In Vitro Effects of Aged Garlic Extract and Other Nutritional Supplements on Sickle Erythrocytes

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ABSTRACT In the circulation of sickle cell anemia patients, a certain population of erythrocytes has an elevated density. These abnormally dense cells are believed to be at the root of the painful crisis and anemia of the patients. We have developed an in vitro method for the preparation of these heavier erythrocytes by a repeated deoxy-oxy cycling of erythrocytes from sickle cell anemia patients. By using this method, we studied whether certain nutritional supplements would inhibit the formation of dense cells in vitro. It was found that aged garlic extract (AGE) as well as its components with antioxidant activity, i.e., S-allylcysteine and Nα-(1-deoxy-D-fructos-1-yl)-L-arginine (fructosyl arginine), inhibited the formation of dense cells in vitro. Vitamin C, vitamin E and the spin-trapping agents, 5-diethoxyphophoryl-5-methyl-1-pyrroline-N-oxide and α-(4-pyridyl-1-oxide)-N-t-butylnitrone were all found to inhibit the formation of dense cells in vitro. These results suggest that, when extremely stretched sickle-shaped cells are formed by the repeated deoxy-oxy cycling, the erythrocyte membrane becomes susceptible to oxidative injury by reactive oxygen species. The protection of the erythrocyte membrane from such an oxidative injury would prevent the membranes from becoming leaky to the calcium ion, thus inhibiting the activation of the calcium-activated potassium efflux channel and the formation of dense cells. We also developed a new ex vivo method of studying the possible efficacy of antioxidants taken orally on the dense cell formation in sickle cell patients. It involved the use of blood plasma taken from a healthy donor (with normal hemoglobin) of AB blood type who had consumed different types of antioxidants orally. By suspending sickle erythrocytes in such plasma and exposing them to the deoxy-oxy cycling, the degree of dense cell formation was determined. The degree of inhibition in vitro by antioxidants taken orally may be related to their efficacy in inhibiting dense cell formation in the patients. On the basis of these in vivo and ex vivo studies, we propose that a cocktail of antioxidants would have beneficial effects in lessening the incidence and severity of crisis and reducing anemia in sickle cell disease.

KEY WORDS: • sickle cell anemia • deoxy-oxy cycling method • oxidative injury • free radicals
• antioxidants • aged garlic extract • S-allylcysteine • fructosyl arginine

Sickle cell anemia is a genetic disease caused by abnormal hemoglobin (called sickle hemoglobin; HbS),3 which polymerizes under physiologically encountered deoxygenated con-

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3 Abbreviations used: AGE, aged garlic extract; DEMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide; DMPO, 5,5-dimethylpyrroline-N-oxide; D-O cycling, deoxy-oxy cycling; fructosyl arginine, Nα-(1-deoxy-D-fructos-1-yl)-L-arginine; HbF, fetal hemoglobin; HbS, sickle hemoglobin; POBN, α-(4-pyridyl-1-oxide)-N-t-butylnitrone; ROS, reactive oxygen species; RSC, reversibly sickling cells; SAC, S-allylcysteine.

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suffers from sickle-cell disease. In Africa, 1 of 50 people suffers, and their average life span is < 20 y. Currently, the only therapy that provides some relief to these patients is oral administration of hydroxyurea. This compound increases the content of fetal hemoglobin (HbF), which does not polymerize like HbS. This compound has been shown to have beneficial effects in patients, but it may have side effects including neutropenia and possible influence on brain development. Therefore, long-term administration, especially to children, should be carefully monitored.

Ohnishi (1983 and 1986) developed a method to prepare dense cells and permanently deformed cells (irreversibly sickled cells) in vitro by a repeated deoxy-oxy cycling method. Subsequently, his group found that a cause for the formation of dense cells was calcium-activated potassium efflux and concomitant dehydration (Ohnishi et al. 1989). This method provided a tool with which to study the mechanisms of formation of dense cells and methods of inhibiting their formation. This line of study would be clinically important because such dense cells seem to play a key role in inducing the painful sickle cell crisis (Ballas and Smith 1992, Fabrey et al. 1984). By using this method, we have shown that several membrane acting drugs, calcium entry blockers and inhibitors of the calcium-activated potassium channel could inhibit the formation of dense cells in vitro (Ohnishi et al. 1986a, 1986b and 1989).

