Sickle Cell Anemia: A Potential Nutritional Approach for a Molecular Disease

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A certain population of red blood cells in patients with sickle cell anemia has an elevated density and possesses an abnormal membrane. These "dense cells" have a tendency to adhere to neutrophils, platelets, and vascular endothelial cells, and, thus, they could trigger vasoocclusion and the subsequent painful crisis from which these patients suffer. We developed a laboratory method of preparing such dense cells and found that nutritional antioxidant supplements, hydroxyl radical scavengers, and iron-binding agents could inhibit the formation of dense cells in vitro. The concentrations at which effective nutritional supplements could inhibit dense cell formation by 50% were 4.0 mg/mL for aged garlic extract, 0.38 mg/mL for black tea extract, 0.13 mg/mL for green tea extract, 0.07 mg/mL for Pycnogenol, 930 μM for α-lipoic acid, 270 μM for vitamin E, 45 μM for coenzyme Q_{10}, and 32 μM for β-carotene. Both an ex vivo study and a pilot clinical trial demonstrated that a cocktail consisting of daily doses of 6 g of aged garlic extract, 4–6 g of vitamin C, and 800 to 1200 IU of vitamin E may indeed be beneficial to the patients. *Nutrition* 2000;16:330–338. ©Elsevier Science Inc. 2000

Key words: sickle cell anemia, dense cells, membrane-acting drugs, antioxidant cocktail, nutritional therapy

INTRODUCTION

Sickle cell anemia (SCA) is a serious disease generally found in a specific ethnic group: African Americans and inhabitants of the African continent and nearby countries. In America, 1 of every 500 people of African descent suffers, but in Africa, the ratio is 10 times higher. Approximate patient numbers are near 100 000 in the United States but are several millions in Africa. In sickle cell crisis, patients experience severe pain caused by the occlusion of blood vessels jammed with red blood cells. Because the average life span of their red blood cells is only about 2 wk as opposed to about 120 d for normal subjects, the patients suffer from chronic anemia. Frequently observed symptoms are splenic infarction; cardiomegaly; neurological disorders such as hemiplegia, convulsions, coma, and stupor; pathologic bone abnormalities such as marrow expansion, avascular necrosis, and osteomyelitis; and leg ulceration.[1] In Africa, SCA causes high mortality in infants and children. The survival rate to adulthood in Africa in the 1950s was reported to be 14–30%, and in Jamaica in the 1960s it was 39.2%.[1] Today, in the United States and the West Indies, many of the deleterious factors in infancy have been controlled and survival to adulthood is not uncommon. Nevertheless, SCA is a disastrous disease.

A half-century ago, Linus Pauling discovered that SCA was a "molecular disease" caused by genetically abnormal sickle hemoglobin (HbS) that polymerizes under deoxygenated conditions to deform the red blood cells.[2] Based on his finding, many drugs have been studied and proposed to inhibit HbS polymerization. Whereas many of them were effective in in vitro experiments, none of them could be used in vivo because of their toxicity. Gene therapy for SCA is a challenging goal, but it would take some years before patients might benefit from it. Although bone-marrow transplantation was found to be effective, this is still limited because of strict type-match requirements between the donor and the recipient.[3]

Considering the demographics of SCA, the best hope for the majority of patients would be a low-cost self-administered oral therapy. Currently, one such hope for these patients is oral administration of hydroxyurea. This is designed to increase the level of fetal hemoglobin, which does not polymerize under deoxygenation.[4] Hydroxyurea therapy has been shown to have beneficial effects,[5,6] but it is still not free of side effects including bone-marrow suppression. Because SCA is a genetic disease, any drug would have to be tolerated for life. It is yet to be determined how long hydroxyurea could be tolerated without long-term side effects.

We have considered a different approach to this genetic disease and proposed that SCA could be regarded as a membrane-linked disease.[7] Instead of targeting the polymerization of the HbS molecule as the only site for therapy, we have studied agents that interact with the red cell membrane. A first-generation drug was cetiedil,[8–10] which was proposed to have an antisickling effect through its interaction with the red cell membrane[8] and was shown to have some therapeutic effect.[10] Because cetiedil changed the red cell shape to that of stomatocytes (cup-shaped cells) in vitro, it was first theorized that cetiedil increased the red cell volume (i.e., lowering hemoglobin concentration), thereby inhibiting hemoglobin polymerization.[8] Ohnishi subsequently found that many other membrane-acting drugs, such as some antipsychotic drugs, also formed cup-shaped cells in vitro and concomitantly inhibited sickling but that a volume change with therapeutic doses of these drugs under physiologic conditions was negligible.[11]

The second-generation drugs were inhibitors for red cell dehy-

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dration. Ohnishi proposed that inhibitors for calcium-activated potassium ion efflux and calcium-entry inhibitors could inhibit dense-cell formation in deoxy-oxy cycling (D-O cycling) experiments.[12–16] It was found that first-generation membrane-acting drugs could inhibit cell dehydration at much lower concentrations than what was required for inhibiting sickling. This seemed to support the usefulness of the membrane-acting drugs, but these drugs may still confront us with a problem. Because most cellular membranes have calcium- and potassium-related channels, the inhibition of such channels could cause serious side effects.

We now propose that the third-generation membrane-acting drugs are antioxidant cocktails that may protect the red cell membrane from free radical-mediated oxidative injury. This approach may be more favorable because oxygen-free radicals are related to the causes of many other diseases; therefore, any attempt to reduce such radical effects may bring additional benefits to SCA patients. To avoid side effects that are always associated with chemotherapy, we decided to search for nutritional supplements the patients could take for life with minimal adverse effects.

