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The effects of cholesterol oxidation products in sickle and normal red blood cell membranes

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The oxysterol content in normal and sickle red blood cell (RBC) membranes was assessed using thin-layer chromatography and capillary gas chromatography / mass spectrometry. Several more oxysterols were present in sickle RBCs compared to normal RBCs. Sickle RBC membranes had a higher concentration of 5α,6α-epoxycholesterol, 5α-cholestane-3β,5,6β-triol, 7-ketocholesterol and 19-hydroxycholesterol than normal RBC membranes. The increased oxysterols in sickle RBC may be an effect of the increased oxidative stress which occurs in sickle RBC membranes. Physical characteristics of normal and sickle RBC membrane ghosts with and without inserted oxysterols were examined by Fourier transform infrared spectroscopy. The data are consistent with a greater sterol content in sickle cells compared to normal RBC membranes, and a possible oxysterol-cholesterol synergism.

Introduction

The sickle red blood cell (RBC) membrane has numerous abnormalities, including an altered phospholipid organization upon deoxygenation, a tendency to vesiculate and an abnormal propensity to adhere to vascular endothelial cells, macrophages, and model membranes [1,2]. Changes in the cell membrane such as lessened deformability [3], changes in ion and water transport [4], membrane lipid loss [5], changes in phospholipid asymmetry [6], as well as other alterations [2,7] occur. It is unclear how changes in sickle RBC membrane molecular species may affect these processes.

Autoxidation occurs in sickle red blood cells in which oxygen radicals are produced at approximately 2–3-times the amount formed in normal cells [4]. All components of RBC membrane (i.e., phospholipids, cholesterol and proteins) could be oxidized under these conditions. The effects of oxygen free radicals on membrane cholesterol could be equally or possibly more important then the effects observed on membrane phospholipids or proteins [9]. Oxidation of cholesterol in RBC membranes has been previously induced in controlled experiments [9]. When cholesterol is in a dispersed state, the 5-,6- double bond seems to be the most reactive position, and yields oxidation products such as α- and β-isomers of 7-hydroxycholesterol, 7-ketocholesterol, and 5α-cholestane-3β,5,6β-triol via 5- and 7-hydroperoxide intermediates [10–12]. Smith and Van Lier found 12 oxidation products of cholesterol in atheromata of man suggesting that oxysterols (OS) may exist in humans in biologically significant concentrations [13]. Oxidation of cholesterol would lead to accumulation of oxysterols (OS) and alter membrane fluidity and organization. As such it could have significant effects on cell function, morphology and viability [14].

It has been previously shown that oxidation products of cholesterol affect the phase characteristics of bilayer [15–20] and non-bilayer [21] forming lipids. Since non-lamellar lipid phases have been correlated to processes involving membrane fusion and lysis [22–24], the studies of non-bilayer forming lipids are particularly important. Specifically, 7α-hydroxycholesterol has been shown to decrease the $L_\alpha \rightarrow H_{II}$ phase transition for DEPE when compared to the effect of cholesterol, while 5α-cholestane-3β,5,6β-triol has been shown to

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increase this transition temperature [21]. These observations are consistent with the previous finding that 7α-hydroxycholesterol promotes echinocytosis of red cells [14], a process involving greater membrane curvature, which could result from the greater propensity for lipids to form non-bilayer phases with greater curvatures. Less consistent, is the report that the presence of small amounts of 25-hydroxycholesterol dramatically increased glucose transport in lipid vesicles [20] since this oxysterol appears to minimally affect both bilayer [15–20] and non-bilayer (Lis, L.J. et al., unpublished observation) phase packing and stability. In addition, it has been previously shown, using vibrational spectroscopy, that cholesterol depletion and enrichment of normal human erythrocyte membranes can alter lipid acyl chain mobility and conformation as well as protein secondary structure [25]. We have shown that inserted OS synergistically modulate the fluidizing/condensing properties of cholesterol in the erythrocyte membrane [26]. Further, specific OS were shown to enhance erythrocyte membrane protein helicity [26].

