CHANGES OF OSMOTIC FRAGILITY OF RED BLOOD CELLS DUE TO REPLETION OR DEPLETION OF CHOLESTEROL IN HUMAN AND RAT RED CELLS IN VITRO

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(Received December 24, 1979)

Summary The relation of membrane lipids and osmotic fragility of red cells of human and rat were studied in vitro by repletion and depletion of cell cholesterol. A decrease in the red cell cholesterol corresponded to an increase in osmotic fragility. Conversely, a repletion of red cell cholesterol decreased the osmotic fragility, but the procedure did not significantly change the phospholipid contents of red cells. Accordingly, osmotic fragility of the red cells correlated highly with the cholesterol content of the cell membrane \( r = -0.944, p < 0.001 \) and with the molar ratio of cholesterol to phospholipid in the red cells \( r = -0.887, p < 0.001 \). Red cells incubated with plasma had decreased membrane cholesterol and increased osmotic fragility, but the change was prevented by the inactivation of lecithin cholesterol acyltransferase (LCAT) in the plasma.

The above results confirm that membrane cholesterol stabilizes human and rat red cells in vitro as well as in vivo, and the content of red cell cholesterol is regulated by the exchange of plasma free cholesterol in vitro. LCAT activity in the plasma is an influential factor in controlling the cholesterol content of red cells.

Keywords osmotic fragility, red cell cholesterol, red cell phospholipids, C/P ratio, plasma LCAT

Previously we reported the role of membrane cholesterol in stabilizing rat red cells in vivo.(10), that is, red cells obtained from splenectomized rats have more cholesterol in the membrane and are less fragile than those of intact rats and, conversely, red cells obtained from rats with splenomegaly have decreased cholesterol and increased osmotic fragility. Additionally, we have found that the loss of cholesterol from red cells is closely related to concentration of esterified cholesterol in the plasma.(11). The exchange of cholesterol between red cell and
plasma is accomplished within a few hours (3). Cooper and Jandle (2) reported that the incubation of human red cells in serum obtained from patients with obstructive jaundice whose plasma was high in cholesterol and phospholipids resulted in an increase in cholesterol and a decrease in osmotic fragility with time of the incubation.

Grunze and Deuticke (6) reported a method for depleting the cholesterol of red cells in vitro. The present study was designed to elucidate the relation of membrane cholesterol to fragility of red blood cells by using procedures to change membrane cholesterol in vitro. We repleted or depleted the content of membrane cholesterol in vitro, so that we could examine the relationship between membrane lipids and osmotic fragility of the red cells. Lecithin cholesterol acyltransferase (LCAT) is an enzyme that esterifies cholesterol in the plasma and this enzyme is inactivated by heating the plasma at 56°C for 30 min. Therefore, in this experiment, we incubated red blood cells in LCAT-inactivated plasma and examined the changes in lipid contents and osmotic fragility of the red cells in comparison with those of cells incubated in fresh plasma.

**MATERIALS AND METHODS**

Blood of male Wistar rats, weighing about 250 g, was withdrawn by heart puncture into a heparinized syringe, and blood of a normal human was withdrawn from an antecubital vein into a heparinized syringe in the postabsorptive period. Centrifugation of the blood of both species was carried out at 5°C, and the plasma was pooled cool until use. The red cells were washed three times with cold isotonic phosphate buffer (pH 7.4) containing 45 mM of glucose.

**Cholesterol depletion in red cell.** Membrane cholesterol of rat red cells was depleted by incubating the cells in suspensions of lecithin liposomes for 6 hr at 37°C by the methods of Grunze and Deuticke (6). The packed cell volume in the suspensions was adjusted to 8–10%. The suspensions were prepared by sonicating 200 mg of egg lecithin (Sigma Chemical Co.) in an N₂-saturated medium of the following composition (in mM): NaCl 140; Na₂HPO₄/NaH₂PO₄ 12.5; glucose 45; penicillin 8 mg/100 ml; streptomycin 20 mg/100 ml; pH 7.4. During sonication, the container of the medium was placed in ice water.

**Cholesterol repletion of red cell in plasma-free medium.** For the repletion of cholesterol in red cell membranes, red cells were incubated in medium adding dispersions of cholesterol and lecithin. The dispersions were prepared by sonicating 200 mg of egg lecithin (Sigma Chemical Co) in an N₂-saturated medium of the following composition (in mM): NaCl 140; Na₂HPO₄/NaH₂PO₄ 12.5; glucose 45; penicillin 8 mg/100 ml; streptomycin 20 mg/100 ml; pH 7.4. During sonication, the container of the medium was placed in ice water.

