RED CELLS

Formation of Dense Erythrocytes in SAD Mice Exposed to Chronic Hypoxia: Evaluation of Different Therapeutic Regimens and of a Combination of Oral Clotrimazole and Magnesium Therapies

By Lucia De Franceschi, Carlo Brugnara, Philippe Rouyer-Fessard, Helene Jouault, and Yves Beuzard

We have examined the effect of hydroxyurea (HU), clotrimazole (CLT), magnesium oxide (Mg), and combined CLT+Mg therapies on the erythrocyte characteristics and their response to chronic hypoxia in a transgenic sickle mouse (SAD) model. SAD mice were treated for 21 days with 1 of the following regimens (administered by gavage): control (n = 6), HU (200 mg/d; n = 6), CLT (80 mg/kg/d, n = 5), Mg (1,000 mg/kg/d, n = 5), and CLT+Mg (80 and 1,000 mg/kg/d, respectively, n = 6). Nine normal mice were also treated as controls (n = 3), HU (n = 3), and CLT+Mg (n = 3). Treatment with HU induced a significant increase in mean corpuscular volume and cell K content and a decrease in density in SAD mice. Treatment with the CLT and Mg, either alone or in combination, also increased cell K and reduced density in SAD mice. After 21 days of treatment, the animals were exposed to hypoxia (48 hours at 8% O_2) maintaining the same treatment. In the SAD mice, hypoxia induced significant cell dehydration. These hypoxia-induced changes were blunted in either HU- or Mg-treated SAD mice and were completely abolished by either CLT or CLT+Mg treatment, suggesting a major role for the Gardos channel in hypoxia-induced dehydration in vivo.

A POTENTIAL THERAPEUTIC approach for sickle cell disease involves the use of drugs that reduce or block the dehydration of sickle erythrocytes. This strategy is based on the extreme dependence of hemoglobin (Hb) S polymerization on Hb S concentration and on the presence of dense dehydrated erythrocytes in the blood of patients with homozygous sickle cell (SS) disease.[1-3] The presence of dense cells containing polymerized Hb S has been linked to the clinical severity of various sickle syndromes.[4] Two cation transport pathways play a prominent role in sickle cell dehydration: the K-Cl cotransport[5,6] and the Ca^{2+}-activated K transport (Gardos channel).[7-10]

The K-Cl cotransport promotes loss of K and Cl with consequent erythrocyte dehydration when cells are exposed to pH values less than 7.4 or when the red blood cell (RBC) magnesium (Mg) content is decreased. We have demonstrated, both in the transgenic sickle (SAD) mouse model, in SS patients, and in patients with β thalassemia intermedia, that oral Mg supplementation ameliorates erythrocyte dehydration by increasing erythrocyte Mg and K contents and reducing K-Cl cotransport activity.[11-13] We have also shown that long-term (6 months) oral Mg supplementation (using Mg pidolate salts) induces a significant reduction in the incidence of acute painful crises in patients with SS disease.[14]

The Ca^{2+}-activated K transport induces K loss and erythrocyte dehydration when cytosolic free Ca^{2+} increases, as occurs upon deoxygenation of sickle cells.[15] We have shown that treatment with clotrimazole (CLT), a specific inhibitor of the Gardos channel,[16,17] can prevent erythrocyte dehydration both in the SAD mouse model and in SS patients.[18,19]

Although the percentage of circulating dense cells does not predict disease severity,[20] an inverse correlation has been demonstrated between the percentage of irreversibly sickled cells (ISC) and erythrocyte survival.[21] α Thalassemia and an increased cellular content of fetal Hb (Hb F) have been shown to be associated with a reduction in the number of circulating dense cells.[3,22,23] Dense cells have also been shown to increase before or in the very first phase of painful crises and to decrease significantly thereafter.[24,25] Dense ISC have been shown to play an important role in the trapping of cells in postcapillary venules[26] and associated microvascular obstructions.[27]

The availability of an animal model for sickle cell anemia offers a useful tool for studying the pathophysiology of the disease and for evaluating the effectiveness of therapeutic agents in vivo. Several different transgenic mouse models for SS disease are available.[28-34] Many of these models show (to different degrees) significant RBC sickling upon deoxygenation in vitro and the presence of circulating ISC in vivo. The 2 more recent models[33,34] seem to mimic closely the clinical and pathologic features of the human disease. The SAD mouse model has been widely used, especially for studies on ion transport and cell dehydration, although these mice do not have anemia, have only mild reticulocytosis, and have normal RBC survival (C. Joiner, personal communication, December 1998). The ion transport pathways of SAD erythrocytes have been characterized in detail,[35] and their response to either oral CLT or Mg therapies reproduces that seen in patients with SS disease.[11,18]

Hydroxyurea (HU) therapy induces macrocytosis, leukopenia, and an increase of the synthesis of the β minor globin chain, with improvement of anemia in a mouse model of human β

From the Department of Internal Medicine, University of Verona, Verona, Italy; the Experimental Laboratory of Gene Therapy, Hopital St Louis, Paris, France; the Laboratory of Hematology, INSERM U91, Hopital Henri Mondor, Creteil, France; and the Departments of Laboratory Medicine and Pathology, Children's Hospital, Harvard Medical School, Boston, MA.

Submitted April 23, 1998; accepted August 11, 1999.

Supported by National Institutes of Health grants from the Heart, Lung and Blood Institute (P60-HL15157 and HL 58930); from the Diabetes, Digestive, and Kidney Diseases Institute (R01-DK50422); and from the "Associazione Filippo Collerone," Caltanissetta, Italy.