We previously employed the D-O cycling method to prepare dehydrated sickle cells in vitro.[12–16] Such dehydrated red cells are called "dense cells" and adhere to vascular endothelial cells,[17] neutrophils,[18,19] and platelets.[20] They are known to play important roles in the pathogenesis of sickle cell crisis in these patients.[21,22]

We previously found that aged garlic extract (AGE; Wakunaga Pharmaceutical Co., Mission Viejo, CA, USA) and S-allyl cysteine (a water-soluble component of AGE; Wakunaga) have antioxidant activity[23,24] and protect cell membranes from oxidative injury.[25] Therefore, we first tested these compounds and found they could inhibit dense cell formation in vitro.[26] In the present study, we report that other antioxidants, hydroxyl radical scavengers and iron-binding agents, also inhibited dense cell formation but with different mechanisms. An ex vivo experiment (preclinical test) and a pilot clinical trial demonstrated that the nutritional approach to this genetic disease may be promising.

MATERIALS AND METHODS

Human Study

The protocol of the human study at the Philadelphia Biomedical Research Institute was approved by the Institutional Review Board, and all participants signed the consent form before participation. Blood was withdrawn at the Noguchi Medical Research Institute, Philadelphia, PA (USA). The clinical trial procedure at the Ibadan University was approved by the Internal Review Board of the university, and all SCA patients signed the consent form before starting the test. Blood was withdrawn at the University Health Services, University of Ibadan (Nigeria).

Blood Collection

Blood samples were drawn by venipuncture using a heparinized vacutainer. The vacuum in the vacutainer was immediately broken after collection because sickle red cells would sickle in the collection tubes. For the in vitro study, we added to the blood one-seventh volume of a solution containing 60 mM of sodium citrate, 10 mM of citric acid, 10 mM of NaH_2PO_3, 110 mM of glucose, 1 mM of adenine, and 1 mM of inosine (pH adjusted to 6.2 and the osmolarity to 290 mOsm/kg). The tube was then slowly tumbled (1 rpm) at 4°C. By this method, blood could be stored for several days.[27]

Reaction Medium

This medium consisted of 109 mM of NaCl, 6 mM of KCl, 5 mM of $CaCl_2$, 1.2 mM of $MgSO_4$, 20 mM of HEPES buffer, 25 mM of $NaHCO_3$, 2.4 mM of Na_2HPO_4, 1 mM of adenine, 1 mM of inosine, 10 mM of glucose, 0.05 mg/mL each of penicillin and streptomycin, and 2% bovine serum albumin. The osmolarity of the medium was adjusted to 290 mOsm/kg and the pH was adjusted to 7.4 after equilibriation with 95% air/5% CO_2. Because sickling is sensitive to the pH and osmolarity, these values were adjusted to the standard conditions after the addition of any inhibitors.

Density Gradient Centrifugation

A Percoll stock solution was prepared as follows. To a 100-mL Percoll solution (Pharmacia-LKB, Piscataway, NJ, USA), 117 mM of NaCl, 20 mM of KH_2PO_4, 0.005 g each of penicillin and streptomycin were added. The pH was adjusted to 7.4. The osmotic pressure of this solution was approximately 290 mOsm/kg as determined by a freezing-point method. The density as determined with a picnometer was approximately 1.137. A diatrizoic-acid stock solution was prepared as follows. To 85 mL of water, 7.03 g of diatrizoic acid (Sigma Chemical Co., St. Louis, MO, USA) was added. Then 8 mL of 2 M NaOH was added to solubilize the compound. Then 0.27 g of KH_2PO_4, was added, and the pH was adjusted to 7.4 with 1 M of HCl. Finally, the volume of the solution was made to 100 mL. The osmolarity was approximately 290 mOsm/kg, and the density was approximately 1.048. A density gradient solution with a density of 1.104 was prepared by mixing these two solutions. The solution was spun in an angle rotor at 11 000*g* for 5 min to create a density gradient. Then 0.1 to 0.2 mL of blood suspension was layered on top of the preformed density gradient and spun in a swing rotor at 2000*g* for 5 min. The top and the bottom layers were collected separately, wash-centrifuged with a phosphate-buffered saline solution, and hemolyzed, and the hemoglobin contents in each layer were assayed with a spectrophotometer to determine the percentage of dense-cell formation.[12,28]

Deoxy-Oxy (D-O) Cycling Experiments

A small aliquot of patients' blood was layered over the preformed density gradient containing 2% bovine serum albumin and spun to separate existing dense cells. Normal density cells in the top layer were then collected and washed with the reaction medium and diluted to make a hematocrit value (HCT) of 2–5%. One to 1.5 mL of the suspension was pipetted into each of the test tubes in a gas equilibrium device,[12,28] and tubes were rotated at the speed of approximately 30 rpm at 37°C. The tubes were purged with 95% N_2/5% CO_2 (11 min) and 95% air/5% CO_2 (1 min) alternately for 1–6 h.[12–16]

Nutritional Supplements for In Vitro Tests

AGE, S-allyl cysteine, and fructosyl arginine were supplied by Wakunaga Pharmaceuticals. For in vitro tests, these compounds were dissolved in the nitrogen-purged reaction medium (without bovine serum albumin), and the pH of the stock solution was adjusted to 7.4. Because a liquid form of AGE with a small amount of alcohol was used, it was vacuum dried before dissolving. All stock solutions were stored frozen at −80°C until use. Ascorbic acid and dehydroascorbic acid (both purchased from Sigma Chemical Co.) were dissolved into nitrogen-bubbled water, the pH was adjusted to neutral, and the solution was used immediately.