**Cholesterol repletion of red cell in the plasma.** The human plasma was divided
into two groups; one was fresh plasma which was stored at 1°C until use, and the other was heated plasma which was placed in a water bath at 56°C for 30 min to inactivate plasma LCAT (5). Each plasma was again divided into two groups, one was control plasma and the other was plasma with cholesterol dispersions prepared by the methods described above. Two-fold of the individual level of cholesterol was added to the corresponding plasma. Therefore, each individual plasma was finally divided into 4 groups. In each plasma, washed homologous red cells were added to reach the hematocrit value of 10%, and they were incubated for 24 hr at 37°C with gentle shaking. Change in pH of the blood during incubation was found to be very slight (within 0.09 pH unit) at this hematocrit value.

Determination of lipids and osmotic fragility of the red cells. After incubation, the red cells were washed three times with cold isotonic phosphate buffer (pH 7.4). Osmotic fragility of the red cells was measured by a series of hypotonic saline solutions (7). Results are expressed as the concentration of NaCl giving 50% hemolysis, here named the half-hemolysis rate (HHR).

Washed red cells for lipid extraction were resuspended in 0.9% NaCl at a density corresponding to the original hematocrit value, and samples were taken to measure the packed cell volume and lipid content. Red cell and plasma lipids were extracted with a mixture of two volumes of chloroform and one volume of methyl alcohol (4), and portions of the extracts were used to measure total cholesterol, free cholesterol (14), and total phospholipids (12). Values for cell lipids were expressed on the basis of the volume (in ml) of packed cells.

RESULTS

Figure 1 shows the changes in cholesterol, phospholipids and osmotic fragility of red cells in the rats after incubation of red cells in medium with dispersed lecithin. Cholesterol in the membrane decreased by 28.1% from control value \( (p < 0.01) \), but red cells incubated in lecithin-free medium did not change their cholesterol. A decrease in membrane cholesterol corresponded with an increase in osmotic fragility, while no significant change in the phospholipids of red cells was observed due to incubation. Figures 2 and 3 exhibit changes in osmotic fragility and lipids by repletion of cholesterol in human red cells. Figure 2 exhibits a gradual increase in membrane cholesterol with an increase in cholesterol content in the incubation medium, and reversely a decrease in osmotic fragility. However, as shown in Fig. 3, no significant change in membrane phospholipids was observed. Accordingly, the molar ratio of cholesterol to phospholipid (C/P ratio) was increased with increase in cholesterol in the medium. Figure 4 shows the relation of the C/P ratio to the fragility of red cells \( (r = -0.887, p < 0.001) \). The osmotic fragility of red cells decreased gradually with increase in membrane cholesterol. As shown in Fig. 5, cholesterol contents of the red cells incubated in the medium with free cholesterol had a linear correlation with the osmotic fragility of the red cells \( (r = -0.944, p < 0.001) \). Table 1 shows the changes in membrane cholesterol and

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Fig. 1. Changes in lipids and osmotic fragility of rat red cells after incubation in phosphate buffer and lecithin liposome. Results are means ± SD in 4 rats. Level of significance was examined by paired t-test from unincubated red cells: **, p<0.01 and ***, p<0.001.

Fig. 2. Changes in cholesterol content and osmotic fragility of human red cells after incubation in medium with added free cholesterol. Points and bars are means ± SD in 4 subjects. Level of significance was examined by paired t-test from cholesterol-free medium; *, p<0.05, ** p<0.01, and ***, p<0.001.

osmotic fragility of human red cells which were incubated for 24 hr in the plasma with or without addition of cholesterol. Incubation of red cells in the plasma

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Fig. 3. Changes in phospholipid contents and molar ratio of cholesterol to phospholipid in human red cells after incubation in medium with added free cholesterol. Points and bars are means ± SD in 4 subjects. Level of significance was examined by paired t-test from cholesterol-free medium; **, p<0.01.

Fig. 4. Relationship between molar ratio of cholesterol to phospholipid and osmotic fragility.

resulted in a significant increase in osmotic fragility and decrease in membrane lipids, and the changes in the value were greater in red cells incubated in fresh plasma than those of cells incubated in heated plasma. By adding free cholesterol to the plasma the increase in osmotic fragility of the red cells became small and not significant. Moreover, the cholesterol content of the red cells became greater than...
initially. The C/P ratio changed in parallel with the changes in membrane cholesterol in red cells.

