A Novel Phospholipid in Irreversibly Sickled Cells: Evidence for In Vivo Peroxidative Membrane Damage in Sickle Cell Disease

By Sushil K. Jain and Stephen B. Shohet

In individuals with sickle cell disease, a variable number of irreversibly sickled cells (ISC) is present that are highly dehydrated and undeformable. These cells may contribute to the pathophysiology of sickle cell anemia. The present study was undertaken to determine the possible role of membrane lipid peroxidation in the genesis of ISC. After 24 hr of simple aerobic incubation, sickle cells accumulated 2-3 times more malonyldialdehyde (MDA), an end product of lipid peroxidation, than normal cells. To assess the possibility of peroxidative damage in ISC in vivo, ISC were separated from sickle blood using Stractan density gradients. Lipid extracts of the untreated ISC-enriched fraction of sickle blood showed significant fluorescence and contained a novel phospholipid:MDA adduct that was not seen in control cells. Taken together, these observations suggest that ISC have previously undergone lipid peroxidative damage and the accumulation of MDA in vivo.

In individuals with sickle cell disease, a variable number of irreversibly sickled cells (ISC) is present that are highly dehydrated and undeformable. These cells may contribute to the pathophysiology of sickle cell anemia. Several factors, such as accumulation of cellular calcium, metabolic depletion, membrane loss during repeated deoxygenation and oxygenation, and polymerization of membrane proteins, have been suggested to be important in the formation of ISC. However, none of these proposed factors has been shown to have an unequivocal role in the ISC formation. At present, the primary membrane damage responsible for ISC formation in sickle cell patients remains unknown.

Considerable evidence has suggested a major role for calcium accumulation in the formation of ISC. The concentration of calcium in sickle cells is substantially elevated over the normal values, suggesting an impairment of the calcium pump that maintains a low calcium concentration in normal cells. However, both reduced and elevated calcium adenosine triphosphatase (ATPase) activity have been reported in sickle cells. Because of the multiplicity of effects that calcium accumulation may have on cellular properties, several different pathways have been suggested for calcium's putative influence on irreversible sickling. Clark et al. and Glader and Nathan have reported a predominant role of calcium-induced cellular dehydration (the Gardos effect) in the formation of ISC. Our recent study has suggested yet another role for calcium in ISC formation. We found that loading of erythrocytes with calcium increased vulnerability of their membranes to exogenous peroxidant threat.

The present study was undertaken to determine if the sickle cell membrane shows residual effects of peroxidative damage and to consider the possibility that peroxidative damage might have a role in the genesis of irreversibly sickled cells.

MATERIALS AND METHODS

Blood was drawn into heparinized tubes and then passed through a cotton-wool column to remove white cells. Red cells were separated from plasma by centrifugation at 1,500 g for 5 min in a refrigerated centrifuge. The residual cells were similarly washed 3 times with cold isotonic saline. After obtaining their informed consent, we drew sickle blood from untransfused sickle cell patients and control blood from normal volunteers.

Preparation of Malonyldialdehyde (MDA)

MDA was prepared by acid-hydrolysis of malonaldehyde bis(dimethyl acetal) (Aldrich Chemical Co., Milwaukee, WI). Fifty microliters of the stock were diluted to 1 ml with 0.9% sodium chloride and subsequently hydrolyzed by the addition of 30 μl of 6N HCl. MDA was freshly prepared before each use.

Treatment With MDA

Red cell suspensions [5% in buffered saline containing glucose and potassium (BSKG), pH 7.4, 290 mosmole] were treated with various concentrations of MDA (0.5-5.0 mM) in Erlenmeyer flasks. The flasks were incubated in a shaking water bath at 37°C for

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various times, as specified in the legends to the figures or tables. Flasks incubated overnight contained 5% (v/v) penicillin-streptomycin solution (Grand Island Biological Co., Grand Island, NY). Acid-hydrolysis of 1 mole of malonaldehyde bis-(dimethyl acetal) yields 1 mole of MDA and 4 mole of CH$_2$OH. In our preliminary experiments, we saw no effect of CH$_2$OH treatment of red cells on formation of phospholipid: MDA adduct, or appearance of fluorescence products.