Ohnishi (1994b) further proposed that protecting the RBC membrane from oxidative stress may improve the clinical manifestations of the patients. Because sickle cell anemia is a genetic disease, any medication would have to be taken for life. This complicates enormously the development of an effective drug therapy with minimal side effects. Thus, we undertook this study to develop a “nutritional” means to manage the disease, namely, to find a food or a food supplement that is safe and efficacious in protecting the sickle cell membrane. The patients would be able to take it daily for life and would benefit from less frequent and less severe crises and less anemia. In this paper, we propose the possibility that aged garlic extract (AGE) may be such a food supplement.

**MATERIALS AND METHODS**

**Nutritional compounds used in vitro studies.** AGE, S-alllylcysteine (SAC) and Nα-(1-deoxy-d-fructos-1-yl)-l-arginine (fructosyl arginine) were provided by Wakunaga Pharmaceuticals (Mission Viejo, CA). Because AGE contains a small amount of alcohol, it was dried under vacuum and suspended with the reaction medium (see below for the content) used for the experiments; the pH was adjusted to 7.4 after equilibration with 95% N2/5% CO2. Both SAC and fructosyl arginine were dissolved into the reaction medium to a concentration of 20 g/L and the pH adjusted to 7.4 under 95% N2/5% CO2. These compounds were stored at ~80°C. Ascorbic acid (vitamin C) and dill-α-tocopherol acetate (vitamin E) were purchased from Sigma Chemical (St. Louis, MO). The latter was dissolved in ethyl alcohol.

**Nutritional compounds used in ex vivo studies.** Kyolic capsules (300 mg AGE/capsule; Wakunaga), vitamin C tablets (1000 mg/tablet) and Vitamin E soft gels (200 IU dill-α-tocopherol/gel) were purchased from a health food store.

**Chemicals.** Percoll solution was purchased from Pharmacia (Piscataway, NJ). Spin trap agents were obtained as follows: 5,5-dimethylpyrroline-N-oxide (DMPO) and α-(4-pyridyl-1-oxide)-N-t-butylnitroxide (POBN) were from Sigma Chemical; 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) was from Oxis International, (Portland, OR). All other chemicals were purchased from Sigma Chemical.

**Solutions.** The reaction medium consists of: 109 mmol/L NaCl, 6 mmol/L KCl, 5 mmol/L CaCl2, 1.2 mmol/L MgSO4, 20 mmol/L HEPES buffer, 25 mmol/L NaHCO3, 2.4 mmol/L NaH2PO4, 1 mmol/L adenosine, 1 mmol/L inosine, 10 mmol/L glucose, 0.05 g/L each of penicillin and streptomycin and 2% bovine serum albumin. The osmolality of the medium was adjusted to 290 mOsm/kg and the pH was adjusted to 7.4 after equilibration with 95% air/5% CO2. Because the sickling is pH sensitive and the addition of testing compounds tends to change pH, the pH of the reaction medium was always readjusted to 7.4 ± 0.01 under gas flow of 95% air/5% CO2 after the addition of any testing compounds.

A Percoll stock solution was prepared as follows: 117 mmol/L NaCl, 20 mmol/L KH2PO4, 0.005 g each of penicillin and streptomycin were added to a 100-mL Percoll solution. The pH was adjusted to 7.4. The osmotic pressure of this solution was usually ~290–300 mOsm as determined by a freezing point method. If the value did not fall between these values, the osmolality was readjusted. The density as determined with a picnometer was ~1.1365.

A diatrizoic acid stock solution (renografin solution) was prepared as follows: 115 mmol/L diatrizoic acid was added to 80 mL water and 8 mL of 2 mol/L NaOH was added to solubilize the compound. Then, 20 mmol/L KH2PO4 was added and the pH adjusted to 7.4 with 1g HCl. Finally, the volume of the solution was brought to 100 mL. The osmolality was ~290–300 mOsm/kg. Again, if the value did not fall inside that range, it was adjusted. The density was ~1.0475.

