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Sickle cell membranes and oxidative damage

Catherine RICE-EVANS, Savvas C. OMORPHOS and Erol BAYSAL
Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

Sickle erythrocytes and their membranes are susceptible to endogenous free-radical-mediated oxidative damage which correlates with the proportion of irreversibly sickled cells. The suppression of incubation-induced oxidative stress by antioxidants, free radical scavengers and an iron chelator suggest that oxidation products of membrane-bound haemoglobin contribute towards the pathology of the disease.

INTRODUCTION

The importance of membrane damage as a secondary factor contributing towards the pathophysiology of sickle cell anaemia is suggested by the retention of the sickled morphology in irreversibly sickled cell (ISCs) even when the haemoglobin is completely reoxygenated.

The altered characteristics of sickle cell membranes compared with those of normal erythrocytes include: increased haemoglobin binding to the membrane (Shaklai *et al.*, 1981), decreased deformability (Palek, 1977), elevated intracellular calcium levels (Eaton *et al.*, 1973), cellular dehydration (Glader *et al.*, 1978), decreased lipid fluidity and altered membrane surface charge (Rice-Evans *et al.*, 1978), defective cytoskeleton (Lux *et al.*,1976, Platt *et al.*, 1985) and altered phospholipid asymmetry (Chiu *et al.*, 1979). The modified levels or activities of the scavengers and enzymes normally involved in the antioxidant defences of erythrocytes are well-documented, namely decreased activities of glutathione peroxidase and catalase (Das & Nair, 1980), decreased levels of vitamin E in plasma and erythrocytes (Chiu & Lubin, 1979), and increased levels of superoxide dismutase (Das & Nair, 1980).

Asakura *et al.* (1977) have reported that irreversible haemichromes formed from denatured haemoglobin S bind to sickle cell membranes and that there is no correlation between reticulocytosis and increased levels of membrane-bound denatured haemoglobin. Our recent works suggests that fresh sickle erythrocytes contain endogenous products of lipid peroxidation (Rice-Evans & Omorphos, 1983). Other workers (Chiu *et al.*, 1979; Das & Nair, 1980), have demonstrated that exposure of sickle cells to exogenous oxidative stress in the form of added H_2O_2 results in the accumulation of lipid breakdown products. It has also been shown that after a 20 h incubation sickle erythrocytes generate twice as much superoxide, peroxide and hydroxyl radicals (Hebbel *et al.*, 1982). Their interpretation is that hydroxyl radical generation may be greatly facilitated by membrane-bound haemichrome-catalysed reaction between the superoxide radical and H_2O_2 in the same way that traces of transition metals have been shown to catalyse the Haber–Weiss reaction (Haber & Weiss, 1934), as follows:

$$O_2^{-\cdot} + H_2O_2 \xrightarrow{\text{iron-catalysed}} O_2 + OH^{\cdot} + OH^-$$

Native haemoglobin, methaemoglobin and cytochromes, etc., will not catalyse hydroxyl radical formation (Halliwell, 1978) via the Haber–Weiss reaction. For hydroxyl radical production via these reactions an open co-ordination site on the iron ion or a site only loosely associated with ligands is necessary (Graf *et al.*, 1984). However, although lipid hydroperoxides are fairly stable at physiological temperatures, transition metals in the form of haem, haemoglobin, methaemoglobin, cytochromes, nonhaem iron proteins and other components have a role in their decomposition to alkoxy or peroxy radicals (O'Brien, 1969). Ascorbate can act as a chain terminator of these radical species (Halliwell & Gutteridge, 1984).

Our work has demonstrated that sickle erythrocytes and their membranes are subjected to varying extents of free-radical-mediated oxidative damage and that the metabolic properties are modified. This supports the classification of the sickle erythrocytes into two groups: one group of samples with a higher proportion of ISCs and containing a higher level of endogenous products of oxidative damage, resulting in pronounced modifications in the membrane properties, and the other group with a lower proportion of ISCs and with apparently more normal characteristics. This classification is consistent with our earlier observations (Rice-Evans *et al.*, 1978), which showed a correlation of the proportion of ISCs and the degree of haemoglobin retention in the sickled erythrocytes with the altered membrane properties. Furthermore, many of the changes observed reflect those in our model system for oxidant stress in erythrocytes (Rice-Evans *et al.*, 1985*a*).

