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RED BLOOD CELL [^{14}C]CHOLESTEROL EXCHANGE AND PLASMA CHOLESTEROL ESTERIFYING ACTIVITY OF NORMAL AND SICKLE CELL BLOOD

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The present study performed on density fractions of sickle and normal erythrocytes prepared on Stractan density gradient shows that dense erythrocytes have consistently decreased uptake of [^{14}C]cholesterol from plasma in comparison to young, less dense erythrocytes. Plasma of sickle cell patients also shows a reduction in cholesterol-esterifying activity in comparison to normal controls. A possible effect of these processes in the increased cholesterol to phospholipid molar ratio of irreversibly sickled cells has been suggested.

Introduction

Cholesterol constitutes nearly 40% of the membrane lipids and is vital in the maintenance of its structural and functional integrity [1–3]. In contrast to reticulocytes, mature erythrocytes lack enzymes for de novo lipid synthesis [1,2]. The major pathway for the renewal of membrane cholesterol in mature erythrocytes is through its exchange with plasma cholesterol [4,5]. In our previous study we have reported that a portion of aminophospholipids of sickle cells are cross-linked through malonyldialdehyde, an end product of lipid peroxidation [6]. In order to determine if cross-linking of membrane phospholipids might affect the cholesterol exchange process, we have studied the exchange rate of [^{14}C]cholesterol between various Stractan density gradient separated sickle cell fractions and normal plasma and compared with the exchange rate in normal cell fractions. We have also studied plasma cholesterol-esterifying activity and erythrocyte cholesterol levels of blood from normal and sickle cell patients.

Methods

Blood was drawn from volunteers or sickle cell patients into heparinized tubes and then passed through a cotton wool column to remove white cells. The blood was then centrifuged at $1500 \times g$ for 5 min at 4°C. The residual cells were similarly washed three times with cold isotonic saline.

Separation of irreversibly sickled cells. Washed sickle cells were suspended in phosphate-buffered saline containing potassium and glucose (20 mM, pH 7.4, 290 mosM) to approx. 30% hematocrit and layered on a continuous Stractan gradient as described by Clark et al. [7]. The top-most layer in the gradient (approx. 5%) containing reticulocytes was discarded. The layer just below the top-most layer (now called the top layer) and the bottom layer of the gradient, each of which constituted about 5% of the total red cells, were then removed with a Pasteur pipet. These cells were washed four times with saline before their incubation with the [^{14}C]cholesterol plasma or lipid extraction. This

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method produced enrichment of irreversibly sickled cells in the bottom fraction of the gradient to approx. 70–80%. There were no irreversibly sickled cells or reticulocytes in our top layer of discocytes. Normal blood was also processed simultaneously and top and bottom fractions were collected to serve as controls.

Lipid extraction and quantitation. The lipids of the washed erythrocytes and plasma were extracted by the methods of Rose and Oklander [8] and Folch et al. [9], respectively. Phospholipid-phosphorus was quantitated by the method of Fiske and SubbaRow [10]. Cholesterol was determined by the method of Zlatkis et al. [11].

Preparation of [^{14}C]cholesterol plasma. [^{14}C]Cholesterol plasma was prepared by incubating 10 μCi of [^{14}C]cholesterol (ICN Chemical and Radioisotope Division, Irvine, CA) (specific activity, 58.4 mCi/mmol) with 10 ml of heat-inactivated normal human plasma (55°C for 30 min) in a shaking water bath. Heat-inactivated plasma containing [^{14}C]cholesterol was then centrifuged for 10 min at 15000 × *g* in a refrigerated centrifuge and a clear supernatant was used for cholesterol exchange studies with erythrocytes or as a substrate for plasma lecithin-cholesterol acyltransferase assay.

Plasma lecithin-cholesterol acyltransferase activity determinations. 100 μl of the test plasma was incubated with 400 μl of the substrate for 2 h in a water bath at 37°C. At the end of incubation, the reaction was stopped by adding 2 ml each of methanol and chloroform. The tubes were allowed to stand for 1 h at room temperature, during which period contents of the tubes were vortexed three times. The tubes were then centrifuged at 800 × *g* for 10 min and 2 ml of the chloroform-methanol extract was transferred to another tube and dried with nitrogen. The free and esterified cholesterol in the lipid extract were separated by thin-layer chromatography on the Silica gel G plates (Brinkman, Inc., Westbury, NY) using solvent system consisting of petroleum ether/ether/acetic acid (90:10:1, v/v) as described previously [12]. The free cholesterol and cholesterol ester silica gel spots were scraped into counting vials and the ^{14}C-radioactivity was determined using a Packard Scintillation Spectrometer, Model 3375.