A density gradient solution with the density of 1.104 was prepared by mixing 1 volume of the Percoll stock solution and 0.575 volume of the diatrizoic acid stock solution.

**Blood.** For in vitro tests, blood was obtained from adult sickle cell patients (using citrate-phosphate-dextrose-adrenaline as an anticoagulant); in these patients, the hemoglobin F content is < 1% and the content of irreversibly sickled cells is < 10%. The blood could be used for ~1 wk as long as the air space of the container was equilibrated with 95% air/5% CO2 and the container was gently tumbled (1 to 2 rpm) at 4°C (Ohnishi et al. 1983, Ohnishi 1994a).

The RBC were separated into a light density fraction (density < 1.104) and heavy dense cells (density > 1.104) by a Percoll-renografin density gradient centrifugation as described below (Ohnishi 1983, 1986 and 1994a). Then, the RBC in the lighter layer (reversibly sickling cells; RSC) were collected, washed with the reaction medium, resuspended in the same medium and used for the experiments.

For ex vivo tests, blood was withdrawn from one of the authors (using heparin as an anticoagulant) and the plasma was separated with centrifugation (2000 g for 10 min) and stored at ~80°C. When the stored plasma was thawed, a small amount of aggregates had formed and was removed by a light centrifugation (600 rpm for 5 min).

**Deoxy-oxygen (D-O) cycling method.** Washed RSC were suspended in the reaction medium to obtain a hematocrit value of 1–2%. Then, 1-mL aliquots of this suspension were added to 8 test tubes, rotating at 27 rpm by a motor-gear mechanism in a thermostatic bath at 37°C ± 0.2°C (Fig. 1). Using a timer, two gas mixtures (95% N2/5% CO2 and 95% air/5% CO2) were alternately flashed inside each tube at the flow rate of 30 mL/(min × tube). The period of cycling was 9 min 40 s for N2/CO2 and 2 min 20 s for air/CO2 (Ohnishi 1994a).

**Density gradient separation.** The density gradient solution (1g/mL) was poured into a 10 × 75-mm glass test tube and inserted into a plastic centrifuge tube (i.d. 12.5 mm, length 100 mm) to which 2.5 mL water had been added to help support the test tube. Then, the tubes were spun at 11,000 × g for 5 min, which automatically formed a density gradient. Then, 50 μL of a blood suspension that had been exposed to the D-O cycling was layered on top of the preformed density gradient, and was spun for 10 min at 2000 g using a low speed centrifuge with a swing rotor. As shown in Figure 2A, if the blood suspension undergoes only deoxygenation or only oxygenation, it forms only a single layer (top layer). However, if the suspension is exposed to repeated D-O cycling, two layers are formed. The bottom layer has a higher density, and is referred to as “dense cells.” Each layer was carefully collected, wash-centrifuged once with the reaction medium, and the precipitates were hemolyzed with a solution containing 5 mmol/L sodium phosphate buffer (pH 7.4), 1 mmol/L EDTA and 0.3% Brij35. From the hemoglobin content of each layer
as measured spectrophotometrically, we obtained the percentage of RBC in the bottom layer.

When sickle red cells were exposed to deoxygenation alone, we did not see the typical sickle shape, but rather, a “maple-leaf” shape (Fig. 2B, left photo). However, after exposure to repeated D-O cycling, the cells took on the typical sickle shape (Fig. 2B, middle photo) (Ohnishi 1983, 1986 and 1994a).