In this report, we used two-dimensional thin-layer chromatography (TLC) and gas chromatography/mass spectrometry (GC/MS) for the first time to characterize the OS present in lipid extracts of normal and sickle red blood cell membranes. Differences in lipid conformation and packing in normal and sickle red blood cell membrane ghosts were examined using Fourier transform infrared (FTIR) spectroscopy. It was expected that moieties within lipid molecules of a normal or diseased red blood cell membrane would vibrate, rotate and sometimes translate in a variety of ways. Correlations are made with normal and sickle red blood cell membrane ghosts with various inserted oxysterols [25].

Materials and Methods

Freshly drawn blood was washed three times in phosphate-buffered saline (0.15 M NaCl/0.001 M PO_4 (pH 7.5)) to remove the plasma and buffy coat. Samples were spun for 15 min at $1500 \times g$ in an IEC-SW bucket after each wash. Hypotonic phosphate buffer (pH 8.0) was added to the pelleted sample in order to lyse the red blood cells. The solutions were then centrifuged at $2000 \times g$ for 10 min. The lysed cells were washed 3–5 times in the same buffer until the pellet was light pink to colorless in appearance. Hemoglobin retention was found to be a problem in the production of both normal and sickle RBC membrane ghosts. Ghost pellets were then centrifuged at $350000 \times g$ for 1 h at 4°C. The pelleted ghost sample was then stored at approx. 0°C before FTIR spectroscopic examination.

Oxidized sterols were inserted into the red cell membrane as described in Rooney et al. [26]. Freshly drawn red blood cells, as well as those with inserted oxysterol compounds, were subsequently washed three times in phosphate-buffered saline (4°C), and ghosts were prepared as previously described. Ghosts were then centrifuged at $350000 \times g$ for 1 h at 4°C. The additional oxysterol content of red blood cell membrane ghosts, with inserted oxysterols, prepared in this manner increased to approx. 10% of the total membrane sterol. It is expected that oxysterols make up typically less than 10 mol% of the total sterol content of normal or sickle RBC membranes.

This final ultracentrifugation was omitted if lipid extracts were to be obtained. In this case chloroform/methanol (1:2, v/v) was added to the red blood cell membrane ghost pellet until a final volume of four times the RBC ghost pellet volume was achieved. The solution was stirred for an hour at room temperature. A 1:1 (v/v) solution of chloroform/methanol was mixed with the extraction solution in a separatory funnel. The aqueous and organic phases were allowed to separate overnight. The bottom phase containing the chloroform/lipid phase was then collected.

The anti-oxidant BHT was added to some samples prior to lipid extraction to determine if oxidation of cholesterol was significant during our sample preparation procedure. Similar results were obtained for samples with and without BHT added.

Thin-layer chromatography

All solvents used for chromatography were analytical reagent grade and used without further purification. Silica-gel and fluorescent silicon-gel chromatography plates (20 cm × 20 cm × 0.25 mm) were obtained from Sigma (St. Louis, MO). Samples were applied to the start line 1 cm from one end as chloroform solutions for one-dimensional chromatography. Irrigation in a closed glass chamber lined with paper was conducted at room temperature, ascending with the solvent system ethyl acetate/heptane (1:1, v/v) for 30–40 min. Irrigated plates were dried momentarily in air prior to multiple irrigation with the same solvent or for visualization.

Samples for two-dimensional irrigation were spotted at a point 1 cm above the lower edge and 1 cm from the left edge of a 20 × 20 cm chromatoplate and irrigated in one dimension with ethyl acetate/heptane (1:1, v/v) in the usual manner. The chromatoplate was dried momentarily and reirrigated with the same solvent system, after which it was irrigated in the second dimension with acetone/heptane (1:1, v/v) twice. Further irrigations improved resolution.

Air dried chromatoplates were routinely examined under ultraviolet light for the presence of 7-ketocholesterol prior to the standard visualization procedure which consisted of spraying the chromatoplate with $FeCl_3$ followed by heating at 100°C for 10 min.

Gas chromatography / mass spectrometry

Gas chromatography of derivatized sterol samples and lipid extracts was performed on two HP-5890 gas chromatographs equipped with flame ionization detectors. Two 60-m HP Ultra II capillary columns were used for the analysis. Gas chromatography/mass spectrometry of these samples employed a JEOL OX505 double focusing mass spectrometer also equipped with a HP-5890 gas chromatograph. A 60-m HP Ultra II capillary column was directly interfaced into the mass spectrometer.