[^{14}C]Cholesterol exchange between erythrocytes and plasma. 500 μl of the packed cells were suspended in 1 ml of [^{14}C]cholesterol plasma in test tubes. Contents were incubated at 37°C in a shaking water bath for various times. At the end of incubation time, 250 μl of the cells were washed with 4 ml of cold saline three times. The washed cells were subjected to lipid extraction as described above. [^{14}C]Cholesterol was counted in an aliquot of the lipid extract and results were expressed as [^{14}C]cholesterol counts/mg of total red cell cholesterol.

Results

The rate of uptake of [^{14}C]cholesterol by normal and sickle cells is given in Fig. 1. All fractions of sickle cells had appreciably decreased uptake of [^{14}C]cholesterol when incubated in [^{14}C]cholesterol serum in comparison to corresponding normal cell fractions. The rate of cholesterol exchange was nearly 25% less in irreversibly sickled cell-enriched bottom fraction in comparison to discoid sickle top or bottom fraction of normal cells.

Irreversibly sickled cells and bottom fraction of normal erythrocytes are known to contain significantly more malonyldialdehyde cross-linked between aminophospholipids in comparison to correspondingly less dense fractions of erythrocytes [6,13]. To determine if cross-linking of membrane

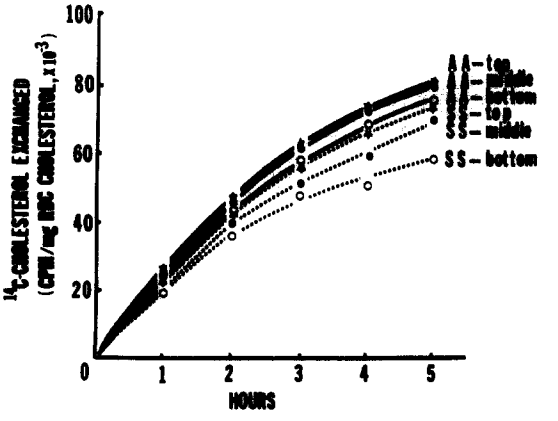

Fig. 1. The rate of [^{14}C]cholesterol exchange between normal and sickle cell fractions with normal plasma. Conditions of incubations are as given in Materials and Methods. A duplicate experiment gave similar results. 1000 CPM are equivalent to 10.21μg cholesterol.

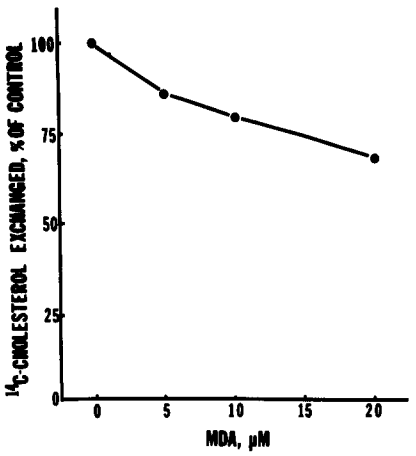

Fig. 2. The effect of malonyldialdehyde treatment to normal erythrocytes on their cholesterol exchange with normal plasma. Normal erythrocytes were treated with malonyldialdehyde (MDA) for 1 h [13]. Washed malonyldialdehyde-treated erythrocytes were then incubated with [^{14}C]cholesterol plasma for 2 h at 37°C in a shaking water bath. Other conditions are as given in Materials and Methods. A duplicate experiment gave similar results.

TABLE I

PLASMA LECITHIN-CHOLESTEROL ACYLTRANSFERASE (LCAT) ACTIVITY OF NORMAL AND SICKLE CELL PATIENTS

Values are mean±S.D. Number of samples are given in parentheses. Differences between the values were statistically significant ($P<0.01$).

	LCAT (μg cholesterol esterified per ml plasma per hour)
Normal (6)	39.2±5.0
Sickle cell (10)	27.7±5.4

phospholipid or proteins has any effect on the cholesterol exchange process, we have studied the [^{14}C]cholesterol exchange in normal erythrocytes treated with malonyldialdehyde. Fig. 2 shows that [^{14}C]cholesterol uptake was reduced after treatment of erythrocytes with malonyldialdehyde.