**Ex vivo method of testing the efficacy of oral intake of antioxidants.** This method involves the intake of antioxidants by one of the authors (nonsickler) whose blood type was AB. After several days of consuming the particular nutritional supplement(s), blood was withdrawn and the plasma separated by centrifugation at 2000 g for 10 min. Using the plasma as a “reaction medium,” the D-O cycling of reversible sickle cells was performed. The blood with AB type has an advantage in that it could accept other types of blood. The inhibition of the formation of dense cells in such plasma may be related to the efficacy of oral intake of antioxidants in inhibiting the in vivo formation of dense cells in patients. The following combination of nutritional supplements were tested: 1) no drugs; 2) intake of daily doses of 4 g vitamin C + 800 IU vitamin E; 3) 6 g AGE; and 4) 4 g vitamin C + 800 IU vitamin E + 6 g AGE. The daily doses were split into four parts; each was taken orally 4 times a day, i.e., after each meal and at bed time.

**Human subjects.** This study was performed in accordance with the revised Declarations of Helsinki, and the protocol was approved by the Institutional Review Board of the Philadelphia Biomedical Research Institute. All participants provided written informed consent before the study.

**RESULTS**

**Inhibition of the formation of dense cells by AGE and other compounds.** Figure 3 shows the result of density gradient centrifugation in which different concentrations of AGE.

**FIGURE 1** A schematic illustration of the method to prepare dense cells in vitro by repeated deoxy-oxy cycling. 1) Stirring device for thermostatic bath; 2) motor; 3) gear system; 4) rotating test tube (27 rpm); 5) teflon tubing; 6) individual humidifier; 7) flow control valve; 8) manifold; 9) flow switch; 10) timer; 11) flow meter; 12) a humidifier for each gas.

**FIGURE 2** (A) A density gradient experiment showed that neither deoxygenation nor oxygenation alone produced dense cells, but deoxy-oxy (D-O) cycling did. (B) Scanning electron microscopy showed that deoxygenation produced “maple leaf-shaped” cells, whereas the D-O cycling produced “sickle-shaped” cells.

**FIGURE 3** An example of dense cell formation (as seen by the formation of the “bottom layer”) and its inhibition by aged garlic extract (AGE). AGE was added to each tube 30 min before the start of deoxy-oxy (D-O) cycling. The concentrations of AGE were (from left to right) 0, 3, 6 and 20 g/L, respectively.
(from 0 to 20 g/L) were added to blood suspensions and incubated for 30 min before the start of the D-O cycling. The percentage of dense cells (bottom layer) decreased when the concentration of AGE increased. Figure 4 shows dose-related inhibition of dense cell formation by AGE, SAC and fructosyl arginine. The metal element, zinc, was reported to decrease the content of irreversibly sickled cells in patients (Brewer et al. 1977, Muskiet et al. 1991). However, in this in vitro test, zinc had no effect.

Figure 5 shows the inhibitory effects of 0.6 mmol/L vitamin C, 0.05 mmol/L vitamin E and 20 mmol/L NaCN on dense cell formation. Figure 6 presents the effects of watersoluble spin-trapping agents, such as DMPO, POBN and DEPMPO (all 50 mmol/L) on dense cell formation. DMPO had no effect, POBN inhibited to some degree, but DEPMPO almost completely inhibited dense cell formation. Figure 7 shows the results of ex vivo experiments in which one of the authors had consumed different nutritional supplements orally in the following manner for 5 d: 1) daily doses of 4 g vitamin C + 800 IU vitamin E; 2) 6 g AGE (daily); and 3) a combination thereof. Using his plasma as a “reaction medium,” an in vitro a D-O cycling test was performed. As shown in the figure, the “cocktail” containing all compounds had the greatest inhibitory activity.

DISCUSSION

After the discovery of Pauling et al. (1949) that sickle cell anemia is caused by genetically abnormal hemoglobin (HbS), many therapies were proposed. Most of the search centered around finding an agent that would bind with HbS to inhibit the deoxygenation-induced polymerization. However, because there is an enormous amount of hemoglobin in the human...
body, it has been difficult to find a compound that inhibits the polymerization, but has no serious side effects.