DISCUSSION

This work showed that the content of cholesterol and osmotic fragility of red cells could change by incubation in medium addition of sonicated lipid dispersions. Incubation of red cells with lecithin micelles resulted in depletion of the red cell cholesterol, because the phospholipid formed liquid crystals with membrane cholesterol. The increase in osmotic fragility was pronounced as a result of a decrease in the membrane cholesterol. Heating the plasma at 56°C for 30 min inactivates LCAT which esterifies free cholesterol in plasma. Murphy reported that the incubation of normal human red cells in plasma heated for 24 hr at 37°C (pH, 7.5; Hct, 10%) showed no change in osmotic fragility or red cell cholesterol. In the present study, however, an increase in osmotic fragility and a decrease in red cell cholesterol were observed in similar conditions. However, changes in osmotic fragility and red cell cholesterol in heated plasma were smaller than those of cells in fresh plasma. Addition of cholesterol to fresh plasma at two-fold of the initial value prevented changes in osmotic fragility and cholesterol level of the red cells, and addition of cholesterol to heated plasma resulted in an increase in cholesterol of red cells and made the cells less fragile. Since cholesterol in the membrane exchanges rather quickly with free cholesterol in the plasma, reaching equilibrium in 8 to 12 hr either in vivo or in vitro, we speculated that addition of cholesterol to plasma in our experiment led to a new equilibrium by transferring plasma cholesterol to red cells. The above results showed that red cell...
Table 1. Effect of incubation on osmotic fragility and lipids of human red cells.

| Condition                              | Osmotic fragility (HHR, %NaCl) | Cholesterol (mg/ml cells) | Phospholipids (mgP/ml cells) | C/P (M/M) |
|----------------------------------------|---------------------------------|---------------------------|-------------------------------|-----------|
| Red cells, unincubated (initial)      | (5) 0.421 ± 0.009               | 1.812 ± 0.262             | 0.144 ± 0.005                 | 1.08 ± 0.15 |
| Red cells, incubated in:               |                                 |                           |                               |           |
| Phosphate buffer                       | (5) 0.449 ± 0.012               | 1.673 ± 0.220             | 0.133 ± 0.004***              | 1.08 ± 0.14 |
| Fresh plasma                           | (5) 0.504 ± 0.019***            | 1.424 ± 0.232***          | 0.138 ± 0.004**               | 0.89 ± 0.15 |
| Heated plasma                          | (5) 0.470 ± 0.013***            | 1.664 ± 0.291             | 0.134 ± 0.004**               | 1.07 ± 0.18 |
| Fresh plasma + cholesterol             | (5) 0.419 ± 0.029               | 1.868 ± 0.320             | 0.138 ± 0.005**               | 1.17 ± 0.22 |
| Heated plasma + cholesterol            | (5) 0.412 ± 0.025               | 2.019 ± 0.390             | 0.139 ± 0.006                 | 1.25 ± 0.21 |

Values are means ± SD. Numbers in parentheses are numbers of subjects examined. HHR is half-hemolysis rate (percentage concentration of NaCl giving 50% hemolysis). Level of significance was examined by a paired t-test from unincubated red cells: **, p < 0.01 and ***, p < 0.001.
cholesterol correlated with changes in osmotic fragility of red cells as observed \textit{in vivo} (11). However, no detectable change in phospholipid content of red cells during incubation was observed, and this is explained by the fact that phospholipids in the membrane exchange more slowly, lecithin having the highest turnover of less than 10\% in 12 hr (9). Previously, we observed the relation of diameter to cholesterol content of rat red cells, and that a decrease in red cell cholesterol diminished the diameter and surface area of red cells and increased osmotic fragility (11). In this study, we did not measure morphological changes of red cells during incubation, but the surface area of red cells was expected to increase as a result of the accumulation of cholesterol.

The present work showed that red cells incubated with lipid dispersions changed the membrane cholesterol without changing the phospholipid content of red cells. From the above results we conclude that the level of cholesterol in red cells \textit{in vitro} is directly related to the osmotic fragility of red cells, and that free cholesterol in the plasma is readily exchangeable with cholesterol in the cell membrane. Activity of LCAT in the plasma is also an influential factor in changing the level of red cell cholesterol \textit{in vitro}.

A part of this work was carried out in the Department of Nutrition School of Medicine, Tokushima University, Tokushima, Japan.

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