Separation of Irreversibly Sickled Cells

Washed sickle cells were suspended at approximately 30% hematocrit and layered on the top of a continuous Stratagel gradient, as described by Clark et al.$^{11}$ The topmost layer in the gradient (~5%), containing reticulocytes, was discarded. The layer just below this discarded layer was designated as the top layer. The top and bottom layers of the gradient, which each constituted about 10% of the total red cells, were removed with a Pasteur pipette. These cells were washed 4 times with saline before lipid extraction. The bottom layer of the density gradient generally contained approximately 70% ISC. For control experiments, normal blood was processed identically, and top and bottom fractions were collected.

Lipid Extraction and Quantitation

Erythrocyte lipids were extracted according to the method of Rose and Oklander$^{12}$ using isopropanol-chloroform. Fluorescence in the lipid extracts was measured using an Amicon Spectrofluorometer with excitation at 400 nm and emission at 460 nm and band pass values of 4 and 8, respectively. Phospholipid classes in the lipid extract were separated by thin-layer chromatography (TLC) on silica gel H plates (Brinkman, Westbury, NY) using the chloroform-methanol-acetic acid-water (50:25:7:3, v/v) solvent system described by Skipski et al.$^{13}$ Lipid spots on ultra-TLC plates were visualized using iodine vapors, ninhydrin, and molybdenum spray (Applied Science Laboratory, State College, PA), as described previously.$^{14}$ The phospholipid-phosphorus in the silica gel spots was quantitated by the method of Fiske and Subbarow.$^{15}$

Measurement of Lipid Peroxidation

Lipid peroxidation was assessed by measuring MDA, an end-product of peroxidation reactions. MDA was measured directly by its reactivity with thiobarbituric acid (TBA), as described by Stocks and Dormandy,$^{16}$ and indirectly by measuring lipid fluorescence in the lipid extracts, as described previously.$^{17}$ The data in Table 1 give delta change in lipid fluorescence units between before and after incubations of cells, because we wanted to examine the formation of lipid fluorescence after in vitro incubation of cells. However, data in Table 3 give the absolute fluorescence units present in the untreated cells.

Acid Hydrolysis of Novel Lipid (MDA Adduct)

The silica-gel-containing new lipid spot, which moved between phosphatidylethanolamine and phosphatidylserine in one-dimensional TLC, was scraped into a tube. The lipid in the silica gel was then eluted by vortexing and filtering with successive portions of chloroform, chloroform-methanol (2:1), chloroform-methanol (1:1), and methanol. The eluate was dried with nitrogen and redissolved into a known amount of chloroform for quantitation of its glycerol, phosphorus, and amino acids content. A portion of the chloroform extract was dried in a Teflon-stoppered tube and hydrolyzed with 1 ml of 6N HCl in a boiling water bath for 4 hr. In control tubes, standard serine and ethanolamine (Sigma Chemical Co., St. Louis, MO) were simultaneously treated with acid. Separation of amines in the acid hydrolysate was done by thin-layer chromatography using cellulose-precoated Eastman Kodak plates with a solvent system consisting of Butanol-pyridine-acetic acid-water (30:30:10:10, v/v). The amines were located on the plates by spraying with ninhydrin, as described by Pataki.$^{18}$ The colored complexes were then eluted from the cellulose with aqueous phosphate buffer and quantitated spectrophotometrically.$^{19}$

Glycerol was quantitated by the method of van Handel and Zilversmit.$^{20}$

RESULTS

Table 1 shows the accumulation of MDA that occurs after simple aerobic incubation of untreated normal and sickle erythrocytes in saline buffer. After 24 hr, sickle cells accumulated 2–3 times as much MDA as normal cells, as measured either by the thiobarbituric acid (TBA) reactivity of free MDA formed from peroxidation of membrane lipids or the specific fluorescence of lipid peroxidation products.$^{1}$ These latter fluorescent products are formed by cross-linking of MDA with amino-phospholipids, and their accumulation provides a sensitive measure of MDA.