D-α-tocopherol, α-lipoic acid, melatonin, coenzyme Q_{10} and β-carotene (all from Sigma Chemical Co.) were dissolved with ethanol and used immediately. The final concentration of ethanol was below 0.2%, which did not affect the results (data not shown). Tea polyphenols such as green tea extract (*Epigallocatechin gallate*; Sigma Chemical Co.) and black tea extract (*Theaflavin gallate*; Sigma Chemical Co.) were dissolved with water by boiling for 30 s and used immediately. Pycnogenol (tablet form; Sundown

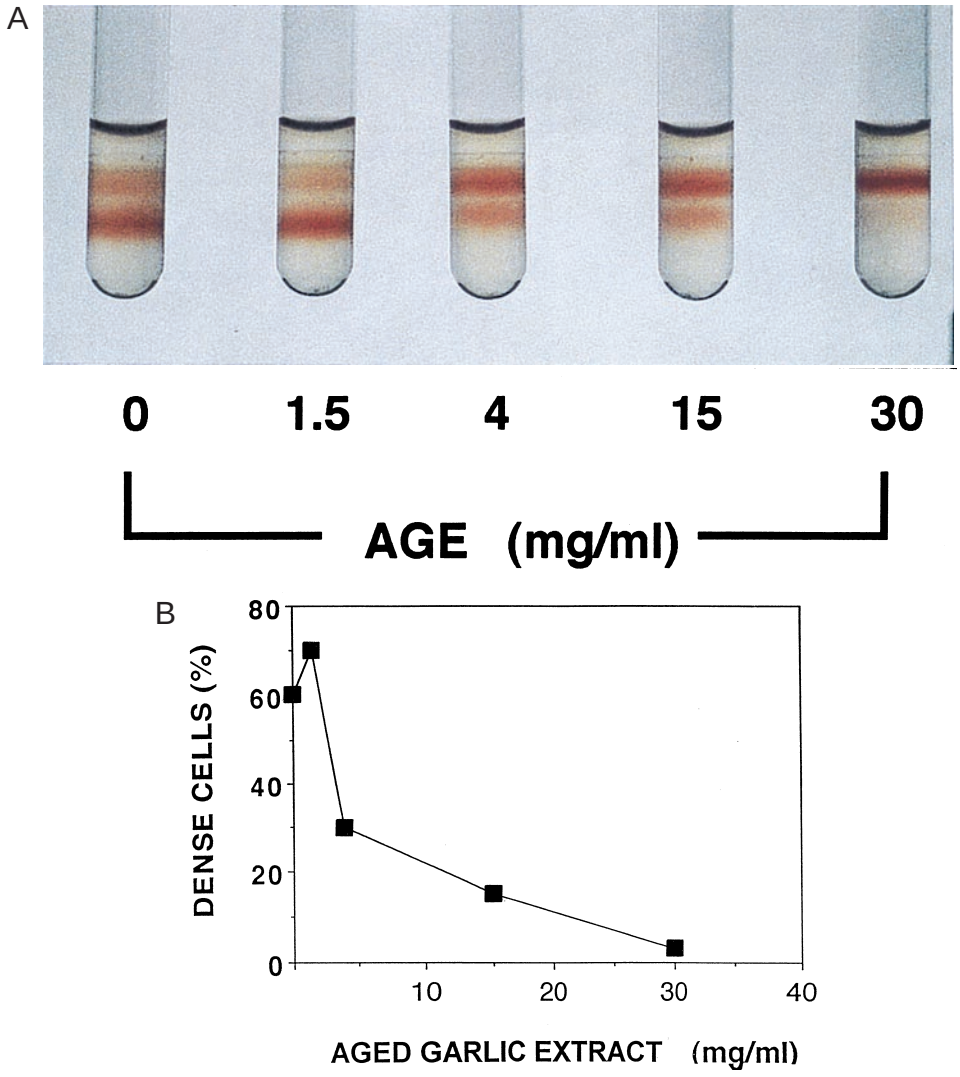

FIG. 1. (A) Percoll-diatrizoic-acid density gradient centrifugation showing the formation of dense cells (bottom layer) and its inhibition by AGE. (B) Dose-response curve of the inhibition by AGE. Sickle cell suspensions (hematocrit value = 2%) were exposed to five deoxy-oxy cyclings at 37°C. Each data point represents the average of two measurements. AGE, aged garlic extract.

Vitamins, Boca Raton, FL, USA) was purchased from a pharmacy. A tablet was crushed and homogenized in water with a teflon-glass homogenizer, boiled for 30 s, and centrifuged, and the supernatant was used immediately. Ginseng and ginkgo biloba (both in liquid extract form; Windmill Consumer Products, West Caldwell, NJ, USA) were purchased from a pharmacy and used as furnished. All other chemicals were obtained from Sigma Chemical Co.

Nutritional Supplements for Oral Administration

For the ex vivo tests and clinical trials, 600-mg capsules of AGE were used. Vitamin C (ascorbic-acid tablets) and vitamin E (D-α-tocopherol capsules) were purchased from Windmill.

Statistical Analysis for the Clinical Trials

The hematocrit (HCT) data for the non-supplement and supplement groups were analyzed using analysis of variance, and the statistical significance between measurements was assessed by Fisher's protected least-significant difference test. The numbers of crisis of patients in both groups during the 6-mo trial were averaged, and the statistical significance between the two groups was assessed by Student's *t* test. The feeling of energy and vitality was analyzed by a nonparametric rank test (Mann-Whitney). All analysis was done using a computer and software. $P < 0.05$ was considered significant.

