetuses before 17 wk of gestation, but the third trimester red cells contained CHIP/spec-trin ratios below adult, and ratios of second trimester red cells were still lower. Therefore, it is likely that earlier stage red cells may have still less CHIP.

Identification of a patient with a novel form of CDA provided evidence in support of the hypothesis that CHIP is first expressed during early human fetal development. This patient’s red cells were markedly deficient in CHIP and had persistent embryonic and fetal globins, suggesting a defect in erythroid development. This putative defect is not absolute, since her peripheral blood contained a small number of red cells that reacted with anti-CHIP (Fig. 3 B). Nevertheless, her red cells exhibited a marked reduction in osmotic water permeability, which was not further reduced by treatment with HgCl₂, and had a high Arrhenius activation energy (Fig. 6, Table II). These measurements are consistent with a lack of membrane water channels from most of the CDA red cells, supporting the paradigm that CHIP is the major water transporter of red cell membranes.

Our patient was recognized as having CDA by morphological studies (23) and was later found to have a unique blood group phenotype, since In(a−b−) individuals are rare, and Co(a−b−) are extremely rare (25). The power of world-wide blood group referencing permits analysis of individuals with the rarest combinations of blood group phenotypes, and some of these individuals may suffer from specific clinical syndromes which might not otherwise be examined. Three types of CDA have been defined (31). Our patient apparently suffers from a clinically distinct form of the disorder with moderate to severe normocytic anemia, but, unlike CDA type II, her red cells lack the rapid SDS-PAGE electrophoretic mobility characteristic of glycoproteins with the fetal polylactosamino-glycan structure (i antigens). Also, detailed electronmicroscopic analyses demonstrated that her erythroblasts lack the characteristic ultrastructural features of the defined CDA types (23). Should other patients with this form of CDA exist, the unique In(a−b−), Co(a−b−) phenotype should allow their rapid and unambiguous identification.

In spite of detailed analyses, the genetic explanation for our patient’s clinical problems is not obvious. The In and Colton blood group antigens reside on different membrane proteins, In on CD44 (32) and Co on CHIP (30). CHIP has been shown to be a homotetramer and is not associated with another protein, so it is unlikely that CD44 and CHIP are both deficient because they can only exist as a complex. CD44 is a hemagglutinin adhesion molecule (33, 34) encoded by a gene located on human chromosome 11p13 (35). Our patient lacked CD44 on her red cells and their progenitors, but her lymphocytes and granulo-cytes expressed normal amounts of intact CD44 protein, so it is very unlikely that this patient is suffering from a primary mutation in the CD44 gene. Nevertheless, it is possible that the lack of CD44 on erythroid progenitors may contribute to the dyserythropoiesis, since monoclonal antibodies to CD44 are known to inhibit the development of hematopoietic precursors in culture (36). Although some CD44-positive cells are present in human fetal liver (37), it is also possible that the molecule is of greater importance for hematopoiesis within the bone marrow. Simple deficiency of CD44 is unlikely to be the only factor causing our patient’s CDA, since depression of In antigens is known in the rare In(Lu) phenotype which lacks Lutheran antigens and has reduced CD44 without CDA. S.F. is not the In(Lu) type, since her red cells have normal Lutheran antigens (25). Moreover, unlike the Co(a−b−) phenotype (30), other null red cell phenotypes including In(Lu) Lu(a−b−), Rhnull, and McLeod (Kx-) all contain adult levels of CHIP by immunoblot (not shown). CHIP is encoded by Aquaporin-1, a single structural gene on human chromosome 7p14 (38). Our patient did not express any obvious renal, neurological, ocular, or endothelial disorder and therefore may only express a deficiency of CHIP in red cells. Also, sequencing of all four exons PCR-amplified from her leukocyte DNA failed to demonstrate a mutation (not shown), but the existence of an erythroid-specific mutation in the CHIP promoter cannot be excluded. It is not clear if the lack of red cell water channels is related to the hemolytic component of this patient’s anemia, since hemolysis has not been associated with the Co(a−b−) phenotype, although such individuals are exceedingly rare.

**Table II. Osmotic Water Permeability of Novel CDA Red Cells and Normal Adult Red Cells**

<table>
<thead>
<tr>
<th>Red cell sample</th>
<th>No.</th>
<th>Pₑ±SD (cm/s at 37°C)</th>
<th>Hg-Pₑ (cm/s in 1 mM HgCl₂)</th>
<th>ΔHₑ (kcal/mol, 8–39°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novel CDA</td>
<td>1</td>
<td>0.0096</td>
<td>0.014</td>
<td>7.3</td>
</tr>
<tr>
<td>Normal adult</td>
<td>11</td>
<td>0.037±0.007</td>
<td>0.009±0.005</td>
<td>4.6±0.8</td>
</tr>
</tbody>
</table>

The Pₑ of CDA red cells and normal adult red cell travel controls were each analyzed as described (see Methods and Table I).
Our patient also expressed other erythroid abnormalities. She had persistent expression of ϵ and ζ embryonic globins, which are the predominant hemoglobins in the first 7 wk of gestation but are not expressed after birth. She also expressed large amounts of αS and αF fetal hemoglobins which comprise > 90% of hemoglobin until 36 wk of gestation, after which their expression declines to < 3% by 6 mo after birth (20).

These proteins are encoded by a series of genes in the α cluster on human chromosome 16p13 and the β cluster at 11p15. Red cells from the novel CDA patient also lack AnWJ and have weakened LWJ, but the significance is less notable since AnWJ is thought to be linked to CD44, and LW is also reduced in several other erythroid phenotypes.

The absence of other tissue or organ dysfunction, the coexistence of embryonic globins, and the lack of two different red cell membrane proteins suggest a fundamental defect in erythroid ontogeny. Several observations from the literature endorse this hypothesis and further suggest that a related defect may be involved in the pathogenesis of certain hematopoietic malignancies. Juvenile chronic myelogenous leukemias are known to exhibit fetal and embryonic erythroid features (39, 40). Patients with monosomy 7 and myelogenous leukemia have been identified who also lack Colton blood group antigens (41, 42). A likely explanation would be that our patient is a homozygote for a mutated form of an erythroid transcription factor which is normally needed to suppress embryonic and fetal globin genes but will induce expression of CD44 and CHIP. If such a transcription factor exists, mutations in the gene from which it is transcribed may be one step in the multistep process of leukemogenesis. Comparison of the sequences of the CD44 and CHIP promoters may reveal shared binding sites which may permit identification of a putative transcription factor essential to the expression of these proteins and the development of normal erythroid progenitors. This suggests an approach to the molecular defect underlying this novel form of CDA.

Acknowledgments

We thank R. Sue Shirey for valuable discussions and resources. Support for this study was provided by National Institutes of Health grants HL-33991, HL-48269, and DK-43955. R. Baumgarten was supported by the medical student exchange program between Erasmus University, Rotterdam and Johns Hopkins University. M. L. Zeidel was supported by a Clinical Investigator Career Development Award from the Department of Veteran’s Affairs.

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