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High-cholesterol Diets Induce Changes in Lipid Composition of Rat Erythrocyte Membrane Including Decrease in Cholesterol, Increase in α-Tocopherol and Changes in Fatty Acids of Phospholipids

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Effects of high dietary cholesterol on erythrocyte membrane lipids were studied. Feeding rats with a diet containing 0.5% cholesterol and 0.15% sodium cholate for two weeks induced changes in erythrocyte membrane lipids including a decrease in cholesterol, an increase in α-tocopherol (α-Toc) and changes in the fatty acid composition of phospholipids. Oleic acid and linoleic acid increased, while arachidonic acid decreased in phosphatidylcholine. Saturated fatty acids decreased and unsaturated fatty acids increased in phosphatidylethanolamine. Almost the same changes in membrane lipids were also noted after six weeks of feeding rats with the diet. A diet containing 0.5% cholesterol but without sodium cholate caused a decrease in erythrocyte cholesterol and an increase in erythrocyte α-Toc after two weeks of feeding, as compared to the basal diet, indicating that high dietary cholesterol, but not sodium cholate, was responsible for these changes in the erythrocyte membrane.

Key words: high cholesterol diets; rat erythrocyte; cholesterol; fatty acids of phospholipids; tocopherol

Hypercholesterolemia has consistently been shown to be risk factor for cardiovascular diseases.[1)] High dietary cholesterol may affect lipid metabolism in various organs. Changes in cholesterol content as well as changes in the fatty acid composition of phospholipids (PL) of erythrocyte membranes affect fluidity and enzyme activities, and may change the deformability of erythrocytes through the capillary vessels. Tocopherols (Toc) are the major antioxidant in erythrocyte membranes, and there is a report that the erythrocyte Toc concentration is associated with thickening of the carotid artery intima-media.[2)] However, the effects of high cholesterol diets on erythrocyte membrane lipids are unclear.

Diets supplemented with both cholesterol and cholic acid have been used in many experiments on hypercholesterolemia in rats.[3–8)] In this study, we also used high cholesterol diets supplemented with cholic acid, and we observed changes in erythrocyte lipids, including a decrease in cholesterol and changes in the fatty acid composition of phospholipids (PL). Dietary fatty acids may induce extensive modification in the fatty acid composition of cell membranes, including erythrocytes.[9–12)] However, to our knowledge, it has not been reported that a high cholesterol diet induces a decrease in the cholesterol content and significant modification in the fatty acid composition of the erythrocyte membranes.

Materials and Methods

Materials. 9-Anthryldiazomethane (ADAM) and standard PL were purchased from Funakoshi Co. (Tokyo, Japan). Authentic fatty acids and tocopherols (Toc) were obtained from Sigma Chem. Co. (Tokyo, Japan). Other reagent grade chemicals and HPLC grade solvents were purchased from Wako Pure Chemical Co. (Osaka, Japan).

Animals and diets. All rats were purchased from KBT Oriental Co. (Tosu City, Saga Prefecture, Japan). Sixteen male Wistar rats, aged 10 weeks,

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Abbreviations: ADAM, 9-anthrildiazomethane; BHT, butylhydroxytoluene; HDL-chol, high density lipoprotein cholesterol; LDL-chol, low density lipoprotein cholesterol; α-Toc, α-tocopherol; HPLC, high pressure liquid chromatography; PL, phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PLTP, phospholipid transfer protein; SM, sphingomyelin; SR-BI, scavenger receptor class B type I; TG, triglyceride; Toc, tocopherols; UV, ultraviolet light

Table 1. Composition of the Experimental Diets (%)

Ingredients	Basal diet	Cholesterol diet	Cholesterol-cholate diet
Casein	20.0	20.0	20.0
Lard	10.0	10.0	10.0
Vitamin mixture[1]	1.0	1.0	1.0
Mineral mixture[1]	3.5	3.5	3.5
Choline bitartrate	0.2	0.2	0.2
DL-methionine	0.3	0.3	0.3
Cellulose	5.0	5.0	5.0
Corn starch	15.0	15.0	15.0
Sucrose	45.0	44.5	44.35
Cholesterol	0	0.5	0.5
Sodium cholate	0	0	0.15