RESULTS

In Vitro Tests

Figure 1A shows the formation of dense cells by the D-O cycling as revealed by density gradient centrifugation. The appearance of a bottom layer (heavier layer) indicates the formation of dense cells, which was inhibited by the addition of AGE. Figure 1B shows the dose-response curve of inhibition by AGE. Figure 2A and B shows inhibition by S-allyl cysteine and fructosyl arginine, both of which are water-soluble antioxidant components of AGE. Figure 2C shows examples of inhibition by Pycnogenol, green tea extract, and black tea extract, all of which were less water soluble, because we could obtain more concentrated stock solutions by

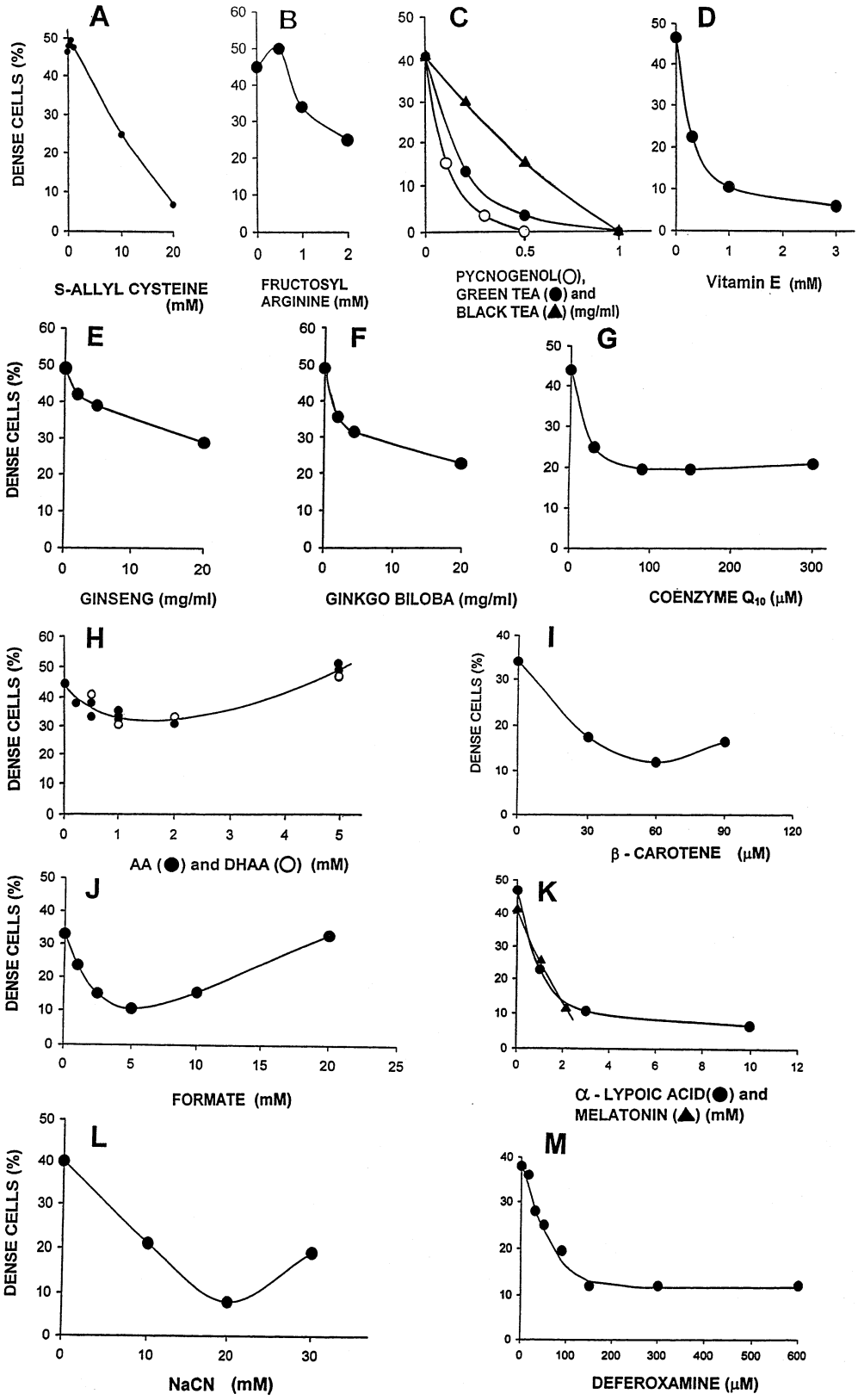

FIG. 2. Dose-related inhibition by five different types of inhibitors, each of which was added 20 min before the start of deoxy-oxy cycling. Type A (strong inhibitors): (A) S-allyl cysteine, (B) fructosyl arginine, (C) Pycnogenol extract, green tea extract, and black tea extract, and (D) vitamin E. Type B (medium inhibitors): (E) ginseng extract, (F) Ginkgo biloba, and (G) coenzyme-Q_{10}. Type C (biphasic inhibitors): (H) ascorbic acid (filled circle) and dehydroascorbic acid (open circle) and (I) β-carotene. Type D (hydroxyl radical scavengers): (J) formate and (K) α-lipoic acid and melatonin. Type E (iron-binding agents): (L) NaCN and (M) deferoxamine. Sickle cells were exposed to five deoxy-oxy cyclings at 37°C.

boiling the compounds in water for 30 s. Vitamin E was an example of a lipid-soluble inhibitor (Fig. 2D). Each of these compounds could completely inhibit dense-cell formation if we added a sufficient dosage. Therefore, we categorized them as TYPE A (STRONG INHIBITORS). Other types of inhibitors are described below.