Address reprint requests to Carlo Brugnara, MD, Department of Laboratory Medicine, The Children's Hospital, 300 Longwood Ave, Bader 760, Boston, MA 02115; e-mail: brugnara@A1.TCH.HARVARD.EDU.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/99/9412-0039$3.00/0

thalassemia intermedia.[36,37] In normal mice, 30 days of HU therapy induce macrocytosis and leukopenia, with no changes in reticulocyte counts.[36] Because there is no clearly demonstrable equivalent of Hb F in mice, studies with HU in SAD mice may be helpful to identify effects that are not related to increased cellular concentration of Hb F. Clinical studies in patients with SS disease have identified cellular changes that are independent of Hb F levels and may explain some of the beneficial effects of HU therapy.[38]

The human and mouse studies indicate that both Gardos channel and K-Cl cotransport are involved in the in vivo generation of dense sickle cells, as recent in vitro studies suggest.[9,10,39-42] The objectives of this study using the SAD mouse model are to determine whether chronic hypoxia (48 hours) induces in vivo changes in erythrocyte features, including the formation of dense cells; what the effects are of different pharmacological regimens, including either HU, CLT, or Mg on the cellular changes induced by hypoxia; and what is the added benefit of combining CLT and Mg therapies.

MATERIALS AND METHODS

Drugs and chemicals. NaCl, KCl, ouabain, bumetanide, Tris (hydroxymethyl) aminomethane (Tris), 3(N-morpholino) propanesulfonic acid (MOPS), choline chloride, and Acationox were purchased from Sigma Chemical Co (St Louis, MO). $MgCl_2$, dimethylsulfoxide (DMSO), n-butyl phthalate, and all other chemicals were purchased from Fisher Scientific Co (Fair Lawn, NJ). Microhematocrit tubes were purchased from Drummond Scientific Co (Bromall, PA). All solutions were prepared using double-distilled water.

Animals and experimental design. Transgenic $Hbb^{single/single}$ SAD1 (SAD) mice were used for the experiment, whereas the control group consisted of nontransgenic litter mates. All of the mice were obtained from breeding performed in the animal facility of INSERM at Henri Mondor Hopital (Creteil, France).[31] Males between 4 and 6 months of age (weight, 28 to 30 g) were used for this study. Twenty-eight SAD mice were divided into 5 different groups: control (n = 6), HU (200 mg/d, n = 6[37]), CLT (80 mg/kg/d, n = 5),[18] Mg (1,000 mg/kg/d, n = 5),[11] and CLT+Mg (80 and 1,000 mg/mg/d, respectively, n = 6).

Nine normal control mice were divided into 3 groups, which were treated for 21 days with 1 of the following regimens: control, HU (200 mg/d), and CLT+Mg (80 and 1,000 mg/kg/d, respectively).

HU was suspended in water (0.2 mL). CLT was suspended in a solution containing deoxycholate (5 mg/mL) and cellulose (0.6%) to a final concentration of 20 mg/mL. Mg supplementation was achieved by adding an additional 600 mg/kg body weight/d for a total Mg of 1,000±20 mg Mg/kg body weight/d to the Mg contained in the regular mouse feed. The Mg supplement consisted of magnesium hydroxide dissolved in water. HU as well as CLT and Mg were administered by gavage. HU and Mg were administrated once daily, whereas CLT was administered twice daily.

The different mouse groups were studied at baseline, at 21 days of therapy, and after 48 hours of hypoxia. No changes in body weight were observed during the treatments. A total of 200 µL of blood was drawn from each animal at the specific times and used for Rb^+ influx measurements, erythrocyte phthalate density distribution curves, cell morphology, erythrocyte cation content, and other hematological parameters. It is our experience that 200 µL of blood can be drawn from mice without incurring significant reticulocytosis.

Hypoxia studies. Treated and untreated SAD and control mouse groups were maintained at 8% oxygen for 48 hours. Oxygen pressure inside the enclosed cage was monitored with an oxygen electrode. Hematological parameters, cell morphology, RBC density patterns, Gardos channel, and erythrocyte cation content were examined before and after 48 hours of hypoxic exposure. The different therapeutic regimens were continued during the exposure to hypoxic conditions.[11,18,37,43]

Hematological data and cation content. Blood was collected from ether-anesthetized mice by retro-orbital venipuncture into heparinized microhematocrit tubes. Hb concentration was determined by spectroscopic measurement of the cyanmet derivative. Hematocrit (Hct) was determined by centrifugation in a micro-Hct centrifuge. Reticulocytes were counted on a Coulter EPICS profile II (Coulter Electronics, Hialeah, FL) using thiazole orange staining: 2.5 µL of whole blood was incubated for 20 minutes with 0.1 mg of thiazole orange dissolved in 1 mL of filtered phosphate-buffer saline (PBS) buffer. The fluorescence of 50,000 erythrocytes was collected with log amplification.[44] White blood cells (WBCs) were measured on a Coulter STK-S hematology analyzer.

Density distribution curves were obtained according to Dannon and Marikovsky,[45] using phthalate esters in microhematocrit tubes, after washing the cells 3 times with PBS solution (330 mosmol/L) at 25°C in 2-mL tubes. The remaining cells were washed 4 additional times with choline washing solution (170 mmol/L choline, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-Mops, pH 7.4, at 4°C, 330 mosmol/L) for measurements of internal Na and K content by atomic absorption spectrometry.

Measurements of Ca^{2+}-activated Rb^+ influx in mouse RBCs. Whole blood was incubated for 30 minutes at room temperature in the presence of 1 mmol/L ouabain, 10 µmol/L bumetanide, and 20 mmol/L Tris-Mops, pH 7.4. The ionophore A23187 was added to the mouse blood to a final concentration of 80 µmol/L, followed by an additional 6 minutes of incubation under stirring at 22°C. At 0 time, RbCl was added to the cell suspension to a final concentration of 10 mmol/L in plasma and incubated at 37°C. Aliquots were removed after 0, 2, 3, and 5 minutes; transferred to a 2 mL medium containing 150 mmol/L NaCl and 15 mmol/L EGTA, pH 7.4, at 4°C; washed 3 times at 4°C with the same solution; and lysed in 1.5 mL of 0.02/Acationox. The lysate was then centrifuged for 10 minutes at 3,000*g*. Rb^+ content was measured in the supernatant by atomic absorption spectrophotometry.