$^{†}$There was 3%–5% loss of total phospholipid-phosphorus in 24-hr incubated sickle cells as compared to unincubated sickle cells.

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>Normal Cells</th>
<th>Sickle Cells</th>
</tr>
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<tbody>
<tr>
<td>O$_2$ (aerobic)</td>
<td>11 ± 2$^a$</td>
<td>21 ± 4$^b$</td>
</tr>
<tr>
<td>N$_2$ (anaerobic)</td>
<td>3 ± 1$^c$</td>
<td>4 ± 2$^c$</td>
</tr>
<tr>
<td>O$_2$ + vitamin E</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are mean ± SD of four observations. Differences between $'a'$ and $'b'$, $'a'$ and $'c'$, and $'b'$ and $'c'$ are statistically significant (p < 0.01).

Unwashed treated RBC were suspended to 10% hematocrit in phosphate-buffered saline containing glucose and incubated at 37°C in a shaking water bath for 24 hr. Lipids from incubated red cells were extracted by adding 3 ml of isopropanol and 2 ml of chloroform as described earlier.$^{17}$ Fluorescence in the lipid extracts were read at excitation wavelength of 400 nm and emission wavelength of 460 nm, using Amicon spectrofluorometer. Details of relative lipid fluorescence and thiobarbituric acid reactivity measurements to quantitate malonyldialdehyde production are given in Materials and Methods.
production. These observations suggest that sickle cells are more susceptible to even the minimal peroxidative stress of aerobic incubation in comparison to normal cells. Accumulation of MDA was blocked in the presence of added vitamin E, an antioxidant. (Fluorescent chromolipids could not be assayed in the samples incubated with vitamin E because of the vitamin's native fluorescence.) In addition, MDA accumulation was appreciably reduced during anaerobic incubation. These two ancillary findings emphasize the peroxidative origin of MDA accumulation in vitro.

To delineate the cellular effects of MDA generated during peroxidation, we conducted experiments in which MDA was added directly to normal cell suspensions. The effect of MDA treatment of normal erythrocytes on the appearance of lipid fluorescence in their lipid extracts is illustrated in Fig. 1. There was a small amount of fluorescence even in untreated erythrocytes, but treatment of the cells with increasing concentrations of MDA resulted in a progressive increase of fluorescence, which was also time-dependent.

Figure 2 illustrates a thin-layer chromatograph of the lipids of erythrocytes incubated for 24 hr with and without varying concentrations of MDA. As can be seen, a new lipid spot appeared between phosphatidylserine (PS) and phosphatidylethanolamine (PE). The intensity of this spot was proportional to the concentration of MDA used. This spot, marked “adduct,” was phosphorus-positive and ninhydrin-negative. A chromatographically identical adduct could be formed in vitro when a mixture (1:1) of pure PS and PE was treated with MDA. Other nonamino phospholipids were ineffective. Further acid hydrolysis of the adduct released nearly 0.5 mole each of ethanolamine and serine, and 1 mole of glycerol per mole of phosphorus (see Table 2). Taken together, these studies suggest that the new lipid spot is the anticipated heterologous Schiff’s base adduct formed by crosslinking amino groups of PE and PS with aldehyde groups of MDA, according to the following reaction scheme:

Phosphatidylserine – NH₂ + 0

– CH – CH₂ – HC – O

(MDA)

+ H₃N – phosphatidylethanolamine →

phosphatidylserine – N = CH – CH₂ – CH = N

– phosphatidylethanolamine + 2H₂O

In order to confirm this reaction, we determined the molar ratio of serine, ethanolamine, and glycerol to phosphorus, after acid hydrolysis of the isolated putative adduct, as described in Materials and Methods. As can be seen from Table 2, the molar ratios of serine:phosphorus, ethanolamine:phosphorus, and glycerol:phosphorus are all consistent with the proposed reaction scheme.