[1] These were based on AIN-76[18] and the diets were supplied by Oriental Yeast Co., Chiba, Japan.

received the basal diet for 10 days, and they were then divided into two groups of eight rats each. One group (basal diet group) was fed the basal diet for an additional six weeks, and the other group (cholesterol-cholate diet group) received the basal diet supplemented with 0.5% cholesterol and 0.15% sodium cholate for six weeks. In another experiment, 18 male Wistar rats, aged 10 weeks, received the basal diet for seven days and they were then divided into three groups of six rats each. The first group (basal diet group) was fed with the basal diet for a further two weeks. The second group (cholesterol diet group) received the basal diet supplemented with 0.5% cholesterol for two weeks, and the third group (cholesterol-cholate diet group) received the basal diet supplemented with 0.5% cholesterol and 0.15% sodium cholate for two weeks. The compositions of the experimental diets are listed in Table 1. The diets were purchased from Oriental East Co., Chiba, Japan. All rats were housed in stainless steel wire cages and maintained in a temperature-controlled room (23°C) with 12-h cycles of light and dark. They had free access to water and the diets. The experimental protocols followed standard criteria for humane care of experimental animals and met the guidelines of the Kyushu University Animal Policy and Welfare Committee.

The rats were deprived of food for 14 to 16 h, and after ether anesthesia, blood was collected from the abdominal artery into citrated syringes. Serum was separated by centrifugation at $1000 \times g$ for 10 min. After removing the buffy coats and a small amount of the top layer of the erythrocytes, the remaining erythrocytes were washed three times in Hank's balanced buffer with centrifugation at $1000 \times g$ for 10 min at 4°C. A small portion of the top layer was removed at each washing. The washed erythrocytes were re-suspended in an isotonic buffer so that the final concentration was a hematocrit (Ht) of 2%. The Ht was measured with an automated blood counter (Sysmex F-820, Kobe, Japan). Ten ml of the suspension was centrifuged at $1000 \times g$ for 10 min and the packed erythrocytes were used for analysis of lipid composition.

Extraction of lipids from erythrocytes. The extraction of the total lipids including Toc from the packed erythrocytes was done immediately after the preparation of the washed erythrocytes, and as described in our previous report.[13] Briefly, the packed erythrocytes were hemolyzed by addition of 1 ml of 5 mM phosphate buffer (pH 7.4) containing 5 mM ascorbic acid, which was followed by addition of 1 ml of 80 mM sodium dodecyl sulfate. Then, 2 ml of ethanol containing 1.2 mM BHT was added, and the mixture was left at room temperature for 60 min. Next, 2 ml of *n*-hexane containing 1.2 mM BHT was added to the mixture and it was mixed vigorously using a Vortex mixer. After centrifugation at $1000 \times g$, the *n*-hexane layer was transferred to another tube. The remaining aqueous layer was washed again with 2 ml *n*-hexane. This hexane layer was combined with the first extract, and the combined hexane layer was dried under a stream of nitrogen gas.

Measurement of cholesterol and tocopherol. The dried total lipid extract was dissolved in 200 μl of *n*-hexane/isopropanol (3:1, by vol), and after filtration through a 0.45 m filter a portion of the solution (usually 5 μl) was injected into a HPLC system. Cholesterol and Toc were measured simultaneously by the HPLC method as described in our previous reports.[13,14] The HPLC system (HP-1100, Yokogawa Electric Co., Tokyo, Japan) was connected in series to a fluorescence detector (FS-8020, Tosoh, Tokyo, Japan) and an evapolative light scattering detector (ELSD) (SEDEX-55, Vitry sur Seine, France).