Before lipid extraction an external standard of cholesterol butyrate was added to all RBC samples. Preparatory thin-layer chromatography of the lipid extracts of RBCs was done and the regions below and above the cholesterol spot were scraped off the chromatoplate and washed before submitting them for gas chromatography. The presence of large amounts of cholesterol in normal and sickle RBC membranes results in the presence of a cholesterol tail in the TLC region descriptive of oxysterols, and a cholesterol peak dominance in the GC pattern. The scraping of the TLC regions above and below cholesterol allow us to minimize the influence of cholesterol on the gas chromatogram and to maximize the identification of oxysterols. However, this results in an inability to determine the absolute amount of oxysterols present in our samples. A standard curve of cholesterol butyrate was generated for quantitation of relative oxysterol content for GC tracings.

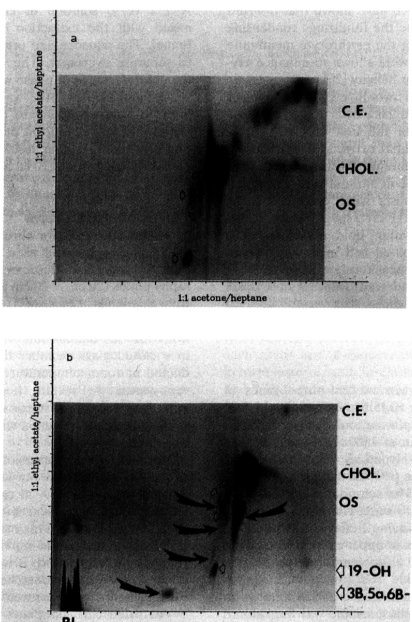

Fig. 1. Typical two-dimensional thin-layer chromatograms of the total lipid extracts of (a) normal and (b) sickle RBC membranes. Open arrows show oxysterol spots present on both normal and sickle samples and solid arrows show oxysterols present only on sickle sample; CHOL denotes cholesterol, OS oxysterol, C.E. cholesterol esters, PL phospholipids, 19-OH 19-hydroxycholesterol and 3B,5α,6B cholestane-3β,5α,6β-triol).

Fourier transform infrared spectroscopy

Approximately 50 μl of sample was transferred at 20°C to a sample cell equipped with AgBr windows. Spectral recording was performed on a Nicolet 7199 Fourier transform infrared spectrometer using parameters previously described [25,26]. A liquid nitrogen cooled HgCdTe detector was used to increase the signal to noise ratio for these samples. Wave number stability was accurate to ± 0.01 cm^{-1}. Band positions were determined at peak maxima by a Nicolet computer algorithm using ten transform points per wave number. The techniques for background subtraction have been previously described [26]. Typically 3–15 samples were examined for each system studied.

Results and Discussion

Thin-layer chromatography

Lipid extracts from normal and sickle RBC membranes were examined using two-dimensional TLC with irrigating systems that only allow non-polar lipids to migrate. Representative two-dimensional thin-layer chromatograms for each type of lipid extract are shown in Fig. 1. Comparisons with standard oxysterols were made in identification of spots obtained from the total lipid extracts (TLE) of normal and sickle RBC membranes on the chromatoplates. The derived position for the standards relative to cholesterol were consistent with previous reports [10,11]. The spot mobilities in each solvent were found to be reproducible between samples if determined relative to the mobility of cholesterol. Sixteen or fewer spots were observed for normal RBC lipid extracts, while 21 or fewer spots were observed for sickle RBC lipid extracts. Currently, we have identified eight of the spots: 5α,6α-epoxycholesterol, cholestane-3β,5α,6β-triol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, 20α-hydroxycholesterol and 25-hydroxycholesterol. We would expect that oxysterols are dynamically exchanged between the RBC membrane and free or bound cholesterol in blood plasma. However, it is expected that an equilibrium is reached between the various molecules involved in the process.