Concomitantly with formation of the adduct, levels
of PS and PE decreased (Fig. 2). At concentrations of 0.5, 1.0, and 2.0 mM of MDA, phospholipid-phosphorus in the adduct was 1.5% ± 0.2%, 2.8% ± 0.4%, and 5.1% ± 0.2% of the total lipid-phosphorus, respectively. PS and PE spots showed an equivalent decrease in their lipid-phosphorus with the increasing concentrations of MDA. Moreover, there was no net loss of phospholipid-phosphorus and we could account for all the phospholipid-phosphorus in the MDA-treated erythrocytes. Lipids extracted from normal cells that had not been exposed to MDA, but which were otherwise handled identically, did not show any new lipid spot, nor could any lipid-phosphorus be detected in the silica gel in the chromatographic region where the adduct was found in extracts from treated cells. The percentage composition of phosphatidylcholine (PC) and sphingomyelin (SM) was not affected in the MDA-treated erythrocytes. It is likely that homologous adducts among PE, MDA and PE or PS, MDA and PS would also have been formed in the MDA-treated red cells. However, TLC fails to separate these homologous adducts from their parent compounds.

To determine whether these changes were present in untreated irreversibly sickled cells (ISC), we separated ISC from sickle blood using Stractan gradients. For controls, top and bottom fractions of normal blood were also subjected to lipid extraction and fluorescence determinations. There was consistently more fluorescence in the lipid extracts of untreated sickle cell fractions than in untreated normal cell fractions (Table 3). This fluorescence was increased nearly 75% in the most dense ISC fraction of sickle blood, in comparison to the most dense fraction of normal blood. This suggested that the ISC may have previously undergone lipid peroxidation and accumulation of MDA in vivo. To further consider this possibility, we analyzed these extracts by thin-layer chromatography. Table 4 gives phospholipid composition of normal and sickle cells. The TLC of the lipid extract of the ISC-enriched fraction of sickle blood, as shown in last two lanes of Fig. 2, contained a new lipid that moved identically to the adduct obtained after in vitro MDA treatment of normal cells. This spot was also phosphorus-positive and ninhydrin-negative and fluoresced under ultraviolet light. This adduct in the ISC-enriched and discoid sickle cell fractions constituted 1.04% ± 0.58% (SD) and 0.28% ± 0.11% (SD) of the total phospholipids, respectively. There was no difference in the other phospholipid classes between normal and sickle cell fractions.

The effect of MDA treatment of discoid sickle cells on the subsequent genesis of ISC by overnight deoxygenation is shown in Fig. 3. After an initial incubation with 0.5 mM MDA, more than 50% of the discoid sickle cells retained their sickled shape after reoxygenation. Prolonged oxygenation or dilution of the suspending buffer to decrease the osmolality of the medium to 150 mosmole did not reverse this sickled state. It should be noted that these sickled forms were spiked, unlike the smooth ISC found in vivo. At higher concentrations of MDA, cells were shrunken, and less than 15% of the cells were found in sickled shape. At concentrations of MDA above 2.0 mM, the cells did not sickle at all. The amount of phospholipid:MDA adduct present in untreated ISC fraction of sickle blood was 1.04% ± 0.58% and the amount of phospholipid:MDA adduct formed after in vitro treatment of normal RBC with 0.5 mM concentrations of MDA was 1.5% ± 0.2% of the total phospholipids. Thus, the concentration of MDA used (0.5 mM) in the shape stabilization of sickle cells is about the same as that could be present in untreated ISC fractionated from sickle blood.