When one of the authors (STO) started working on this topic, he was puzzled by one simple question: "In the patients circulation, red blood cells could take a 'sickled' shape. But when the same red blood cells were deoxygenated in vitro, they took a 'maple-leaf' shape. Why?" In an attempt to prepare typical sickle-shaped cells in vitro, he found that repeated deoxygenation-oxygenation (D-O cycling) of sickle RBC formed elongated sickled cells with an elevated density (Ohnishi 1983, 1986 and 1994a). He interpreted this as follows: if polymerized HbS (during the deoxy phase) is depolymerized in the subsequent oxy phase, the polymers do not depolymerize completely to single Hb molecules, but stay as many oligomers. Then, when the next deoxy phase occurs, all of these oligomers serve as nuclei for polymerization, thus enhancing the speed and degree of polymerization. As a result, after repeated D-O cycling, a bundle of elongated HbS fibers is formed whose length is larger than the diameter of the RBC. Thus, red cells were stretched to form "sickle-shaped" cells instead of "maple leaf-shaped" cells (Ohnishi 1986). When these cells were oxygenated, they still had a normal, biconcave shape. However, their density had already increased, showing that dehydration took place as a result of repeated stretching of the cell membrane. It was also found that if D-O cycling was continued for several hours, the density increased further, leading to the formation of permanently deformed cells known as irreversibly sickled cells (Ohnishi 1983).

Because RBC with higher density are believed to play an important role in the clinical manifestations of these patients (Ballas and Smith 1992, Fabrey et al. 1984), research using this in vitro method may have significant clinical relevance. Therefore, in this paper, we used this method in the search for food and food supplements that are effective for the treatment of sickle cell patients.

We focused on the effects of reactive oxygen species (ROS) on dense cell formation. ROS (superoxide, hydroxyl radical and other oxygen-derived free radicals) have serious adverse effects and are linked to the cause of many diseases such as cancer, arthritis, stroke, heart diseases, diabetes and arteriosclerosis. Sickle cell patients have decreased levels of vitamin E and glutathione peroxidase (Chui and Lubin 1979), all of which are important compounds in the defense against oxygen free radical attack. The importance of vitamins E (Phillips and Tangney 1992) and C (Lachant and Tanaka 1986) in sickle cell anemia has been emphasized. Several investigators proposed that oxygen stress is a part of the disease (Hebbel et al. 1982, Hebbel 1986, Rice-Evans et al. 1986a). In sickle cell disease, hemoglobin has been found to degenerate and bind to the red cell membrane as hemichrome (Asakura et al. 1977).

There is a good possibility that hemichrome and/or membrane-bound iron could catalyze the production of hydroxyl radicals through the Haber-Weiss reaction, thereby damaging red cell membranes (Hebbel 1986, Rice-Evans et al. 1986a).

There is another observation to suggest that vitamin E could prevent the dehydration of sickle erythrocytes (Jain et al. 1989, Natta et al. 1980). Several nutritional approaches have been tried. The administration of zinc was suggested because it may have an effect on the calcium channel and/or hemoglobin synthesis. It was reported that zinc decreased the content of irreversibly sickled cells (Brewer et al. 1977). Effects of the combination of normal daily supplemental levels of vitamin E, vitamin C and zinc were studied (Musket et al. 1991), but the administration of all these compounds did not increase either the hematocrit value or the hemoglobin level of the patients.

By comparing sickle cell anemia with ischemia/reperfusion injury of the brain and other organs, we introduced the concept of sickle cell anemia as a "membrane-linked disease" (Ohnishi and Ohnishi 1994). It was suggested that we might be able to treat sickle cell patients with "membrane-protecting agents." The search for such compounds came from the study on the antioxidant activity of AGE (Kojima et al. 1994). Ohnishi and Kojima (1997) found that AGE has a strong antioxidant effect. Garlic is known to have various health benefits and has been used as a food or food flavoring for thousands of years. AGE has been marketed as a food supplement for many years and is known to be safe. Therefore, it may be safe for the patients to take AGE for their entire lives.