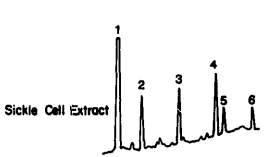

Fig. 2. Typical gas chromatogram of a sickle RBC membrane total lipid extract with cholesterol butyrate added for quantitation. 1 = Cholesterol, 2 = 19-hydroxycholesterol, 3 = 5α,6α-epoxycholesterol, 4 = cholestane-3β,5α,6β-triol, 5 = 7-ketocholesterol, 6 = cholesterol butyrate.

TABLE I

Mean oxysterol levels by gas chromatographic quantitation using cholesterol butyrate as internal standard in sickle (n = 3) and normal (n = 4) red cell lipid extracts (μg / ml RBC)

Oxysterol	Normal RBC	Sickle RBC
Cholestane-3β,5α,6β-triol	0.02	1.20
5α,6α-Epoxycholesterol	0.29	1.10
7-Ketocholesterol	0.23	0.90
19-Hydroxycholesterol	0	0.60

Gas chromatography / mass spectrometry

GC/MS results are consistent with the presence of oxidized sterols in both normal and sickle RBC membranes (Fig. 2). There are significantly greater amounts of 5α,6α-epoxycholesterol, 5α-cholestane- 3β,5,6β-triol, 7-ketocholesterol and 19-hydroxycholesterol, for example, in sickle compared to normal RBC membrane lipids (Table I). It is clear that the increase in sickle red cell membrane oxysterol content must be due to increased oxidant stress. The high levels of increases could not be justified on the basis of the relatively slight increase in cholesterol reported [27] in sickle cell disease. Air oxidation can be ruled out [10,11] as the cause of the appearance of both 5α, 6α-epoxycholesterol and cholestane-3β,5α,6β-triol. Preferential oxidation at the 5- and 6-positions of cholesterol is due to the presence of hydroxyl radicals [9,10]. Thus, our results suggest that the greatest oxidant effect in sickle RBC membranes is due to hydroxyl radicals. Currently, we are in the process of identifying and quantitating the remaining unidentified oxysterol peaks of the GC tracing using oxysterol standards and mass spectrometry.

Fourier transform infrared spectroscopy

Vibrational spectroscopy (i.e., Fourier transform infrared and Raman spectroscopy) have been used to study molecular conformation and organization of erythrocyte membranes [25,26,28–31]. The frequency, intensity and linewidth of particular vibrational spectral features are extremely sensitive to packing constraints, conformational changes, and chemical variations of lipids and proteins which form the RBC membranes. Bands which have been previously used in the study of lipids [32–34] are the CH_2 stretch (2850 and 2920 cm^{-1}), bending or scissoring (1450–1480 cm^{-1}), and wagging modes (1180–1350 cm^{-1}), the lipid fingerprint region containing phosphate and diester stretch modes (100–1450 cm^{-1}) and the C=O stretch (1700–1750 cm^{-1}). Changes in the peak positions of the methylene vibrations can be used to imer changes in lipid hydro-

carbon chain packing, while changes in the fingerprint region and head group carbonyl stretch vibrations can be used to infer changes in molecular packing involving the lipid head groups.

The lipid packing in red blood cell membrane ghosts has been previously examined as a function of sterol content using FTIR spectroscopy [25]. It was shown that for all temperatures, RBCs with an enhanced cholesterol content had a lower frequency, while RBCs with a depleted cholesterol content had a higher frequency for the C-H stretch band when compared to the native RBC. These results would indicate that increased cholesterol content decreased the mobility of the lipid acyl chains in the ghost membrane, while a decrease in the cholesterol content increased the mobility of the lipid acyl chains. This result was consistent with previous studies of the effect of cholesterol on biological and lipid model membranes. In addition, comparison between membrane lipid packing could be made at a single temperature, where the higher the sample temperature (and the disorder in the membrane), the greater the difference between C-H stretch band positions.

In the present study, RBC membrane ghosts and lipid extracts were obtained from at least three normal individuals and three sickle cell patients, and examined using FTIR spectroscopy. The positions of the C-H stretch bands were effectively the same, indicating that the lipid acyl chain packing in the normal and sickle RBC ghost membranes are the same. This is not unexpected since it would be unlikely that changes in lipid and protein packing could be significantly altered to cause one membrane to be less fluid than the other.