Separation and measurement of each phospholipid class. The separation and measurement of each PL class was done by the HPLC method reported previously.[13,15] The column was a Wakosil 5 NH_2, 150 × 4.6 mm (Wako Pure Chemical Co.) and the guard column was a μ-Bondapak NH_2, 20 × 4 mm (Waters Co., Tokyo, Japan). A portion (5 μl) of the total lipid extract, which was used for the detection of Toc and cholesterol, was injected into the HPLC system. PC and PE fractions were collected for analysis of fatty acid composition.

Analysis of fatty acid composition of phospholipids. Fatty acid analysis of the PL was done as described previusly.[15,16] Briefly, the collected PL fraction was dried under a stream of nitrogen gas and hydrolyzed with 2 ml of 0.5 M KOH in 70% methanol at 75°C for 60 min. After acidification with 0.6 ml of 2 M HCl, the fatty acid was extracted with 2 ml of *n*-hexane. The fatty acids were dried under a stream of

nitrogen gas and were labeled with ADAM by mixing with 0.2 ml of 0.05% ADAM solution in methanol. The mixture was left for more than 1 h at room temperature. A portion of the labeled fatty acid was injected into the HPLC system. The column was a TSK-GEL ODS 100 S, 250×4.6 mm, (Tosoh, Tokyo, Japan). The fatty acids were detected with a fluorescence detector (FS 8020, Tosoh, Tokyo, Japan) which was set at 418 nm for emission and 365 for excitation.

Measurement of serum lipids. Serum total cholesterol, TG, and HDL cholesterol (HDL-chol) were measured by enzymatic methods using an automated analyzer (Olympus AU-5200, Olympus Co., Tokyo, Japan), and LDL cholesterol (LDL-chol) was measured by an enzymatic method using an analyzer (Olympus AU-800). All assay reagents for analyzers were purchased from Daiichi Chemical Co., Tokyo, Japan.

Statistics. Statistical analyses were done by Student's *t*-test using Microsoft Excel 2000 (Microsoft Co.). Differences were considered significant at $p<0.05$.

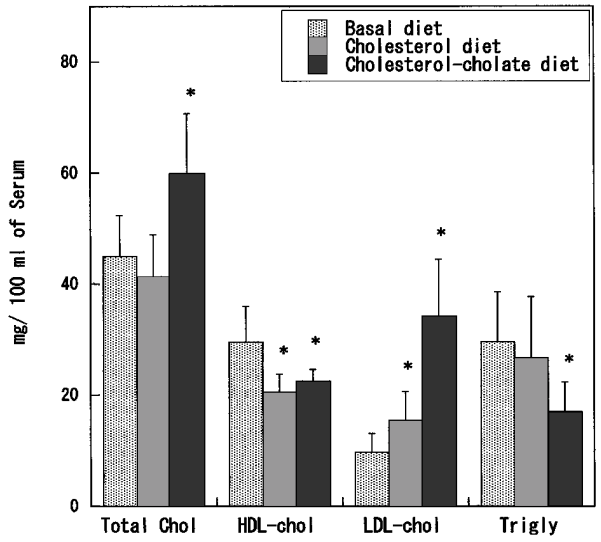

Fig. 1. Changes in Serum Lipids after Feeding Rats with Diets for Two Weeks.

Each bar represents the mean±SD. *Abbreviations*: Total chol, total cholesterol; HDL-chol, high density lipoprotein cholesterol; LDL-chol, low density lipoprotein cholesterol; TG, triglyceride. * indicates $p<0.05$ compared the basal diet group by Student's *t*-test.

Results

Changes in lipid composition in serum

Compared to the basal diet group, the cholesterol diet group showed a decrease in HDL-chol and an increase in LDL-chol, but total cholesterol and TG did not change significantly (Fig. 1). On the other hand, the cholesterol-cholate diet group showed increases in total cholesterol and LDL-chol, and decreases in HDL-chol and TG (Fig. 1). After six weeks of feeding the rats with the cholesterol-cholate diet, changes in serum lipids were similar to those after two weeks of feeding with the same diet (data not shown).