RESULTS

Effects of HU, CLT, Mg, and CLT+Mg treatments on hematological parameters. HU therapy in normal control mice produced no significant changes in Hct and Hb (data not shown). In SAD mice, HU induced an increase in Hct (from 44.4% ± 1.1% to 46.7% ± 1.1%, $P < .005$), mean corpuscular volume (MCV; from 43.1 ± 0.4 fL to 45.8 ± 0.3 fL, $P < .05$), Hb, and reticulocyte counts (Table 1) over their normal baseline values and a decrease in WBC counts (Table 1). A shift in the phthalate density distribution curve towards lower values was also observed (Fig 1B and Table 2).

CLT treatment of SAD mice resulted in a significant increase in Hct (from 43.9% ± 1.3% to 47.0% ± 1.2%, $P < .05$) and a decrease in cell density (Table 2 and Fig 1C). Hb, reticulocyte, and WBC counts were unchanged after CLT therapy (Table 1).

Mg treatment of SAD mice resulted in significant increases in Hct (from 43.6% ± 0.7% to 45.5% ± 0.2%, $P < .05$) and Hb (Table 1) and decreased cell density (Table 2 and Fig 1D), as described in our previous report.[11] Mg treatment did not induce significant changes in either reticulocyte or WBC counts (Table 1).

A combination of CLT+Mg treatments in normal control mice resulted in significant increases in Hb (from 14.1 ± 0.4 to 15.3 ± 0.3 g/dL, $P < .02$) and Hct (from 45.8% ± 1.3% to 48.2% ± 0.6%, $P < .02$). Because we have previously demonstrated that CLT administration did not affect the hematological parameters of normal mice,[18] whereas Mg increased Hb

Table 1. Effects of HU, CLT, Mg, and CLT + Mg Treatments Under Ambient and Hypoxic Conditions on Hematological Parameters in SAD Mice

	Untreated (n = 6)			HU (n = 6)			CLT (n = 5)			Mg (n = 5)			CLT + Mg (n = 6)		
	Hb	Retics	WBC	Hb	Retics	WBC	Hb	Retics	WBC	Hb	Retics	WBC	Hb	Retics	WBC
Baseline	12.8 ± 0.6	7.2 ± 3.2	12.2 ± 1.5	12.1 ± 0.5	7.4 ± 4.3	16.8 ± 0.8	12.3 ± 0.6	6.8 ± 1.2	14.7 ± 1.5	12.4 ± 0.6	5.9 ± 1.8	11.4 ± 1.4	13.1 ± 0.9	6.2 ± 1.9	11.4 ± 2.0
21 days	—	—	—	13.6 ± 0.6*	12.4 ± 1.1*	5.9 ± 0.2†	13.1 ± 0.6	6.1 ± 0.8	12.8 ± 2.4	13.8 ± 0.2*	6.2 ± 0.7	10.2 ± 0.8	14.8 ± 0.6†	5.0 ± 1.5	9.4 ± 1.2
Hypoxia	12.5 ± 0.4	5.8 ± 2.1	10.9 ± 1.4	12.6 ± 0.2‡	10.8 ± 0.7	4.1 ± 0.4†	12.6 ± 0.3	4.8 ± 1.9	13.7 ± 0.2	12.8 ± 0.2§	4.9 ± 1.3	12.3 ± 1.3	13.9 ± 0.7	5.6 ± 0.5	10.8 ± 1.4

Data are presented as the means ± SD.

*$P < .05$ compared with baseline.

†$P < .005$ compared with baseline.

‡$P < .02$ for comparison between after 21 days and hypoxia.

§$P < .05$ for comparison between after 21 days and hypoxia.

and Hct,[11] these effects are most likely due to Mg supplementation.

Combined treatment with CLT+Mg of SAD mice resulted in significant increases in Hct (from 44.5% ± 1.0% to 47.6% ± 1.4%, $P < .005$) and Hb (Table 1) and decreased cell density (Table 2 and Fig 1E). No significant changes were observed in either reticulocyte or WBC counts (Table 1).

Effects of HU, CLT, Mg, and CLT+Mg treatments on erythrocyte K and Mg content and Gardos channel activity. SAD mice have a reduced erythrocyte K content and normal activity of the Gardos channel (Table 2 and De Franceschi et al[11,18]).

HU treatment did not modify the activity of the Gardos channel in either normal control (data not shown) or SAD mice (Table 2). The erythrocyte K content was unchanged by HU treatment in normal mice (data not shown), whereas it increased significantly in SAD mice (Table 2).

CLT treatment of SAD mice induced marked inhibition of the Gardos channel and significantly increased erythrocyte K content (Table 2), as described in our previous study.[18]