TYPE B (MEDIUM INHIBITORS). Figure 2E shows the effect of ginseng (glycerin suspension). The effect of ginkgo biloba is shown in Figure 2F. Because ginkgo biloba was in a glycerin–polyethylene glycol suspension, it contained both water-soluble and lipid-soluble components. Figure 2G shows the effect of coenzyme Q_{10}, a lipid-soluble compound.

TYPE C (BIPHASIC INHIBITORS). Figure 2H shows the effect of ascorbic acid (vitamin C, denoted as AA) and dehydroascorbic acid (DHAA; both from Sigma Chemical Co.). Both compounds inhibited the formation at concentrations less than 1 mM but enhanced formation at 5 mM. The prooxidant effect of AA on sickle red cells has been reported.[29,30] The red cell membrane has a glucose transporter that can transport DHAA but not AA. Therefore, AA is oxidized at the membrane to become DHAA, transported into the cell, and reduced by glutathione to return to AA.[31] Thus, it consumes cellular antioxidant capacity and may contribute to the apparent prooxidant activity of AA at its higher level. A lipid-soluble β-carotene behaved similarly (Fig. 2I). It was known that β-carotene could behave as either an antioxidant or a prooxidant.[32]

TYPE D (HYDROXYL RADICAL SCAVENGERS). Figure 2J shows inhibition by a water-soluble scavenger, formate.[33] This is not a supplement, but it was used to study the mechanism of dense-cell formation.[34,35] Figure 2K shows the effects of lipid-soluble scavengers, α-lipoic acid[36] and melatonin.[37] Because melatonin is a hormone that controls the sleep cycle, it may not be appropriate for sickle cell therapy.

TYPE E (IRON-BINDING AGENTS). Thus far, we tested only water-soluble agents. As shown in Figures 2L and 2M, both NaCN (iron ligand) and deferoxamine (DFO; iron chelator) inhibited dense-cell formation. NaCN was used only for study purposes. DFO has been reported to have a lipid chain-breaking antioxidant activity independently of its iron-chelating property.[38] DFO is not a nutritional supplement but has been used in iron-chelation therapy by means of intravenous or continuous subcutaneous administration.[39,40]

To examine whether oxidative reactions are really involved, we measured lipid peroxidation of the sickle cell membrane in terms of thiobarbituric acid–reactive substances.[41] As shown in Figure 3A, a 6-h D-O cycling (30 cycles) produced lipid peroxidation in the membrane. Both water-soluble (AGE and vitamin C) and lipid-soluble (vitamin E and α-lipoic acid) compounds inhibited the reaction.

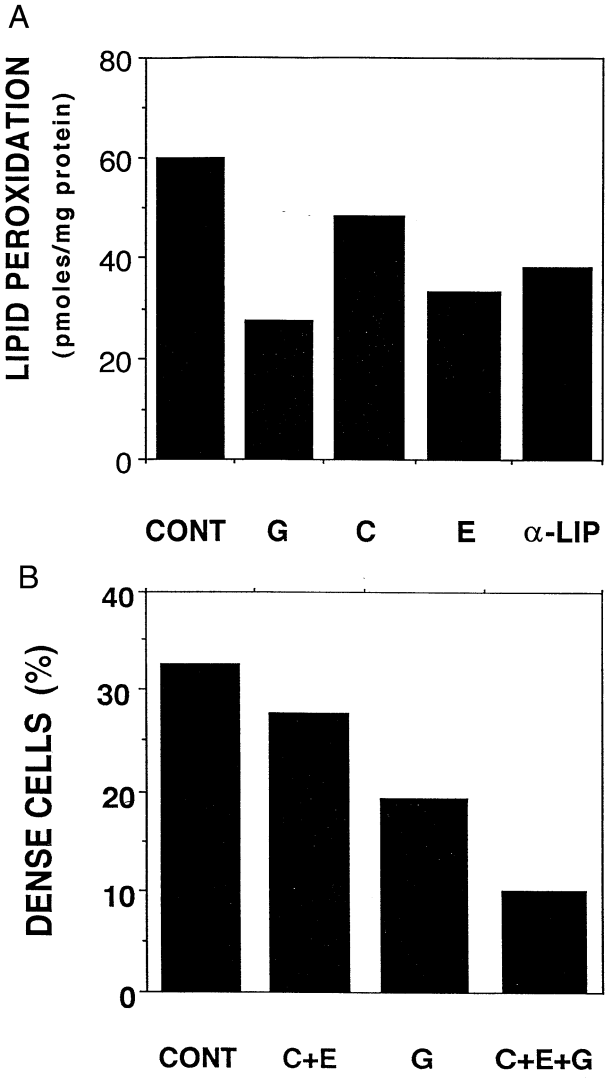

FIG. 3. (A) Lipid peroxidation caused by in vitro deoxy-oxy cycling and its inhibition by different supplements. Sickle cell suspensions (hematocrit value = 5%) were exposed to 30 deoxy-oxy cyclings (for 6 h) at 37°C in the absence and presence of supplements. Their ghosts were then prepared, and the amount of thiobarbituric acid-reactive substances (TBARS) measured using a fluorescence method.[41] The ordinate is expressed in picomoles malondialdehyde per milligram of ghost protein. CONT, control (no addition); G, 10 mg/mL of AGE; C, 1 mM of vitamin C; E, 1 mM of vitamin E; α-LIP, 1 mM of α-lipoic acid. Each bar represents the average of two measurements. (B) Inhibition of in vitro formation of dense cells in the sera that was withdrawn from a volunteer with normal hemoglobin. The volunteer had taken different supplements for 5 d for each experiment. The following daily doses were used. CONT, none; C, 4 g of vitamin C; E, 800 IU of vitamin E; G, 6 g of AGE. Each bar represents the average of two measurements. AGE, aged garlic extract.