Changes in erythrocyte membrane cholesterol and tocopherol

Amounts of γ-Toc detected in the rat erythrocyte membranes were very small and therefore not calculated. The membrane cholesterol decreased significantly and the membrane α-Toc increased significantly in both the cholesterol diet group and the cholesterol-cholate diet group, compared to those in the basal diet group (Fig. 2A). After six weeks of feeding with the cholesterol-cholate diet, the membrane cholesterol was still significantly lower, but the difference in α-Toc was not present (Fig. 2B).

Changes in erythrocyte membrane phospholipids

Each PL class on the chromatogram was identified by the retention time for each standard PL. The chromatogram for rat erythrocytes, unlike human erythrocytes, showed two large peaks in the region of PS, therefore, these were tentatively named PS-1 and PS-2. The erythrocytes of the rats fed either the cholesterol diet or the cholesterol-cholate diet showed a decrease in PC and an increase in PE, compared to those rats fed the basal diet (Fig. 3A). The changes in PL in the cholesterol-cholate diet group after six weeks of feeding were almost the same as those after two weeks of feeding the same diet (Fig. 3B).

Changes in fatty acid composition of erythrocyte membrane phospholipids

The unidentified fatty acids on the chromatograms were omitted from the calculation and the whole of the identified fatty acid was set at 100% (Fig. 4, 5). The fatty acid composition of PC in the cholesterol-cholate diet group after feeding for two weeks showed an increases in oleic acid (18:1) and linoleic acid (18:2), but arachidonic acid (20:4) decreased (Fig. 4). The fatty acid composition of PE in the cholesterol-cholate diet group showed decreases in saturated fatty acids including lauric acid (12:0) and myristic acid (14:0), and increases in all unsaturated fatty acids (Fig. 5). After six weeks of feeding with either the basal diet or the cholesterol-cholate diet, oleic acid and arachidonic acid increased, and stearic acid (18:0) decreased in both PC and PE, as compared to those values after two weeks of feeding with the diets (Fig. 4, 5). The differences in the fatty acids of PC and PE between the basal diet group and the cholesterol-cholate diet group after six weeks of feed-

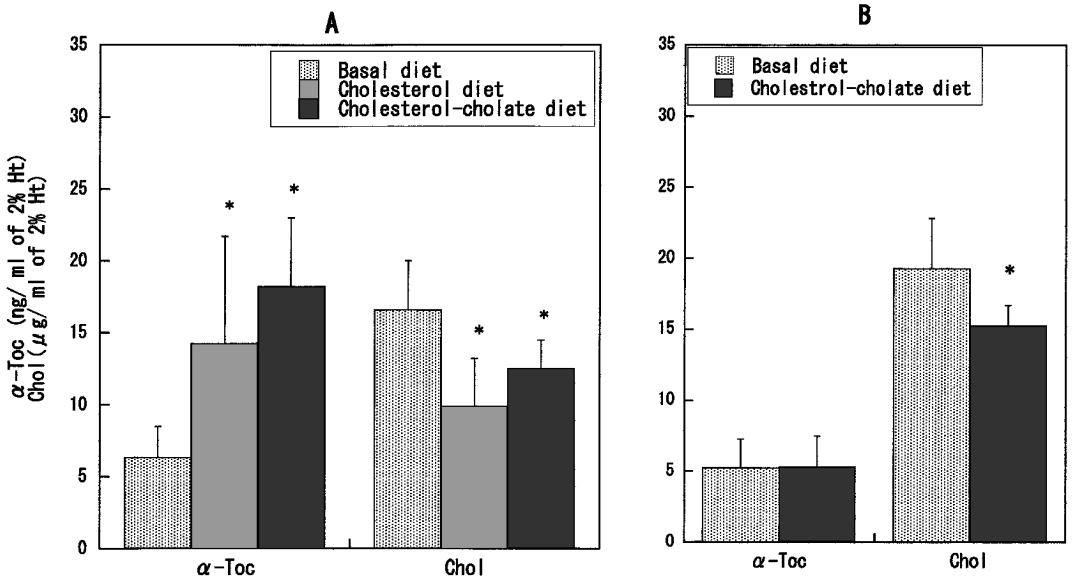

Fig. 2. Changes in α-Tocopherol and Cholesterol of Erythrocyte Membrane after Feeding Rats with Diets for Two Weeks (A) and for Six Weeks (B).