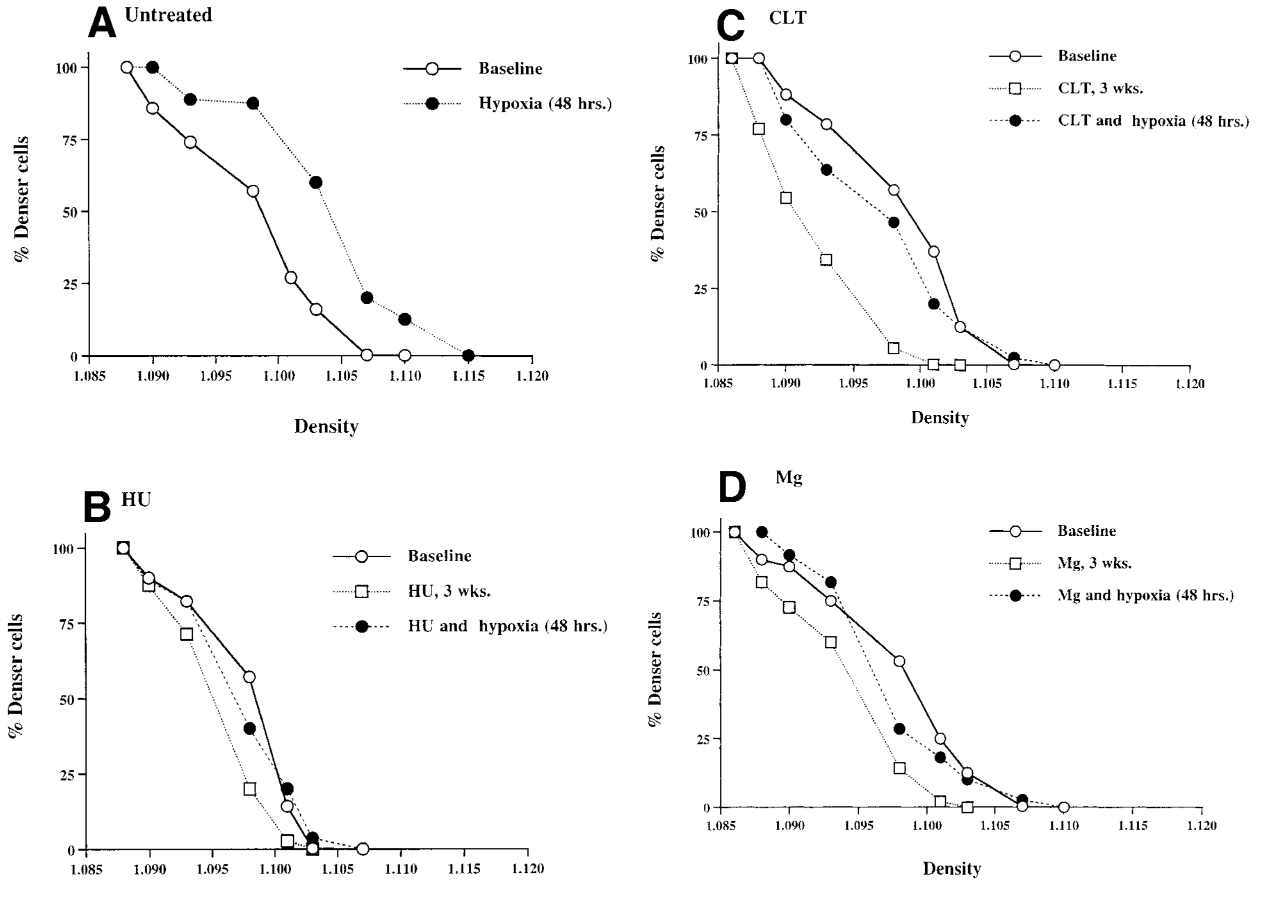
Mg treatment of SAD mice yielded no changes in Gardos channel activity and significantly increased erythrocyte K content (Table 2), as shown before.[11] Erythrocyte Mg content increased from 9.6 ± 0.7 mmol/kg Hb to 13.9 ± 1.1 mmol/kg Hb (n = 5, $P < .05$) and plasma Mg increased from 0.94 ± 0.9 mmol/L to 1.5 ± 0.7 mmol/L (n = 5, $P < .05$).

Combined CLT+Mg treatments of normal control mice inhibited Gardos channel without affecting K content (data not shown). As expected, SAD mice treated with CLT+Mg showed a reduction in the activity of the Gardos channel and increased erythrocyte K content (Table 2). CLT+Mg treatment resulted in an increase in both plasma and erythrocyte Mg levels in SAD mice (plasma: 0.75 ± 0.4 mmol/L at baseline *v* 1.7 ± 0.6 mmol/L, n = 5, $P < .05$; erythrocytes: 8.7 ± 1.2 mmol/kg Hb at baseline *v* 12.9 ± 1.7 mmol/kg Hb after treatment, n = 5, $P < .05$).

Effects of hypoxia. To evaluate the effect of the 4 therapeutic regimens on the changes induced by hypoxia, control and transgenic mice were exposed for 48 hours to an atmosphere containing 8% O_2. No significant changes in Hct or Hb were observed in normal control mice after hypoxia (data not shown).

In untreated SAD mice, hypoxia induced a shift of the phthalate density profiles toward higher erythrocyte density values, indicating that hypoxia exacerbates RBC dehydration (Table 2 and Fig 1A). Erythrocyte K content also decreased significantly with hypoxia (Table 2). No significant changes were observed in either reticulocyte or WBC counts after hypoxia (Table 2).

In HU-treated SAD mice, hypoxia decreased Hb levels (Table 1), MCV (from 45.8 ± 0.3 fL to 43.4 ± 0.2 fL, $P < .05$), and cell K content (Table 2), whereas cell density showed a trend toward higher values (Fig 1B), which, however, was not statistically significant (Table 2). Reticulocyte or WBC counts did not change with hypoxia in HU-treated SAD mice (Table 1). Cell K content and density in HU-treated SAD mice exposed to hypoxia were still significantly different from those of untreated, hypoxic SAD mice ($P < .02$ and $P < .05$, respectively, ANOVA).