In this paper, we first found out that AGE and other components of AGE, such as SAC and fructosyl arginine, could inhibit the formation of dense cells. AGE and SACs have been known to have antioxidant activity. Fructosyl arginine does not contain sulfur molecules like many other garlic components, yet it was found to have antioxidant activity (Ryu et al. 2001). This strongly suggests that other antioxidants could also inhibit dense cell formation. Therefore, we tested other antioxidants such as vitamin C and vitamin E. They also inhibited dense cell formation. Although it was not an antioxidant, we tested zinc because oral zinc was reported to decrease the percentage of irreversibly sickled cells in patients (Brewer et al. 1977, Musket et al. 1991). However, in those studies, the administration of zinc did not produce beneficial effects in the patients. Therefore, we tested to see whether zinc decreases the formation of dense cells. As shown in Figure 4D, zinc had no effect in our in vitro test. Because zinc is not an antioxidant, it may not protect the membrane by a direct action.

The inhibition of dense cell formation by spin traps (agents used in electron spin resonance study to trap free radicals) supports the idea that free radicals are involved in dense cell formation. We tested three spin traps, DMPO, DEPMPO and POBN. All of them are water soluble. As shown in Figure 6, DMPO had no effect, but POBN inhibited dense cell formation to some degree, and DEPMPO was quite inhibitory. It is interesting to note that POBN is known as a good trapping agent for hydroxyl radicals, and DEPMPO is an efficient superoxide trapping agent. Further study is required to determine whether POBN and DEPMPO crossed the red cell membrane to trap these free radicals. Because they are small molecules, their molecular weights are between 200 and 300 with some lipophilic bases, they may actually have entered the red cells, possibly even more readily in the stretched, sickled state.

Because we found that antioxidants and spin-trapping agents could inhibit dense cell formation in vitro, we extended our work to perform an ex vivo study. Instead of a clinical trial which requires the involvement of sickle cell anemia patients, we used the blood of one of the authors who has the AB blood type (Rh positive). The AB blood type has the advantage that it requires the involvement of sickle cell anemia patients, our work to perform an ex vivo study. Instead of a clinical trial which requires the involvement of sickle cell anemia patients, we used the blood of one of the authors who has the AB blood type (Rh positive). The AB blood type has the advantage that it requires the involvement of sickle cell anemia patients, our work to perform an ex vivo study. Instead of a clinical trial which requires the involvement of sickle cell anemia patients, we used the blood of one of the authors who has the AB blood type (Rh positive). The AB blood type has the advantage that it requires the involvement of sickle cell anemia patients, our work to perform an ex vivo study.
lected, erythrocytes were obtained from a sickle cell patient, and D-O cycling experiments were conducted on these erythrocytes in isolated, stored plasma. The result showed that the "cocktail" of all nutritional supplements had the strongest inhibitory effect (Fig. 7). This ex vivo experiment does not actually reflect what would really occur when patients took these nutritional supplements. Because the sickle red cells were exposed to the plasma for 1–2 h during this in vitro test, antioxidants contained in the plasma could induce only a limited effect. Nevertheless, the fact that the formation of dense cells was remarkably inhibited by the oral cocktail of all compounds was very encouraging (Fig. 7).

On the basis of these results, we launched a pilot clinical trial with the collaboration of Dr. G. B. Ogunmola (Ibadan University, Ibadan, Nigeria), the director of a National Sickle Cell Project. Patients in Nigeria (n = 10) consumed daily an oral “cocktail” of 6 g vitamin C + 1200 IU vitamin E + 6 g AGE (the doses are divided into 4 parts, and each is taken after meals and at bed time). The hematocrit value of these patients increased by 20% after 6 mo of oral administration of the “cocktail” (Ohnishi et al. 2000).

Why were all previous clinical trials, which also employed nutritional supplements, not efficacious? We suggest two possible reasons. First, the literature reports that the maximum daily dosages given to the patients were 0.6 g vitamin C + 460 IU vitamin E (Muskiet et al. 1991). Perhaps, the doses were not sufficiently high to produce a beneficial effect.