Ex Vivo Test

The next issue to be addressed is whether these compounds could bring beneficial effects to SCA patients. To answer this question, we devised a new ex vivo method to assess the serum concentrations of antioxidants and their possible efficacy. In this experiment, one of the authors who has type AB blood took relatively large doses of supplements for 5 d on three separate occasions, i.e., daily doses of 1) 4 g of vitamin C (pure AA tablet) and 800 IU of vitamin E (D-α-tocopherol acetate); 2) 6 g of AGE; or 3) a combination of all compounds. Blood was then withdrawn, and the serum separated by centrifugation (2000*g* for 10 min). Using this serum as a reaction medium, D-O cycling of sickle red cells was performed. As shown in Figure 3B, the serum was able to inhibit dense-cell formation in vitro, and the combination of all supplements had the most remarkable effect. The data suggested that a cocktail of large daily doses of different types of antioxidants would have beneficial effects on SCA patients.

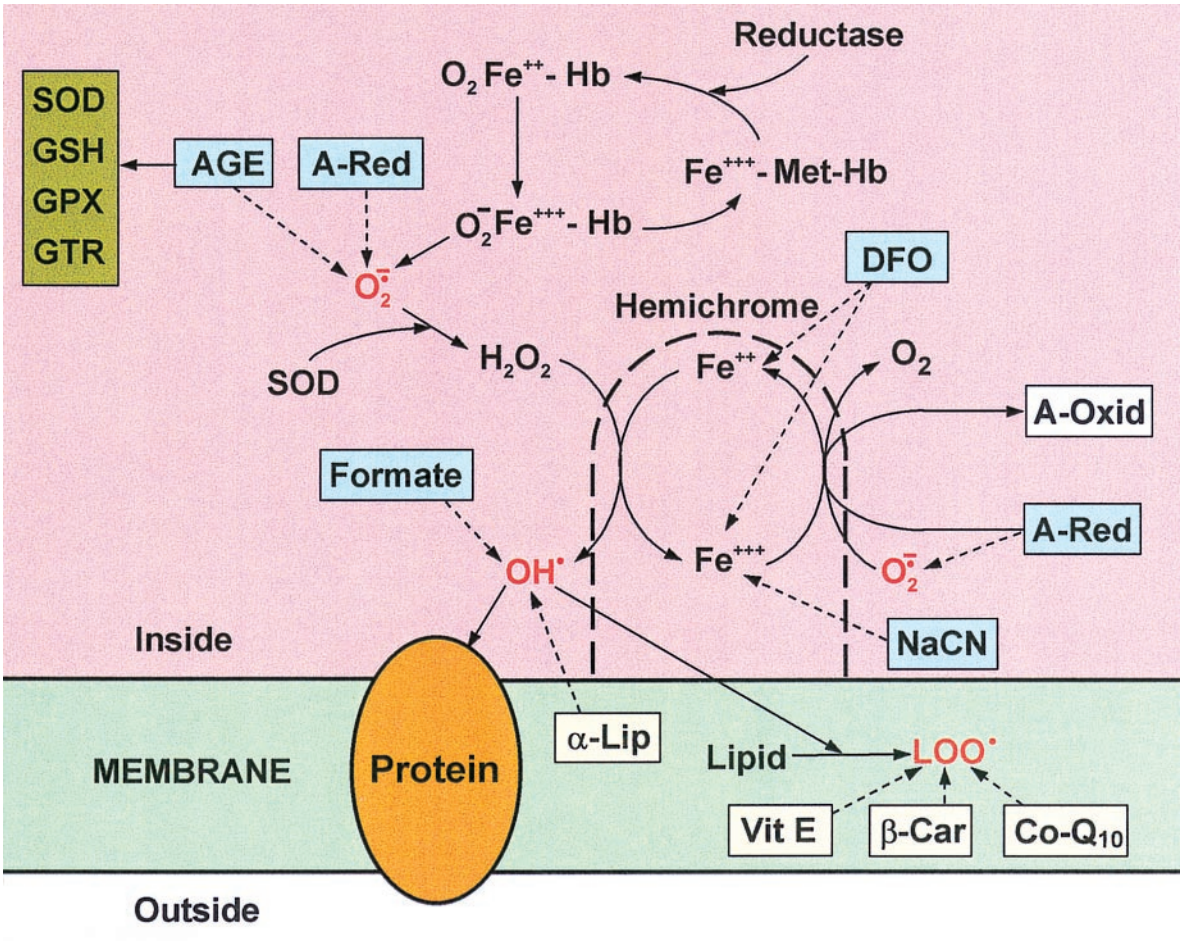

FIG. 4. Schematic presentation of the mechanism of dense-cell formation triggered by superoxide (O_2^-), hydroxyl radical ($OH^\cdot$), and lipid peroxide ($LOO^\cdot$) and the site of action of different inhibitors. "A-oxid," oxidized form of water-soluble antioxidant; "A-red," reduced form of water-soluble antioxidant; DFO, deferoxamine; GSH, glutathione; GPX, glutathione peroxidase; GTR, glutathione S-transferase; Hb, hemoglobin; SOD, superoxide dismutase. Solid arrows show the direction of reactions, and dotted arrows indicate the sites of inhibitors' action.