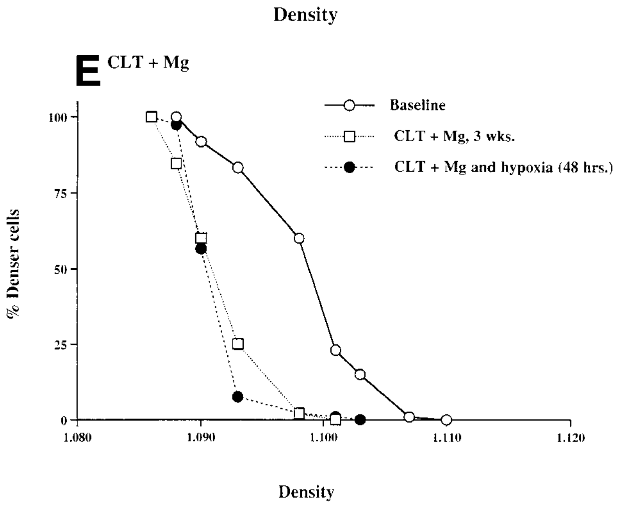


Fig 1. (A) The effect of chronic hypoxia (48 hours at 8% O_2) on the density distribution of erythrocytes in an SAD mouse. This animal is representative of 6 animals. D_{50} was 1.099 ± 0.002 at baseline and increased after hypoxia to 1.107 ± 0.004 ($P < .05$, n = 6). (B) The effect of HU treatment on the changes induced by chronic hypoxia in an SAD mouse. This animal is representative of 6 animals. D_{50} was 1.101 ± 0.002 at baseline, 1.098 ± 0.003 ($P < .05$, n = 6) after 3 weeks of HU treatment, and 1.101 ± 0.004 (not significantly different [NS] from baseline) after hypoxia. (C) The effect of CLT treatment on the changes induced by chronic hypoxia in an SAD mouse. This is representative of 5 animals. D_{50} was 1.097 ± 0.001 at baseline, 1.092 ± 0.01 ($P < .005$, n = 5) after 3 weeks of CLT treatment, and 1.097 ± 0.02 (NS from baseline) after hypoxia. (D) The effect of Mg treatment on the changes induced by chronic hypoxia in an SAD mouse. This is representative of 5 animals. D_{50} was 1.097 ± 0.001 at baseline, 1.093 ± 0.001 ($P < .05$, n = 5) after 3 weeks of Mg treatment, and 1.096 ± 0.002 (NS from baseline) after hypoxia. (E) The effect of CLT+Mg treatment on the changes induced by chronic hypoxia in an SAD mouse. This is representative of 6 animals. D_{50} was 1.102 ± 0.002 at baseline, 1.095 ± 0.002 ($P < .005$, n = 5) after 3 weeks of CLT+Mg treatment, and 1.096 ± 0.005 ($P < .05$ from baseline) after hypoxia.

In CLT-treated mice exposed to hypoxia, discrepant results were obtained between measured cell density, which increased significantly (Table 2 and Fig 1C), and measured cell K content, which was unchanged (Table 2). This unexplained discrepancy does not allow us to determine with certainty how much of the erythrocyte dehydration induced by chronic hypoxia is mediated by the Gardos channel. It should be noted that, with hypoxia, erythrocyte density and cation contents of CLT-treated SAD mice were still significantly different from those of untreated SAD mice (Table 2, ANOVA, $P < .005$), indicating an effect of CLT on hypoxia-induced dehydration.

In Mg-treated mice exposed to hypoxia, a reduction in Hb (Table 1) and cell K content and an increase in cell density were noted (Table 2 and Fig 1D) that almost completely abolished the changes induced by 21 days of Mg therapy. The K loss induced by chronic hypoxia was essentially the same as that of untreated SAD mice, indicating that the K-Cl cotransport plays a minor role in hypoxia-induced dehydration of SAD erythrocytes,

Table 2. Effects of HU, CLT, Mg, and CLT + Mg Treatments Under Ambient and Hypoxic Conditions on Erythrocyte Gardos Channel Activity, K Content, and D_{50} of SAD Mice

	Untreated (n = 6)	HU (n = 6)	CLT (n = 5)	Mg (n = 5)	CLT + Mg (n = 6)
Gardos channel activity (mmol/L cell × min)					
Baseline	12.6 ± 0.7	11.7 ± 1.2	11.8 ± 0.9	10.8 ± 1.3	11.4 ± 0.9
3 wks	11.6 ± 1.2	12.4 ± 0.6	3.2 ± 0.1*	11.9 ± 1.8	2.7 ± 1.3*
After 48 h of hypoxia	12.3 ± 1.2	12.3 ± 1.2	4.4 ± 0.4*	10.4 ± 0.7	3.1 ± 0.6*
Erythrocyte K content (mmol/kg Hb)					
Baseline	350 ± 12	330 ± 8.4	346 ± 12	340 ± 10	358 ± 20
3 wks	348 ± 18.3	394 ± 10.1*	401 ± 10.1*	390 ± 12*	410 ± 3.2*
After 48 h of hypoxia	310 ± 9.7*†	370 ± 11.3*‡	398 ± 8.4*	350 ± 4.7†	407 ± 12*
D_{50}					
Baseline	1.099 ± 0.002	1.101 ± 0.002	1.097 ± 0.001	1.097 ± 0.001	1.102 ± 0.002
3 wks	—	1.098 ± 0.003§	1.092 ± 0.001‖	1.093 ± 0.001§	1.095 ± 0.002*
After 48 h of hypoxia	1.107 ± 0.004§	1.101 ± 0.004	1.097 ± 0.001†	1.096 ± 0.002†	1.096 ± 0.005§

Data are expressed as the means ± SD.
*$P < .02$ compared with baseline.
†$P < .02$ compared with after 3 weeks of therapy.
‡$P < .05$ compared with after 3 weeks of therapy.
§$P < .05$ compared with baseline.
‖$P < .005$ compared with baseline.

which seems to be mostly a Gardos phenomenon. Interestingly, the density and cation content of Mg-treated mice after hypoxia were still significantly different ($P < .005$ and $P < .03$, respectively, ANOVA) than those of hypoxic, untreated SAD mice (Table 2).

In SAD mice treated with CLT+Mg, hypoxia induced no significant changes in either Hb, reticulocyte, or WBC counts (Table 1). Erythrocyte K content and cell density did not change significantly with hypoxia and remained significantly different from baseline values (Table 2) and from untreated SAD mice ($P < .002$ and $P < .005$, respectively, ANOVA, Table 2). These data indicate that CLT+Mg treatment almost completely abolished the density changes induced by chronic hypoxia. Because Mg was ineffective in preventing hypoxia-induced dehydration, it is likely that blockade of the Gardos channel is responsible for these effects. However, due to the discrepancies observed in the CLT-treated group and differences in baseline density among the various groups, the superiority of CLT+Mg treatment compared with the other regimens cannot be convincingly demonstrated.