In the present work we have also investigated the effects of antioxidants, free radical scavengers and an iron chelator on sickle erythrocytes and their membranes as an approach to the suppression of the membrane damage which, although secondary to the genetically defective haemoglobin S, possibly contributes towards the pathology of this disease.

EXPERIMENTAL

Blood from normal donors and patients with sickle cell anaemia was taken into heparin and used within 20 h. The red cells were separated and washed with iso-osmotic phosphate-buffered saline, pH 7.4. Assays for ATP and glucose-6-phosphate dehydrogenase were performed by

Abbreviations used: TBAR, thiobarbituric acid reactive; ANS, 1-anilino-8-naphthalene sulphonate; ISC(s), irreversibly sickled cell(s).

using the test-combination kits from Boehringer. The GSH levels of the red cells were determined spectrophotometrically (Beutler *et al.*, 1963) at 412 nm with 5,5′-dithiobis-(2-nitrobenzoic acid). Erythrocytes were incubated at 37°C for 5 h at a 5% suspension with the following additions: L-ascorbic acid (Sigma) at various concentrations, iron(II) sulphate at 0.1 mM and desferrioxamine mesylate (CIBA-Geigy) at 0.4 mM. Methaemoglobin formation was measured spectrophotometrically by the method of Harley & Mauer (1960).

Lipid peroxidation was assayed by measuring TBAR products by the method of Walls *et al.* (1976): 0.2 ml of 50% (w/v) trichloroacetic acid was added to 2 ml of the 5% erythrocyte suspension after incubation, mixed and centrifuged at 3000 rev./min for 10 min. Then 1 ml of the supernatant was removed and 0.5 ml of 0.75% thiobarbituric acid in 0.1 M-HCl was added. The samples were heated at 90–95°C for 20 min and centrifuged for 10 min. The supernatant was removed and the pink chromophore was assayed spectrophotometrically at 532 nm. The standard curve was prepared with malonaldehyde bis(dimethylacetal) (Aldrich) hydrolysed with 6M-HCl.

Erythrocyte membranes were prepared by the method of Dodge *et al.* (1963) at pH 7.4 and protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard. The content of free thiol sidechains in the membrane was determined by the method of Ellman (1959). Iron levels in erythrocyte membranes were measured by using a Perkin–Elmer model 107 Atomic Absorption Spectrometer.

Normal and sickle cell membranes were incubated for 5 h with additions as above of superoxide dismutase (0.1 mg/ml), catalase (1.3 mg/ml) or vitamin E (0.1 mg/ml). DL-α-Tocopherol was dissolved in ethanol at a concentration of 10 mg/ml. Membrane suspensions (3 ml) were vigorously injected with 30μl of stock solution and mixed thoroughly. Appropriate controls with 30 μl of ethanol were also performed and shown to have no effect. Red cell membrane lipid peroxidation was assayed (*a*) by monitoring the TBAR products from the breakdown of lipid hydroperoxides as for the erythrocytes, adding 1 vol. of membranes to 1 vol. of 10% (w/v) trichloroacetic acid and 1 vol. of 0.75% thiobarbituric acid, and (*b*) by the measurement of the formation of fluorescent chromolipids (Bidlack & Tappel, 1973), which were extracted from the erythrocytes with chloroform/methanol and quantified on the basis of phospholipid phosphorus (Bligh & Dyer, 1959; Bartlett, 1959); the fluorescence spectra from 400 to 500 nm were determined with a Perkin–Elmer MPF44B fluorescence spectro photometer with an excitation wavelength of 350 nm. The fluorescent probe ANS was applied to investigate changes in the polarity and charge at the membrane surface. Membrane samples contained 53.3 μg of protein/ml and 10 μM-ANS; the extrinsic fluorescence was excited at 350 nm and the emission intensity observed between 450 and 470 nm (Kennedy & Rice-Evans, 1976).