Pilot Clinical Trial

Encouraged by this ex vivo test, we launched a small pilot study in collaboration with Dr. B. Balogun at the University Health Services, University of Ibadan. Twenty adult homozygous (S/S) patients (age 18–26 y) were assigned to two groups, each having six males and four females. All patients were given 1 mg of folic acid everyday. Ten patients did not take anything else (non-supplement group). Ten patients (supplement group) orally took daily doses of 6 g of AGE, 6 g of vitamin C, and 1200 IU of vitamin E. This total dose was divided and administered four times a day, at each meal time and at bedtime. This regimen was tolerated well by all participants.

The hematocrit value (HCT) of the supplement group was 20.5 ± 0.22 before the trial but increased during the 6-mo trial to 24.6 ± 0.69 ($P < 0.001$), which was an increase of 20%. HCT of the non-supplement group was 20.2 ± 0.65 but did not change significantly (21.2 ± 0.49) during the test period. At the end of the 6-mo trial, HCT of the supplement group was significantly higher than that of the non-supplement group ($P < 0.001$). During the 6-mo trial period, the average number of painful crises per individual in the supplement group (0.4 ± 0.22) was 36% of that in the non-supplement group (1.1 ± 0.28), but the significance of difference ($P = 0.063$) barely missed the significant level of $P = 0.05$.

DISCUSSION

Our in vitro data suggest that nutritional supplements, hydroxyl radical scavengers, and iron-binding agents inhibited dense-cell formation. The mechanisms of action of these compounds are shown schematically in Figure 4. A small fraction of oxygenated hemoglobin is oxidized to produce superoxide anions and methemoglobin, which is reduced by methemoglobin reductase.[29,34] Superoxide anions are dismuted spontaneously or by superoxide dismutase to form H_2O_2. Then, in the presence of iron, which is abundant in sickle red blood cells as hemichrome or Heinz bodies,[35,42] an iron-catalyzed, superoxide-driven Fenton reaction takes place to produce hydroxyl radicals.[30,35] The radicals, which are highly reactive, subsequently attack ion-channel proteins, Ca-Mg-ATPase protein,[43] and/or phospholipids. This would disturb the ion balance of sickle cells to form dense cells.[11–16,44–46] The sites of action of different types of inhibitors are also shown in Figure 4.

Type-A water-soluble inhibitors (Figs. 1, 2A, and 2B) have the interesting feature that they enhanced dense-cell formation at low concentrations. This could be explained by the antioxidant-driven Fenton reaction because Fe^{3+} may have a high affinity with antioxidants (as shown by A-Red in Fig. 4). However, when the concentration of antioxidants increases, they scavenge superoxide, thereby inhibiting the production of hydroxyl radicals. The involvement of the iron-recycling Fenton reaction was shown by the

inhibitory actions of NaCN and DFO, where NaCN[33] binds with Fe^{3+} and DFO[35] chelates with both Fe^{2+} and Fe^{3+}. DFO has been reported not to enter red cells.[30] It is possible that it entered the cells while the membrane was disturbed by sickling. Supplements such as Pycnogenol, tea extracts, ginseng, and ginkgo biloba are not shown in Figure 4, but they are believed to react with these inhibitory sites.

As a test of the validity of this scheme, we measured lipid peroxidation of the sickle cell membrane. As shown in Figure 3A, the D-O cycling caused lipid peroxidation of the sickle cell membrane in vitro, and the addition of antioxidants or hydroxyl radical scavengers decreased the production of lipid peroxidation.

Our pilot trial was not a double-blind study, the number of patients was small, and the length of the trial was short. Nevertheless, we found that the HCT of the supplement group increased by 20% ($P < 0.001$) during the 6-mo trial period. This increase is remarkable because the increase of hemoglobin content (to which HCT is approximately proportional) produced by 2 y of hydroxyurea administration was 15.4%.[6]

The average number of painful crises in the supplement group was decreased to 36% of that in the non-supplement group. Unfortunately, the statistical significance in the crisis analyses was $P = 0.063$, which barely missed the significant level of $P = 0.05$. This was caused by the small number in our test ($N = 10$). For example, if we had been able to employ 20 subjects, a similar result would have reached the level of $P < 0.01$. In a hydroxyurea trial in adults (which enrolled patients with a history of three or more crises a year), the yearly average number of crises per individual in the placebo group was 4.5, whereas that in the test group was 2.5 ($P < 0.01$); the average number of crises in the hydroxyurea group was 55.5% of that in the placebo group.[5] We had high hopes that the nutritional therapy would be as effective as hydroxyurea therapy.

An interesting observation was that most of the patients in the supplement group felt much more energetic after they had started the regimen. The feeling of energy and vitality in the non-supplement group was 0 (the same as before, eight patients), 1 (feeling a little better, two patients), and 2 (feeling much better, no patients). In contrast, for the supplement group, the result was 0 (two patients), 1 (two patients), and 2 (six patients). The difference between the two groups was significant ($P < 0.01$). Because this was not a double-blind trial, we cannot rule out the possibility that there might have been some "placebo effect." However, the difference was so remarkable that we believe it cannot be explained by a mere placebo effect. Although this was a small-scale pilot trial, all observations favored the notion that a nutritional approach is a promising one for the management of this genetic disease. This study should be expanded.