Second, we used the concept of a “cocktail,” which is the combination of different antioxidants. Our inspiration for this idea came from the work of Hoffer and Pauling (1993) who proposed an antioxidant therapy for terminal cancer patients. They used a “cocktail” consisting of daily doses of 12 g Vitamin C, 800 IU vitamin E, 25,000 IU β-carotene, 0.2 g selenium, 220 mg zinc sulfate (which is equivalent to 50 mg elemental zinc) and vitamins B-3, B-6 and others, all of which are amounts 25–50 times higher than the recommended dietary allowances. With this regimen, these authors reported beneficial effects in 40% of 170 terminal cancer patients; their mean survival time was 4 times longer than that of the control group who did not take this cocktail (Hoffer and Pauling 1993).

The benefit of using a cocktail may be understood by a scheme presented in Figure 8. This depicts possible mechanisms involved in sickle cell anemia. As Hebbel (1986) and Rice-Evans et al. (1986b) proposed, cycling of hemoglobin and methemoglobin produces superoxide anions, which are dismuted either by superoxide dismutase or spontaneously to produce hydrogen peroxide. Superoxide and hydrogen peroxide are catalyzed by membrane-bound hemichrome (Asakura et al. 1977) and/or compartmented iron (Rank and Hebbel 1994) to produce hydroxyl radicals. All of these ROS would attack membrane lipid and proteins to enhance calcium entry and potassium exit (with water following) to form dense cells. It has been known that CN− inhibited the formation of hydroxyl radicals by inhibiting the redox cycling between Fe++ and Fe+++ (Hebbel et al. 1982). Because the addition of potassium ions itself could inhibit dense cell formation (Ohnishi et al. 1986a), we tested the effect of NaCN. As shown in Figure 5, it inhibited dense cell formation, suggesting that the redox cycling of iron seems to be involved in dense cell formation.

On the basis of this scheme, we speculate that the proposed
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“cocktail therapy” may have the following beneficial effects: 1) vitamin C would scavenge ROS in plasma, thus protecting the red cell membrane (Jain and Williams 1985); 2) vitamin E would inhibit membrane lipid peroxidation and protein oxidation; and 3) AGE would not only scavenge ROS in the aqueous phase, but also increase the levels of superoxide dismutase, glutathione, glutathione transferase and glutathione peroxidase mechanisms (Geng and Lau 1997, Hatono et al. 1996, Liu and Milner 1992, Wei and Lau 1998), all of which are important cellular defense components.

Because the Ca-pump (Hebbel 1986, Leclerc et al. 1987, Moore et al. 1992), Ca-activated K channel (Brugnara et al. 1993, Ohnishi et al. 1986a, 1986b and 1989) and K-Cl cotransport (Apovo et al. 1994, Franco et al. 1996, Ohnishi et al. 2001, Schwartz et al. 1998, Vitoux et al. 1989) play important roles in the ion movements in RBC, these sites may be involved in the observed free radical–induced dehydration of sickle red cells. Membrane disturbance caused by sickling may also induce passive Ca and Na entry and K efflux (Etzioni et al. 1993, Joiner 1993) (Fig. 8).

Nitric oxide is known to react with superoxide to form peroxynitrite, which is a highly reactive compound (Beckman 1991). Because nitric oxide causes blood vessel dilation, it would be beneficial to the sickle cell patients. However, this compound may contribute to the injury of RBC. Further study is required to investigate the involvement of nitric oxide in sickle cell disease.

In summary, sickle cell anemia was identified as the first example of a “molecular disease” by Pauling et al. (1949). Fifty years later, sickle cell anemia is now further proposed to be an example of a “membrane-linked disease,” and a new nutritional therapy to protect the red cell membrane is proposed. The recipe employs none other than the “antioxidants,” which Pauling so ardently advocated at the later stage of his career. These nutritional supplements would be safer than any other drugs tested to date on sickle cell disease patients.

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[Note added in proof] After the submission of the manuscript, we further found that green tea extract (GTE) and aged garlic extract (AGE) can inhibit both K-Cl cotransport and Ca-activated K channel, while vitamins C and E cannot inhibit K-Cl cotransport (Ohnishi et al. 2001). This would explain why vitamins C and E were ineffective in the past clinical trials. Further, this suggests the possibility that both GTE and AGE would bring beneficial effects to sickle cell anemia patients. If any clinical institution is interested in a clinical trial to test this possibility, we are willing to collaborate.

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