RESULTS

Fresh erythrocytes from patients with sickle cell anaemia were examined microscopically and the ISC content of oxygenated cells was monitored and divided into two groups related to the proportion of ISCs: those with less than 5% and those with greater than 5%.

Table 1. Membrane abnormalities in sickle erythrocytes

Values are means ± S.D. for the number of samples shown.
*$P < 0.0005$ compared with normal.

Parameter	Normal erythrocytes	Sickle erythrocytes	
		Group 1	Group 2
TBAR products in erythrocytes (nmol/10^{10} cells) (18 patients)	6.95 ± 2.5 ($n = 10$)	6.00 ± 1.4 ($n = 8$)	11.2 ± 2.6* ($n = 10$)
TBAR products in membranes (A_{532}/mg of protein) (24 patients)	0.072 ± 0.01 ($n = 21$)	0.080 ± 0.04 ($n = 5$)	0.150 ± 0.03* ($n = 19$)
Membrane thiol content (nmol of SH/mg of protein)	84 ± 9 ($n = 24$)	80 ± 3 ($n = 10$)	65 ± 5* ($n = 14$)
Decrease in ANS fluorescence intensity (%)	0 ($n = 10$)	0 ($n = 3$)	34 ± 6 ($n = 10$)
Relative lipid fluorescence (fluorescence units/μg of phospholipid phosphorus)	15 ± 2 ($n = 4$)	17 ± 3 ($n = 4$)	

Membrane lipid peroxidation was determined in the form of TBAR products both on fresh sickle erythrocytes and freshly prepared sickle cell membranes from erythrocytes from a different set of patients without first having been subjected to oxidative stress *in vitro*. The data in Table 1 illustrate that sickle erythrocytes and their membranes show a greater tendency to lipid peroxidation and the formation of secondary breakdown products which are TBAR compared with normal control erythrocytes. A smaller group of patients display more normal levels and have lower ISC counts. Examination of lipid extracts of the sickle cell membranes for phospholipid adducts cross-linked via malonaldehyde to form fluorescent chromolipids gives levels as found in normal erythrocytes for all samples studied. This is in contrast with the results of Jain & Shohet (1984). Investigations of the reduced protein thiol sidechains in the membrane are summarised in Table 1. Of the 24 patients homozygous for sickle cell anaemia involved in this particular series of experiments one group ($n = 10$) gives a free thiol content within the range for normal erythrocyte membranes while the other group ($n = 14$) gives significantly lower results.

The amphipathic fluorescent probe ANS was applied to investigate changes in polarity or charge at the membrane surface. The data show (Table 1) that there is a decrease in the fluorescence intensity when ANS binds to sickle cell membranes compared to the control erythrocyte membrane, with no shift in the emission maximum wavelength (460 nm) (Rice-Evans *et al.*, 1978).

Table 2. Metabolic characteristics of sickle cells

Values are means ± S.D. for the number of samples shown. *$P < 0.0005$ compared with normal.

Parameter	Normal erythrocytes	Sickle erythrocytes Group 1	Sickle erythrocytes Group 2
ISC count (%)	–	5	5–25
ATP (mg/100 ml of blood)	23.3 ± 3.7 ($n = 12$)	20.8 ± 1.5 ($n = 5$)	13.5 ± 2.5* ($n = 16$)
Glucose-6-phosphate dehydrogenase (munit/10^9 cells)	117 ± 13 ($n = 9$)	131 ± 15 ($n = 7$)	196 ± 32* ($n = 12$)
GSH (mg/litre of cells)	640 ± 63	731 ± 94 ($n = 7$)	

Table 3. Effects of ascorbate on erythrocytes

Incubation was at 37°C for 5 h. Levels of significance versus incubated controls: *$P < 0.005$; **$P < 0.0005$; NS not significant. Values are means ± S.D. for the numbers of samples shown.