A decade ago, Hebbel and others[34,35] and Rice-Evans and others[29,30] pointed out that oxygen-free radicals play a crucial role in the pathogenesis of SCA. Several investigators have performed clinical trials involving vitamin C and/or vitamin E, but no dramatic effects have been reported.[47–51] Why did previous trials not produce more significant results?

In our view, there were at least three reasons. First, the doses in the previous trials were not high enough. For example, the daily dose of vitamin C in the previous trials was less than 1 g, whereas ours was 6 g.

Second, vitamin C was not a strong inhibitor for dense-cell formation, but AGE was. The combination of strong inhibitors, i.e., AGE (water soluble) and vitamin E (lipid soluble) with a large dose of vitamin C, which supports the recycling of other antioxidants,[52] seems to be the key to our success. In other words, a "cocktail" of different types of nutritional inhibitors worked in a synergistic manner to produce a beneficial effect in SCA.

Third, AGE has additional pharmacologic effects beneficial to the patients, namely AGE increases cellular levels of superoxide dismutase, glutathione, glutathione peroxidase, and glutathione S-transferase,[53–56] all of which are important cellular defense components; and AGE inhibits platelet aggregation.[57] The latter property would help prevent the onset of painful crisis because platelets play a role in vasoocclusion.[58–59]

TABLE I.

PROPOSED DAILY DOSES OF NUTRITIONAL SUPPLEMENTS FOR ADULTS IN DIFFERENT DISEASES

	Hoffer and Pauling[60]*	Prasad et al.[61]†	Ohnishi et al.‡
N-acetylcysteine	—	500 mg	—
β-Carotene	25 000–50 000 IU	30 mg	—
Coenzyme Q_{10}	—	200 mg	—
NADH	—	10 mg	—
Vitamin B3 (non-flushing niacin)	1.5–3.0 g	—	—
Vitamin B6	250 mg	—	—
Other vitamin B	25–50 times the RDA	—	—
Vitamin C	12 g	4 g	2–4 g
Vitamin E	800 IU	600 IU	800 IU
Selenium	0.2–0.5 mg	0.2 mg	—
Zinc	50 mg	30 mg	—
Folic acid	5–10 mg	—	1 mg
Aged garlic extract	—	—	2–6 g
Tea polyphenols (or Pycnogenol)	—	—	200–600 mg (50–150 mg)

* For cancer.

† For Parkinson's disease.

‡ For sickle cell anemia (present study).

NADH, nicotinamide adenine dinucleotide (reduced form); RDA, recommended daily allowance.

We owe the idea of using high doses of several compounds to the work of Hoffer and Pauling who studied a nutritional approach for cancer therapy[60] (Table I). Prasad et al.[61] also proposed the combination of nutritional supplements to treat Parkinson's disease. They emphasized that any free radical scavenger could become a free radical when oxidized; thus, using a high dose of only one scavenger may be health hazardous. From the observations that vitamin C alone or vitamin E alone was not effective to treat Parkinson's disease, they proposed a regimen that combined many supplements[61] (Table I). Because the goal of Hoffer and Pauling was to apply this regimen to terminal cancer patients, their regimen had high doses of antioxidants and other supplements. In contrast, the regimen of Prasad et al. had much lower doses because of concern for safety. We used a cocktail of relatively high doses of vitamin C, vitamin E, and AGE because our goal in this pilot trial was to demonstrate that a combination of different antioxidants would have therapeutic efficacy.

Because our data suggest that the cocktail may be efficacious to SCA patients, our next goal will be to find appropriate doses that have reasonable efficacy with complete safety. The concentrations at which effective nutritional supplements could inhibit dense-cell formation by 50% in vitro were estimated from the data as follows: 4.0 mg/mL of AGE, 0.38 mg/mL of black tea extract, 0.13 mg/mL of green tea extract, 0.07 mg/mL of Pycnogenol, 930 μM of α-lipoic acid, 270 μM of vitamin E, 45 μM of coenzyme Q_{10}, and 32 μM of β-carotene. These values would help nutritionists design more effective regimens, although bioavailability, serum concentration, stability, and toxicity have to be considered.

To administer a large dose of vitamin C to patients with high iron levels would be of concern because of possible free radical production. Although it has been reported that a large dose of vitamin C does not cause a problem in such patients,[62] we still

would like to reduce the daily dose of vitamin C. We believe that vitamin C could be replaced in part by either tea extracts (tea polyphenols) or Pycnogenol because these have potent inhibitory activity, as shown in Figure 2C. Pycnogenol, which was extracted from the bark of French maritima pine because of its strong vitamin C–like activity, was found to belong to procyanidines consisting of monomers and oligomers of catechin and epicatechin units and to have biologic activities.[63,64] Our proposed cocktail for SCA, which we are planning to test, is shown in Table I. Our final goals are to improve the efficacy and long-term safety of our cocktail by incorporating multiple supplements and to establish a safe regimen for administration to infants and children.

We hope that our nutritional approach does not cause serious side effects like other chemotherapeutic agents. A combination of a nutritional approach with hydroxyurea therapy is another avenue worth investigating. In addition to SCA, our cocktail may be efficacious in other diseases linked to "oxidative stress" including cancer and Parkinson's disease.

In summary, our in vitro, ex vivo, and pilot clinical studies point toward a new direction of sickle cell therapy targeting the protection of the red blood cell membrane from oxidative injury. The method employs a cocktail of nutritional antioxidants, an approach to which Linus Pauling had made a pioneering contribution.

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