Plasma lecithin-cholesterol acyltransferase activity of sickle cell patients is given in Table I. Plasma of sickle cell patients also shows a reduction (35%) in cholesterol-esterifying activity in comparison to normal controls. This reduced lecithin-cholesterol acyltransferase activity may be due to the known inhibitory effect of bilirubin on cholesterol esterification. Elevated levels of plasma bilirubin are known to be associated with sickle cell patients (Ballas, S.K. (1981), personal communication).

Cholesterol and phospholipid composition of top and bottom fractions of normal and sickle blood separated on Stractan density gradient is given in Table II. There was consistently more lipid in the top fractions of the normal and sickle blood as compared to bottom fractions. A significant increase in the cholesterol to phospholipid molar ratio was observed in irreversibly sickled cell-enriched bottom fraction as compared to discoid-top sickle cells or normal-bottom cells.

Discussion

The level of erythrocyte membrane cholesterol is regulated by its passive exchange with plasma cholesterol [4,5] and by plasma enzyme lecithin-cholesterol acyltransferase, which esterifies cholesterol to cholesterol ester utilizing un-

TABLE II

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF NORMAL AND SICKLE CELLS SEPARATED ON STRACTAN DENSITY GRADIENT

Values are mean±S.E. of six observations. Differences between 'B' and 'D', and 'C' and 'D' were statistically significant ($P<0.05$).

	Normal cells		Sickle cells	
	Top	Bottom	Top	Bottom
Cholesterol/cell (mg)($\times 10^{10}$)	1.45±0.18	1.20±0.07 [B]	1.52±0.011	1.43±0.08 [D]
Phospholipid/cell (mg)($\times 10^{10}$)	2.78±0.12	2.64±0.17	3.32±0.09	2.53±0.11
Cholesterol/phospholipid molar ratio	1.04±0.05 [A]	0.91±0.06 [B]	0.93±0.04 [C]	1.13±0.06 [D]

saturated fatty acid of erythrocyte phosphatidylcholine. In conditions with decreased plasma lecithin-cholesterol acyltransferase, an elevated level of erythrocyte cholesterol has been reported [14,15].

Concentrations of erythrocyte lipids in patients with sickle cell anemia were first reported by Westerman et al. [16]. They reported that nonfractionated cells have increased cholesterol as well as phospholipid, in comparison to normal cells. Rice-Evans et al. [17] have also found elevated erythrocyte cholesterol but no change in phospholipid level in their sickle cell patients. However, recent studies of Clark et al. [18] and Westerman et al. [19] have shown that lipid profile in the membranes of sickle cells may not be abnormal when corrected for the significant effects of cell age and contaminating reticulocytes.

The present study carried out on the Stractan density gradient fractionated sickle and normal cells shows that dense normal erythrocytes have consistently decreased uptake of [^{14}C]cholesterol from plasma in comparison to young, least dense erythrocytes. Similarly, [^{14}C]cholesterol in dense irreversibly sickled cell-enriched sickle cells is considerably reduced in comparison to discoid-least dense sickle cells and dense normal erythrocytes. Increased cholesterol in dense sickle cells fraction is unlikely to be due to cell age since the phospholipid levels are not increased as would be expected for any major effect due to young cells. One possible explanation for decreased exchange of cholesterol in irreversibly sickled cells is that their membranes have undergone cross-linking of aminophospholipids present in the inner bilayer of the erythrocyte membrane as a consequence of exposing to malonyldialdehyde, an end product of lipid peroxidation [6,20]. This may not facilitate the exchange of cholesterol from the inner bilayer to outer bilayer of the membrane during the exchange process. In fact, decreased uptake of [^{14}C]cholesterol was also observed in in vitro malonyldialdehyde-treated erythrocytes (Fig. 2).

Thus, loss of endogeneous membrane cholesterol known to be associated with cell aging may be slow in sickle cells as compared to normal erythrocytes. This, coupled with the decreased plasma lecithin-cholesterol acyltransferase activity limiting exogenous cholesterol uptake, may result in elevated cholesterol levels in irreversibly sickled cells. Elevated erythrocyte membrane cholesterol has been proposed to cause decreased membrane fluidity and changes in membrane permeability characteristics [1,2]. Hence, altered mechanical and/or permeability properties of irreversibly sickled cell membranes [21,22] may be related to the membrane cholesterol content of these unusual cells.

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