DISCUSSION

We have examined in this study the effect of 4 therapeutic regimens, including either HU, CLT, Mg, or CLT+Mg, on the changes induced by a short-term (48 hours) exposure to hypoxia in the SAD mouse model. These studies were prompted by several in vitro and in vivo studies that have identified a role for the erythrocyte Gardos channel and K-Cl cotransporter in promoting erythrocyte dehydration.[9,46] Combination treatment with CLT and Mg offers the theoretical possibility of interfering with the dehydration of both reticulocytes and mature erythrocytes by inhibiting the 2 major pathways for sickle cell dehydration.

The SAD mouse has shown to be extremely valuable in assessing the cellular effects of therapies aimed at preventing sickle cell dehydration. SAD mouse erythrocytes resemble human sickle erythrocytes in having a reduced K content, normal Gardos channel activity at baseline, and increased K-Cl cotransport.[11,18,35] The response observed in SAD mice to either CLT or Mg therapies is similar to that observed in patients with sickle cell disease.[11,18,19,35] Thus, although SAD mice are not anemic, they exhibit significant RBC dehydration and organ damage and are a valuable model for studies on ion transport and blockade of cell dehydration.

K-Cl cotransport plays a major role in the dehydration of sickle erythrocytes and reticulocytes. Transferrin receptor-positive (Tfr^+) dense reticulocytes have greater K-Cl cotransport activity than Tfr^+ light reticulocytes, suggesting that K-Cl cotransport may mediate dehydration of young sickle cells.[47] K-Cl cotransport activity is modulated by the erythrocyte Mg content, which is markedly reduced both in transgenic SAD mouse and human sickle erythrocytes.[11,12] We have shown that oral Mg supplementation induces an increase in RBC Mg content that, in turn, leads to a reduction in K-Cl cotransport activity and cell dehydration.[11-13] However, although the Gardos channel has been shown to become active with deoxygenation,[17,48,49] the role of K-Cl cotransport in promoting dehydration in conditions of hypoxia is not well established.[10,40,50] For these reasons, we have examined the effect of pharmacological blockade of these ion pathways in the SAD mouse under conditions of chronic hypoxia.

The results presented here indicate that (1) hypoxia induces formation of dense erythrocytes in SAD mice; (2) HU, CLT, Mg, or CLT+Mg therapies improve the hydration state of erythrocytes and blunt the erythrocyte dehydration induced by hypoxia; (3) hypoxia-induced dehydration in the SAD mouse is mediated almost exclusively by the Gardos channel; and (4) combination of CLT and Mg treatments may have an additive effect in protecting from the erythrocyte dehydration induced by hypoxia, but the results presented here are not unequivocal.

Although other studies have demonstrated formation of dense cells by hypoxia in transgenic sickle mice, no information was available on the mechanisms underlying the formation of dense mouse erythrocytes. Rubin et al,[29] using a mouse model expressing both human α and $\beta^{S\ \mathrm{Antilles}}$ (50% of total Hb), exposed the transgenic mice for 10 days at 8.4% O_2 and showed a significant increase in irreversibly sickled cells. Similar results were obtained by Fabry et al,[28] who exposed the human a^H and β^S (β^{MDD}) transgenic mice for 3 or 5 days to hypoxia (8% O_2). This group also observed a significant reduction in urine osmolarity due to compromised renal function.[28] Reilly et al[30] examined 3 lines of transgenic Hb S mice with human β^S contents of approximately 30%, 50%, and 80% relative to mouse β globins. Exposure to hypoxia (7% O_2) for 7 days resulted in increased Hct, Hb, and MCV and significant reticulocytosis, indicating that this level of chronic hypoxia significantly stimulated erythropoiesis. An increase in the percentage of cells residing in the most dense fraction was also noted.[30]

Duration of exposure to hypoxia seems to be a critical variable for these studies. We have observed significant reticulocytosis both in control and β thalassemic mice after 5 days of hypoxic exposition (Y. Beuzard, unpublished data). The shorter period of hypoxia (48 hours) allowed us to study erythrocyte changes primarily due to polymerization of Hb SAD. With our study, we were able to evaluate the effect of 4 different treatments on these erythrocyte changes with no significant changes in reticulocyte counts. Recently, hypoxia has been shown to enhance sickle cell adhesion to both macrovascular and human microvascular endothelial cells via the adhesive receptor vascular cell adhesion molecule-1 (VCAM-1), suggesting that reticulocytes may be involved in the enhanced adherence to the hypoxic endothelium.[51-53] It will be of interest to determine whether adhesion to endothelium of sickle transgenic erythrocytes,[54] in addition to being modulated by hypoxia, can also be affected by either HU, CLT, or Mg therapies.

In this study, HU treatment of SAD mice induced a significant increase in MCV and a decrease in WBC counts (Table 1), as observed in humans. However, because mice do not produce Hb F, the effects of HU on erythrocyte cation content and density of SAD mice are not easily explained. Our data clearly indicate that HU has no effect on the activity of the Gardos channel (Table 2). In addition, HU induced a significant reticulocytosis in SAD mice, whereas it usually decreases reticulocyte counts in SS patients.[38,55] We have described a marked reduction of the in vitro adherence of human sickle erythrocytes to endothelium in the early phase of HU therapy.[38] Whether this effect is present in transgenic sickle mice remains to be determined.

These studies provide experimental evidence for a major role of the Gardos channel in promoting dehydration of SAD mice erythrocytes under conditions of chronic hypoxia. They also demonstrate the beneficial effects of HU, CLT, Mg, and CLT+Mg therapies in preventing or blunting the hypoxia-induced dehydration. Combination therapy with CLT and Mg could in theory be superior to single-agent therapy in preventing cell dehydration. However, under experimental conditions that maximize dehydration via the Gardos channel, this potential additive benefit could not be confirmed.