Conditions	TBAR products (nmol/10^{10} cells)
Sickle erythrocytes	
Control, no incubation	11.2 ± 2.6 ($n = 10$)
Control, incubated	15.2 ± 2.8 ($n = 10$)
0.1 mM-Ascorbate	9.0 ± 2.1** ($n = 6$)
1 mM-Ascorbate	14.9 ± 3.2^{NS} ($n = 6$)
5 mM-Ascorbate	29 ± 3** ($n = 5$)
Normal erythrocytes	
Control, no incubation	6.95 ± 2.5^{NS} ($n = 10$)
Control, incubated	8.33 ± 2.4 ($n = 6$)
0.1 mM-Ascorbate	7.85 ± 2.0^{NS} ($n = 4$)
1 mM-Ascorbate	8.10 ± 2.5^{NS} ($n = 4$)
5 mM-Ascorbate	33 ± 5** ($n = 4$)

Hence this is not simply a polarity effect (Freedman & Radda, 1969) altering the accessibility of the probe to the aqueous environment but rather is indicative of increased negative charge at the membrane surface. A small group of patients studied with low ISC count showed no effect.

Studies on the metabolic properties indicate further that the sickle cells can be divided into two groups (Table 2). For cellular ATP and glucose-6-phosphate dehydrogenase levels the sickle cell samples with the higher proportion of ISCs had modified contents of both, whereas the samples with the lower proportion of ISCs had more normal metabolic properties. Cellular GSH levels were not significantly different from normal in any of the samples studied. Lowered ATP levels have been previously reported in ISC-rich fractions, but our samples were not separated according to age or density (Glader *et al.*, 1978).

Table 4. Effects of iron, ascorbate and desferrioxamine on sickle cells and normal erythrocytes

Incubation was for 5 h at 37°C. Levels of significance versus incubated controls: *$P < 0.005$; **$P < 0.0005$; NS not significant. $n = 5$ for all experiments. Values are means ± S.D.

Conditions	TBAR products (nmol/10^{10} cells)	Methaemoglobin levels (%)
Sickle erythrocytes		
Control, incubated	17.1 ± 3	3 ± 1
+0.4 mM-Desferrioxamine	16.7 ± 3^{NS}	2 ± 1
+0.1 mM-Iron(II)/1 mM-ascorbate	36.1 ± 4**	15 ± 2
+0.1 mM-Iron(II)/1 mM-ascorbate/0.4 mM-desferrioxamine	20.0 ± 3^{NS}	4 ± 1
Normal erythrocytes		
Control, incubated	8.0 ± 2	1.5 ± 1
+0.4 mM-Desferrioxamine	8.2 ± 2^{NS}	1 ± 1
+0.1 mM-Iron(II)/1 mM-ascorbate	14.5 ± 3*	14 ± 2
+0.1 mM-Iron(II)/1 mM-ascorbate/0.4 mM-desferrioxamine	7.2 ± 1.5^{NS}	3 ± 1

Incubation of erythrocytes for 5 h at 37°C slightly increased the release of TBAR products in sickle erythrocytes (Table 3) but had no effect on normal red cells. In the presence of ascorbate during the incubation various responses were measured which were concentration-dependent: 0.1 mM-ascorbate inhibited the increase in concentration of the TBAR products in sickle erythrocytes after a 5 h incubation, but there was no effect on normal erythrocytes, 1 mM-ascorbate treatment was ineffective in both types of cells, whereas elevated concentrations such as 5 mM-ascorbate caused pronounced peroxidation of the membrane lipids and oxidation of the haemoglobin (results for measured methaemoglobin levels not shown).

Treatment of sickle and normal erythrocytes *in vitro* with iron-containing systems for generating oxygen radicals consisting of 0.1 mM-iron(II) and 1 mM-ascorbate caused elevated production of TBAR products and oxidation of haemoglobin after a 5 h incubation at 37°C (Table 4). Sickle erythrocytes with higher endogenous levels of TBAR products at time zero were more susceptible to the externally added oxidizing species compared with normal erythrocytes. In the presence of the iron chelator desferrioxamine the increased oxidative damage to the membrane lipids and to the haemoglobin was suppressed, as expected (Rice-Evans *et al.*, 1985*b*).