**DISCUSSION**

The observation that sickle cells are more vulnerable to peroxidant threat than normal cells was first made by Stocks et al., and, subsequently, has been confirmed by Das and Nair and Chiu and Lubin. These latter authors also reported that untreated sickle cells

| Table 2. Molar Ratio of Amines and Glycerol to Phosphorus in the Novel Lipid |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Molar Ratio     |                 |                 |                 |
| Serine/phosphorus | 0.48            |                 |                 |                 |
| Ethanolamine/phosphorus | 0.43          |                 |                 |                 |
| Glycerol/phosphorus | 0.92            |                 |                 |                 |

Details of acid hydrolysis of novel lipid and serine, ethanolamine, glycerol, and phosphorus analysis are given in Materials and Methods.

| Table 3. Lipid Fluorescence in Untreated Fractions of Normal and Sickle Cells |
|-----------------|-----------------|-----------------|-----------------|
|                 | Relative Fluorescence/μg Phospholipid |                 |                 |
|                 | Normal           | Sickle          |                 |
| Top             | 27 ± 2           | 33 ± 4 (discoid)|                 |
| Bottom          | 40 ± 3           | 69 ± 5 (> 75% ISC)|                 |

Values are mean ± SE (n = 9). Differences are statistically significant (p < 0.01). Top and bottom fractions from normal and sickle blood were separated on Stractan density gradients, as described in Materials and Methods.

| Table 4. Phospholipid Adduct in Untreated Fractions of Normal and Sickle Cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Fraction        | Percent of Total Phospholipids |
|                 | Normal           | Sickle          |                 |
| Top             | 0               | 0.28 ± 0.11'    | 1.04 ± 0.58'    |
| Bottom          | 0.25 ± 0.18'    |                 |                 |

Values are mean ± SD of five samples each. Details of lipid extraction and thin-layer chromatography of phospholipid adduct are given in Materials and Methods. Differences between 'x' and 'z' and 'y' and 'z' were statistically significant (p < 0.05), using paired t test.
have more MDA than normal cells and that these cells are deficient in vitamin E, an important physiologic membrane antioxidant.

The present study shows that sickle cells have a greater tendency to form MDA in comparison to normal cells under the minimal stress of aerobic saline incubation. Increased membrane peroxidation and accumulation of MDA in the sickle cells during incubation of untreated sickle cells could be due to: (1) the autoxidation of unstable sickle hemoglobin, resulting in increased production of H₂O₂; (2) the presence of increased intracellular calcium in sickle cells, which may accentuate membrane peroxidation; and (3) a deficiency of vitamin E in sickle cells. The current studies do not allow us to discriminate between these possibilities. However, all of the conditions mentioned are known to be present in sickle cells, and calcium is known to be present in higher concentrations in dense sickle cells.

The presence of the phospholipid:MDA adduct, as confirmed by the acid hydrolysis stoichiometry, as well as the increased lipid fluorescence in the untreated ISC suggest the prior peroxidative damage in their membranes. More than half of the peroxide-vulnerable fatty acids in the red cell phospholipid are present in PS and PE, which are predominantly inner leaflet lipid and, hence, close to the putative initial peroxidative source—the relatively unstable sickle hemoglobin. MDA formed as a consequence of their peroxidative breakdown could, in turn, crosslink with either the phospholipids themselves or adjacent proteins, such as spectrin. However, one might anticipate that in vivo, MDA generated in the lipid phase would first react with the members of its surrounding lipid milieu before reacting with membrane proteins. Both the presence and the absence of high molecular weight protein complexes have been reported in the untreated sickle cells.

ISC generated following deoxygenation and oxygenation of MDA-treated discoid cells are not exactly the same as those found in the blood of sickle cell patients. The surface of the ISC we produced in vitro is rough and spiked, whereas the surface of ISC found in vivo is smooth. It is possible that “pinching off” or other remodeling of the membrane during the circulation through fine capillaries of the reticuloendothelial system may be responsible for this difference.

In summary, we suggest that accumulation of hydrogen peroxide, generated by the autoxidation of sickle hemoglobin and perhaps augmented by either depletion of vitamin E or increased intracellular calcium, can cause peroxidation of membrane fatty acids and the formation of malonyldialdehyde. Malonyldialdehyde, in turn, can react with the aminophospholipids of the membrane to produce a novel lipid adduct that represents membrane lipid crosslinking. This phospholipid:MDA adduct is seen both in cells treated in vitro with exogenous MDA and in untreated, ISC-enriched sickle cells. This suggests that in vivo peroxidative membrane damage may have occurred in the formation of irreversibly sickled cells.

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