REFERENCES

1. Eaton WA, Hofrichter J: Hemoglobin S gelation and sickle cell disease. Blood 70:1245, 1987

2. Noguchi CT, Rodgers GP, Schechter AN: Intracellular polymerization. Disease severity and therapeutic predictions. Ann NY Acad Sci 565:75, 1989

3. Bertles JF, Milner PFA: Irreversibly sickled erythrocytes: A consequence of the heterogeneous distribution of hemoglobin types in sickle cell anemia. J Clin Invest 47:1731, 1968

4. Brittenham GM, Schechter AN, Noguchi CT: Hemoglobin S polymerization: Primary determinant of hemolytic and clinical severity of the sickling syndromes. Blood 65:183, 1985

5. Brugnara C, Bunn HF, Tosteson DC: Regulation of erythrocyte cation and water content in sickle cell anemia. Science 232:388, 1986

6. Canessa M, Spalvin S, Nagel RL: Volume-dependent and NEM-stimulated K-Cl cotransport is elevated in SS, SC and CC human red cells. FEBS Lett 200:197, 1986

7. Gardos G: The function of calcium in the potassium permeability of human erythrocytes. Biochim Biophys Acta 30:653, 1958

8. Glader BE, Nathan NG: Cation permeability alteration during sickling: Relationship to cation composition and cellular dehydration of irreversibly sickled cells. Blood 51:983, 1978

9. Bookchin RM, Ortiz OE, Lew VL: Evidence for a direct reticulocyte origin of dense red cells in sickle cell anemia. J Clin Invest 87:113, 1991

10. Schwartz RS, Musto S, Fabry ME, Nagel RL: Two distinct pathways mediate the formation of intermediate density cells and hyperdense cells from normal density sickle red blood cells. Blood 92:4844, 1998

11. De Franceschi L, Beuzard Y, Jouault H, Brugnara C: Modulation of erythrocyte potassium chloride cotransport, potassium content, and density by dietary magnesium intake in transgenic SAD mouse. Blood 88:2738, 1996

12. De Franceschi L, Bachir D, Galacteros F, Tchernia G, Cynober T, Alper SL, Platt O, Beuzard Y, Brugnara C: Oral magnesium supplements reduce erythrocyte dehydration in patients with sickle cell disease. J Clin Invest 100:1847, 1997

13. De Franceschi L, Cappellini MD, Graziadei G, Manzato F, Olivieri O, Corrocher R, Fiorelli G, Beuzard Y, Brugnara C: The effect of dietary magnesium supplementation on the cellular abnormalities of erythrocytes in patients with beta thalassemia intermedia. Haematologica 83:118, 1998

14. De Franceschi L, Bachir D, Galacteros F, Tchernia G, Cynober T, Alper SL, Platt O, Beuzard Y, Brugnara C: Dietary magnesium supplementation reduces pain crises in patients with sickle cell disease. Blood 90:264a, 1997 (abstr, suppl 1)

15. Rhoda MD, Apovo M, Beuzard Y, Giraud F: Ca^{2+} permeability in deoxygenated sickle cells. Blood 75:2453, 1990

16. Alvarez J, Montero M, Garcia-Sancho J: High affinity inhibition of Ca2+-dependent K+ channels by cytochrome P-450 inhibitors. J Biol Chem 267:11789, 1992

17. Brugnara C, de Franceschi L, Alper SL: Inhibition of Ca(2+)-dependent K+ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. J Clin Invest 92:520, 1993

18. De Franceschi L, Saadane N, Trudel M, Alper SL, Brugnara C, Beuzard Y: Treatment with oral clotrimazole blocks Ca(2+)-activated K+ transport and reverses erythrocyte dehydration in transgenic SAD mice. A model for therapy of sickle cell disease. J Clin Invest 93:1670, 1994

19. Brugnara C, Gee B, Armsby C, Kurth S, Sakamoto M, Rifai N, Alper SL, Platt O: Therapy with oral clotrimazole induces inhibition of the Gardos channel and reduction of erythrocyte dehydration in patients with sickle cell disease. J Clin Invest 97:1227, 1996

20. Billett HH, Kim K, Fabry ME, Nagel RL: The percentage of dense red cells does not predict incidence of sickle cell painful crisis. Blood 68:301, 1986

21. Serjeant GR, Serjeant BE, Milner PF: The irreversibly sickled

cell: A determinant of hemolysis in sickle cell anemia. Br J Haematol 17:527, 1969

22. Fabry ME, Mears JG, Patel P, Schaefer-rego K, Carmichael LD, Martinez G, Nagel RL: Dense cells in sickle cell anemia: The effects of gene interaction. Blood 64:1042, 1984

23. Horiuchi K, Stephens MJ, Adachi K, Asakura T, Schwartz E, Ohene-Frempong K: Image analysis studies of the degree of irreversible deformation of sickle cells in relation to cell density and Hb F level. Br J Haematol 85:356, 1993

24. Ballas SK, Smith ED: Red blood cell changes during the evolution of the sickle cell painful crisis. Blood 79:2154, 1992

25. Lawrence C, Fabry ME, Nagel RL: Red cell distribution width parallels dense red cell disappearance during painful crises in sickle cell anemia. J Clin Lab Med 105:706, 1985

26. Kaul DK, Chen D, Zhan J: Adhesion of sickle cells to vascular endothelium is critically dependent on changes in density and shape of the cells. Blood 83:3006, 1994

27. Kaul DK, Fabry ME, Nagel RL: Vaso-occlusion by sickle cells: Evidence for selective trapping of dense red cells. Blood 68:1162, 1986

28. Fabry ME, Costantini F, Pachnis A, Suzuka SM, Bank N, Aynedjian HP, Factor SM, Nagel RL: High expression of human bS and a globins in transgenic mice: Erythrocyte abnormalities, organ damage and the effect of hypoxia. Proc Natl Acad Sci USA 89:12155, 1992