The amount of iron bound to the sickle cell membranes compared with that in white normal erythrocyte membranes prepared under the same conditions at the same time was measured by atomic absorption spectroscopy. While the normal membranes

Table 5. Effects of radical-scavenging systems on oxidative damage in sickle cell membranes

Incubation was for 5 h at 37°C. Levels of significance versus incubated controls: $^{*}P < 0.0005$; ^{NS}not significant. Values are means ± S.D. for the numbers of samples shown.

Conditions	TBAR products (A_{532}/mg of membrane protein)
Sickle cell membranes	
Control, no incubation	$0.150 \pm 0.03^{*}$ ($n = 10$)
Control, incubated	0.260 ± 0.03 ($n = 5$)
+ Desferrioxamine (0.4 mM)	$0.111 \pm 0.02^{*}$ ($n = 3$)
+ Ascorbate (5 mM)	$0.141 \pm 0.01^{*}$ ($n = 4$)
+ Ascorbate (5 mM) + desferrioxamine (0.4 mM)	$0.094 \pm 0.03^{*}$ ($n = 3$)
+ Vitamin E (0.1 mg/ml)	$0.104 \pm 0.02^{*}$ ($n = 4$)
+ Superoxide dismutase (0.1 mg/ml)	0.250 ± 0.03^{NS} ($n = 3$)
+ Catalase (1.3 mg/ml)	0.271 ± 0.04^{NS} ($n = 3$)
+ Superoxide dismutase + catalase (at above concentrations)	0.270 ± 0.04^{NS} ($n = 3$)
Normal erythrocyte membranes	
Control, no incubation	0.070 ± 0.01^{NS} ($n = 10$)
Control, incubated	0.069 ± 0.01 ($n = 5$)
+ Ascorbate (5 mM)	0.085 ± 0.01^{NS} ($n = 5$)
+ Desferrioxamine (0.4 mM)	0.074 ± 0.01^{NS} ($n = 5$)

contain very little bound iron (0.02 μg/mg of membrane protein), the data show that within this group of patients the sickle cells contain 50 times more iron (1.78 ± 1 μg/mg of protein, $n = 5$), corresponding to about 0.7% of total haemoglobin bound to the membrane.

In order to assess the possible role of membrane-bound haemoglobin in the propagation of oxidative damage in sickle erythrocytes, experiments were performed on isolated sickle erythrocyte membranes (Table 5). Incubation of membranes in buffer for 5 h with no additions resulted in an increased production of lipid breakdown products in the sickle membrane, as with the intact sickle erythrocyte system, whereas the normal erythrocyte membrane was unmodified in this respect. The addition of 5 mM-ascorbate to this system prevented the increase in lipid peroxidation which would normally have been observed after a 5 h incubation of the sickle cell membranes in its absence. The combination of 0.4 mM-desferrioxamine and 5 mM-ascorbate caused a further decline in lipid peroxidation during the 5 h incubation, and 0.4 mM-desferrioxamine alone also significantly depressed lipid peroxidation below the level observed before incubation. Incorporation of antioxidation enzymes into the membrane system, such as superoxide dismutase, catalase and a combination of both, were ineffective in suppressing the production of TBAR products, whereas the chain-breaking antioxidant vitamin E (Tappel, 1962; Diplock, 1983) decreased lipid peroxidative damage to the same extent as did desferrioxamine (Table 5) after the 5 h incubation.

DISCUSSION

It has been suggested that the initial event in the sickling process is the irreversible binding of deoxyhaemoglobin S to the membrane (Shaklai *et al.*, 1981). Experiments on the extent of such binding have shown that haemichromes bind irreversibly to the membrane to the extent of 0.2% of the total haemoglobin (Asakura *et al.*, 1977) and that this is independent of the degree of reticulocytosis. Within the erythrocyte there normally exists a balance between spontaneous production of methaemoglobin, on autoxidation of oxyhaemoglobin to the superoxide radical and methaemoglobin, and the restoration of this oxidized haemoglobin to its functional state. In cases in which oxidation is followed by the formation of such haemichromes, the reduction mechanisms may not be able to counteract efficiently this conversion leading to the accumulation of oxidation products of haemoglobin, including haemichromes (Graf *et al.*, 1984).