Phospholipid head group vibrations were also examined in the ghost samples in the spectral region 1000–1350 cm^{-1} (Fig. 3). It has been previously shown that the presence of oxidized sterols can change the position of bands from RBC membranes in this region [25]. Specifically, it was shown that the insertion of either 7α-hydroxy- or 20α-hydroxycholesterol causes the position of the asymmetrical P=O stretch band to decrease from a position of $\approx$ 1260 cm^{-1} observed in a normal RBC to a position of $\approx$ 1230 cm^{-1}. This observation would indicate the presence of a greater number of sterol-phospholipid contacts in the membrane. In addition, the insertion of 7α-hydroxycholesterol caused a splitting in the symmetric P=O stretch band from a single band at $\approx$ 1080 cm^{-1} into two bands at 1060 and 1090 cm^{-1}. This observation (as well as the splitting in the C-O-C stretch band at $\approx$ 1170 cm^{-1} observed for oxysterols inserted in model membranes) was considered to be caused by an increase in the packing between lipid head groups at the membrane/water interface in the presence of inserted 7α-hydroxycholesterol resulting in the presence of two preferential vibrational directions.

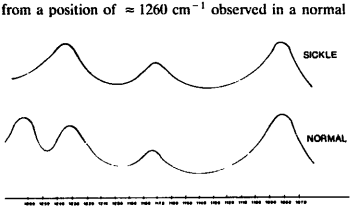

Fig. 3. Infrared phosphate diester stretch bands from normal and sickle red blood cell membrane ghosts at approx. 25°C.

The FTIR lipid head group fingerprint region for membrane ghosts obtained from normal and sickle RBCs in this study is shown in Fig. 3. There was no change in the sym. P=O or asym. C-O-C stretch band positions in the spectra of the sickle and normal RBC ghost membranes, again indicating no difference in the lipid packing within either membrane ghost. A striking change, however, was observed in the position of the sym. P=O stretch band. A band position of $\approx$ 1260 cm^{-1} was observed for the normal RBC membrane as previously reported by Rooney et al. [25]. In addition, in some but not all samples, an additional band was observed at $\approx$ 1230 cm^{-1} possibly due to the presence of sterol rich domains in some of the RBC membranes. In contrast, the P=O sym. stretch band for the membrane ghost from sickle cells appeared at $\approx$ 1230 cm^{-1} with no evidence for a second band at $\approx$ 1260 cm^{-1} in any of the samples studied. In order to probe the possible source of this spectra difference we examined normal and sickle red cell membrane ghosts with inserted oxysterols.

The phospholipid head group vibrations (sym. and asym. P=O, and sym. and asym. C-O-C stretch bands) were thus examined in these samples in the spectral region 1000–1350 cm^{-1}. It was previously shown that the presence of oxysterols can change the position of the RBC membrane spectral features in this region. Specifically, it was shown that the insertion of 7α-hydroxy- or 20-α-hydroxycholesterol causes a downward shift in the frequency of the sym. P=O stretch band from $\approx$ 1260 cm^{-1} observed in normal RBC membranes to $\approx$ 1230 cm^{-1}. This observation would indicate the presence of a greater number of sterol-phospholipid contacts in the RBC membranes with inserted oxysterols. The insertion of 7α-hydroxycholesterol was also shown to cause a splitting in the sym. P=O stretch band at $\approx$ 1080 cm^{-1} into two bands at $\approx$ 1060 and $\approx$ 1090 cm^{-1}. This observation is consistent with a closer packing in the RBC membrane lipids in the presence of 7α-hydroxycholesterol.

The FTIR lipid head group fingerprint region was examined in membrane ghosts obtained from normal

TABLE II

FTIR peak positions for normal RBC membranes with inserted oxysterols at approx. 20°C

Inserted oxysterol	Peak position		
	sym. P=O stretch	asym. C-O-C stretch	asym. P=O stretch
None	1081.5 ± 0.4	1171.2 ± 0.5	1232.1 ± 0.5 1261.0 ± 1.0
5α-Cholestane-3β,5,6β-triol	1081.1 ± 0.7	1173.0 ± 0.4	1228.0 ± 0.7
7α-Hydroxycholesterol	1083.4 ± 1.0	1170.9 ± 0.5	1233.4 ± 1.4
7β-Hydroxycholesterol	1085.4 ± 2.0	1174.3 ± 0.5	1230.8 ± 1.5
7-Ketocholesterol	1082.0 ± 1.0	1171.2 ± 0.5	1228.4 ± 0.6
20α-Hydroxycholesterol	1081.0 ± 0.5	1172.7 ± 0.4	1231.1 ± 2.0
25-Hydroxycholesterol	1083.9 ± 0.5	1173.1 ± 0.4	1226.3 ± 0.7