29. Rubin EM, Witkowska HE, Spongler E, Curtin P, Lubin BH, Mohandas N, Clift SM: Hypoxia-induced in vivo sickling of transgenic mouse red cells. J Clin Invest 87:639, 1991

30. Reilly MP, Chomo MJ, Obata K, Asakura T: Red blood membrane and density changes under ambient and hypoxic conditions in transgenic mice producing human sickle hemoglobin. Exp Hematol 22:501, 1994

31. Trudel M, Saadane N, Garel MC, Bardakdjan-Michau J, Blouquit Y, Guerquin-Kern JL, Royer-Fessard P, Vidaud D, Pachnis A, Romeo PH, Beuzard Y, Constantini F: Toward a transgenic mouse model of sickle cell disease: Hemoglobin SAD. EMBO J 11:3157, 1991

32. Trudel M, De Paepe ME, Chretien N, Saadane N, Jacmain J, Sorette M, Hoang T, Beuzard Y: The sickle cell disease of transgenic SAD mice. Blood 84:3189, 1994

33. Ryan TM, Ciavatta DJ, Townes TM: Knockout-transgenic mouse model of sickle cell disease. Science 278:873, 1997

34. Paszty C, Brion CM, Manci E, Witkowska HE, Stevens ME, Mohandas N, Rubin EM: Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. Science 278:876, 1997

35. De Franceschi L, Beuzard Y, Brugnara C: Sulfhydryl oxidation and activation of red cell K(+)-Cl-cotransport in the transgenic SAD mouse. Am J Physiol 269:C899, 1995

36. Sauvage C, Rouyer-Fessard P, Beuzard Y: Improvement of mouse b thalassemia by hydroxyurea. Br J Haematol 84:492, 1993

37. De Franceschi L, Rouyer-Fessard P, Alper SL, Jouault H, Brugnara C, Beuzard Y: Combination therapy of erythropoietin, hydroxyurea, and clotrimazole in a beta thalassemic mouse: A model for human therapy. Blood 87:1188, 1996

38. Bridges KR, Barabino GD, Brugnara C, Cho M, Christoph GW, Dover G, Ewenstein B, Golan DE, Guttmann CR, Hofrichter J, Mulkern RV, Zhang B, Eaton WA: A multiparameter analysis of sickle erythrocytes in patients undergoing hydroxyurea therapy. Blood 88:4701, 1996

39. Franco RS, Palascak M, Thompson H, Joiner CH: KCl cotransport activity in light versus dense transferrin receptor-positive sickle reticulocytes. J Clin Invest 95:2573, 1995

40. Franco KR, Palascak M, Thompson H, Rucknagel DK, Joiner CH: Dehydration of transferrin receptor-positive sickle reticulocytes during continuous or cyclic deoxygenation: Role of KCl cotransport and extracellular Ca. Blood 88:4359, 1996

41. Franco RS, Thompson H, Palascak M, Joiner CH: The formation of transferrin receptor-positive sickle reticulocytes with intermediate density is not determined by fetal hemoglobin content. Blood 90:3195, 1997

42. Franco RS, Lohmann J, Silberstein EB, Mayfield-Pratt G, Palascak M, Nemeth TA, Joiner CH, Weiner M, Rucknagel DL: Time-dependent changes in the density and hemoglobin F content of biotin-labeled sickle cells. J Clin Invest 101:2730, 1998

43. De Franceschi L, Fumagalli L, Olivieri O, Corrocher R, Lowell CA, Berton G: Deficiency of src family kinases Fgr and Hck results in activation of erythrocyte K/Cl cotransport. J Clin Invest 99:220, 1997

44. Chinn-Yee I, Keeney M, Lohmann RC: Flow cytometric reticulocyte analysis using thiazole orange: Clinical experience and technical limitations. Clin Lab Haematol 13:177, 1991

45. Dannon D, Marikovsky Y: Determination of density distribution of red cell population. J Lab Clin Med 64:668, 1964

46. Lew VL, Freeman CJ, Ortiz OE, Bookchin RM: A mathematical model of the volume, pH and ion content regulation in reticulocytes. J Clin Invest 87:100, 1991

47. Franco RS, Polascak M, Thompson H, Joiner C: K-Cl cotransport activity in light versus dense transferrin receptor-positive sickle reticulocytes. J Clin Invest 95:2573, 1995

48. Lew VL, Ortiz OE, Bookchin RM: Stochastic nature and red cell population distribution of the sickling-induced Ca2+ permeability. J Clin Invest 99:2727, 1997

49. Fathallah H, Coezy E, de Neef RS, Hardy-Dessources MD, Giraud F: Inhibition of deoxygenation-induced membrane protein dephosphorylation and cell dehydration by phorbol esters and okadaic acid in sickle cells. Blood 86:1999, 1995

50. Canessa M, Fabry ME, Nagel RL: Deoxygenation inhibits the volume-stimulated, Cl(-)-dependent K+ efflux in SS and young AA cells: A cytosolic Mg2+ modulation. Blood 70:1861, 1987

51. Setty BN, Stuart MJ: Vascular cell adhesion molecule-1 is involved in mediating hypoxia induced sickle red blood cell adherence to endothelium: Potential role in sickle cell disease. Blood 88:2311, 1996

52. Joneckis CC, Ackley RL, Orringer EP, Wayner EA, Parise LV: Integrin a4b1 and glycoprotein IV (CD36) are expressed on circulating reticulocytes in sickle cell anemia. Blood 823:3548, 1993

53. Gee BE, Platt OS: Sickle reticulocytes adhere to VCAM-1. Blood 85:268, 1995

54. Kaul DK, Fabry ME, Constantini F, Rubin EM, Nagel RL: In vivo demonstration of red cell-endothelial interaction, sickling and altered microvascular response to oxygen in the sickle transgenic mouse. J Clin Invest 96:2845, 1995

55. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, McMahon RP, Bonds DR: Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. N Engl J Med 332:1317, 1995