Our work proposes that erythrocytes from patients with sickle cell anaemia can be divided into two groups: one group of samples with a higher proportion of ISCs (5–25%) and containing higher endogenous levels of secondary products of oxidative damage, the other of samples with a low proportion of ISCs (< 5%) and with apparently more normal characteristics. The observations in the former group are consistent with the presence of lipid hydroperoxides in the membrane. Various extents of endogenous free-radical-mediated oxidative damage are observed in freshly prepared samples without prior incubation or externally added oxidative species. The ensuing modifications of the lipid chains may contribute towards the deceased average bulk lipid fluidity (Rice-Evans *et al.*, 1978) in the membranes of sickle erythrocytes with a higher proportion of ISCs. The oxidative damage observed correlates with the membrane protein modifications in terms of the diminution of the availability of reduced thiol groups, and yet no thiol-cross-linked proteins have been observed by polyacrylamide-gel electrophoresis under non-reducing conditions either by us or by others (Ballas & Burka, 1980). Rank *et al.* (1985) have also reported the altered thiol redox status of sickle cell membranes.

The increased negative charge at the membrane surface may reflect the state of the haemoglobin and its interaction with the membrane and those components which contribute towards the surface charge. Changes both in the cytoskeletal proteins, which control the lateral mobility of certain transmembrane glycoproteins (Fowler & Bennett, 1979), and in membrane lipid asymmetry may be involved here. The decreased cellular levels of ATP may relate to a defective glycolytic pathway and the inability of NADH-dependent methaemoglobin reductase effectively to dispense with the consequences of haemoglobin autoxidation in sickle cells. Many of the altered membrane characteristics in sickle cells reflect those observed in our model systems (Rice-Evans *et al.*, 1985*a*) for studying the susceptibility of normal erythrocytes to oxidative damage.

In the present work we have shown that ascorbate is an effective antioxidant in sickle cell membranes after incubation for 5 h. It is possible that the mechanism of action of ascorbate may involve the scavenging of alkoxy or peroxy radicals by a chain-termination reaction (Halliwell & Gutteridge, 1984) or the decreased initiation of lipid peroxidation.

Ferric ion bound to desferrioxamine is not free to catalyse the decomposition of lipid hydroperoxides nor to participate in other potential iron-mediated reactions (Gutteridge *et al.*, 1979). The suppression of oxidative damage and of breakdown of lipid hydroperoxides in sickle cell membranes after incubation with desferrioxamine may arise by a chelation mechanism of the membrane-bound iron, which would therefore imply that the iron is not in the state of irreversible haemichromes, contrary to the observations of Asakura *et al.* (1977). If this is the case and the state of the ferric ion in the membrane-bound haemoglobin is chelatable, then in the unchelated form it may equally well be available for the Haber–Weiss reaction involving the iron-catalysed production of hydroxyl radicals from H_2O_2 and superoxide radicals, although we have no direct evidence here that the hydroxyl radical is implicated. In the presence of haemoglobin, ascorbate was only effective as an antioxidant in intact sickle erythrocytes at lower concentrations, higher levels having an oxidizing effect on haemoglobin in both normal and sickle erythrocytes. This is in contrast with the reported effect of ascorbate on lipid peroxidation in cortex membranes prepared from rat brains (Muakkassah-Kelly *et al.*, 1982) in which the production of TBAR products was maximal at 0.5 mM-ascorbate and progressively decreased thereafter up to 6 mM-ascorbate. The differential response probably lies in the presence of haemoglobin in the erythrocytes as compared with endogenous iron from other sources in the rat brain membrane preparation. These studies on sickle cell membranes suggest the presence of lipid hydroperoxides and the potential for iron-catalysed production of alkoxy and peroxy radicals by the membrane-bound components of the haemoglobin. These factors cause continued oxidative stress and contribute towards the membrane damage.

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