RBCs into which various oxysterols were inserted. In general, these results (see Table II) are consistent with those previously reported by Rooney et al. [25]. The major observation was a change in the position of the asym. P=O stretch band from ≈ 1260 cm^{-1} for the normal RBC membrane to ≈ 1230 cm^{-1} for RBC membrane with inserted oxidized sterols. In addition, in the spectra from some normal RBC membrane ghosts, a band at ≈ 1230 cm^{-1} was also observed. However, in no RBC membrane samples with inserted oxidized sterols was a 1260 cm^{-1} band observed. It can be reasoned that some native RBC membrane samples have a greater amount of sterol rich domains than others, but that in all cases there are domains where the lipid-sterol contacts are minimized.

The FTIR lipid head group fingerprint region for membrane ghosts from sickle cells into which various oxysterols were inserted were examined with specific band positions listed in Table III. The change in the asym. P=O stretch band from ≈ 1260 cm^{-1} for native RBC's to ≈ 1230 cm^{-1} for sickle RBCs was previously discussed. This result would indicate the presence of a greater number of sterol-phospholipid contacts in sickle RBC membranes as compared to normal RBC membranes by analogy to the previous observations obtained for normal RBC membranes with inserted oxysterols. It is clear that there are no differences in the sickle RBC membrane symmetric P=O stretch band positions upon insertion of any of the oxysterols studied. This was unexpected, since it seemed likely that the insertion of oxysterols would increase the amount of sterol-phospholipid contacts in the sickle RBC membrane and shift this peak position to an even lower frequency. This finding would suggest that a saturation of the membrane sterol content may be present in relation to the contact of sterol and phospholipid molecules in the sickle RBCs. The source of the additional sterol-phospholipid contacts cannot be determined from the spectral data.

It can be concluded from our FTIR spectroscopic studies that there are a greater number of sterol-phospholipid contacts in sickle as compared to normal RBC membranes. This result is consistent [26] with an increase in the sterol content of the sickle RBC either with cholesterol and/or oxysterols. It is less likely that this observation is solely due to an increase in cholesterol which would require a larger increase in content [25] than observed in sickle red cell membranes [27]. However, a synergism [26] between oxysterols and cholesterol could account for this observation.

TABLE III

FTIR peak positions for sickle RBC membranes with inserted oxysterols at approx. 20°C

Inserted oxysterol	Peak position		
	sym. P=O stretch	asym. C-O-C stretch	asym. P=O stretch
None	1082.7 ± 0.5	1171.8 ± 2.0	1231.9 ± 4.5
5α-Cholestane-3β,5,6β-triol	1081.7 ± 0.7	1173.3 ± 1.0	1228.4 ± 4.2
7α-Hydroxycholesterol	1083.9 ± 0.4	1171.2 ± 1.5	1232.0 ± 1.9
7β-Hydroxycholesterol	1082.7 ± 0.6	1173.1 ± 1.0	1229.7 ± 3.1
7-Ketocholesterol	1082.9 ± 0.9	1164.6 ± 2.5	1239.1 ± 3.9
20α-Hydroxycholesterol	1083.9 ± 1.0	1171.7 ± 1.8	1231.6 ± 2.0
25-Hydroxycholesterol	1084.0 ± 1.0	1171.9 ± 1.6	1230.2 ± 2.3

Conclusions

The study shows for the first time that oxysterols are present in both normal and sickle RBC membranes with a higher concentration in the sickle RBC membranes. In addition, lipid packing and organization differ between normal and sickle RBC membranes. These results are compatible with both a greater sterol content and an increased oxidative stress in sickle RBC membranes resulting in a synergism between oxysterols and cholesterol. Further studies are underway to explicitly determine the role of oxysterols in influencing red cell function.

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