Each bar represents the mean ± SD. *Abbreviations*: Ht, hematocrit; α-Toc, α-tocopherol; Chol, cholesterol. * indicates $p < 0.05$ compared to basal diet group by Student's *t*-test.

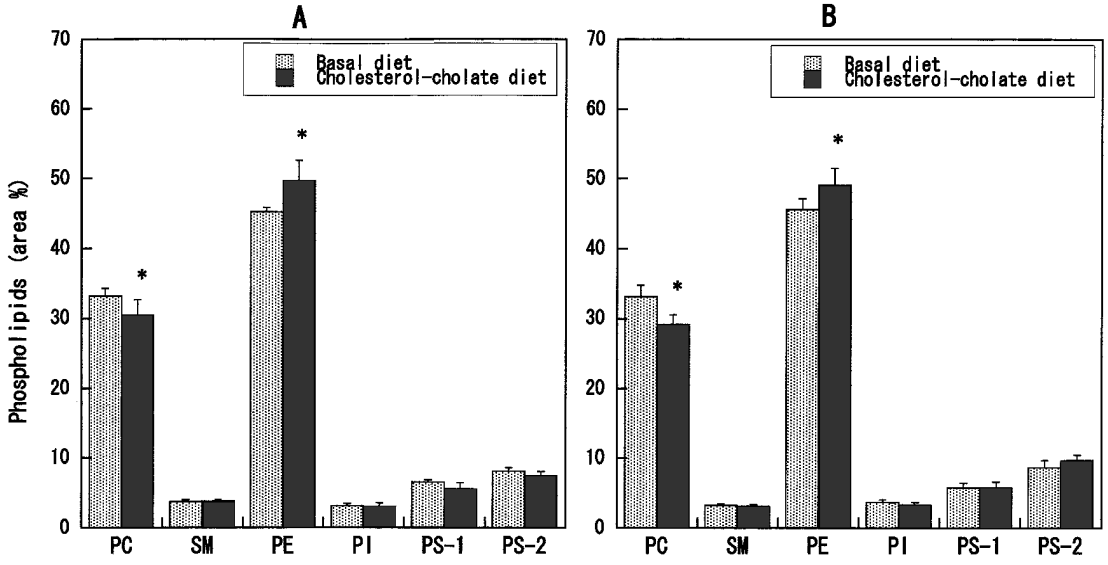

Fig. 3. Changes in Phospholipid Classes of Erythrocyte Membranes after Feeding Rats with Diets for Two (A) and Six weeks (B).

For the measurement of the phospholipid class, a portion of the same lipid extract used for the measurement of cholesterol and α-Toc was injected into the HPLC system. Each bar represents the mean ± SD. *Abbreviations*: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin. * indicates $p < 0.05$ by Student's *t*-test.

ing were almost same to the differences after two weeks of feeding (Fig. 4, 5). Unsaturation index of fatty acids (sum of percentages of individual fatty acids × number of double bonds) decreased in PC from 92.2 ± 8.6 to 84.9 ± 7.0 and increase in PE from 151.2 ± 26.1 to 186.0 ± 30.6.

Discussion

Membrane cholesterol, α-Toc, and PL were measured from the same lipid extract, and the cholesterol and α-Toc were measured simultaneously. The diet supplemented with 0.5% cholesterol and 0.15% sodium cholate induced a decrease in erythrocyte membrane cholesterol and an increase in erythrocyte membrane α-Toc after two weeks of feeding, as com-

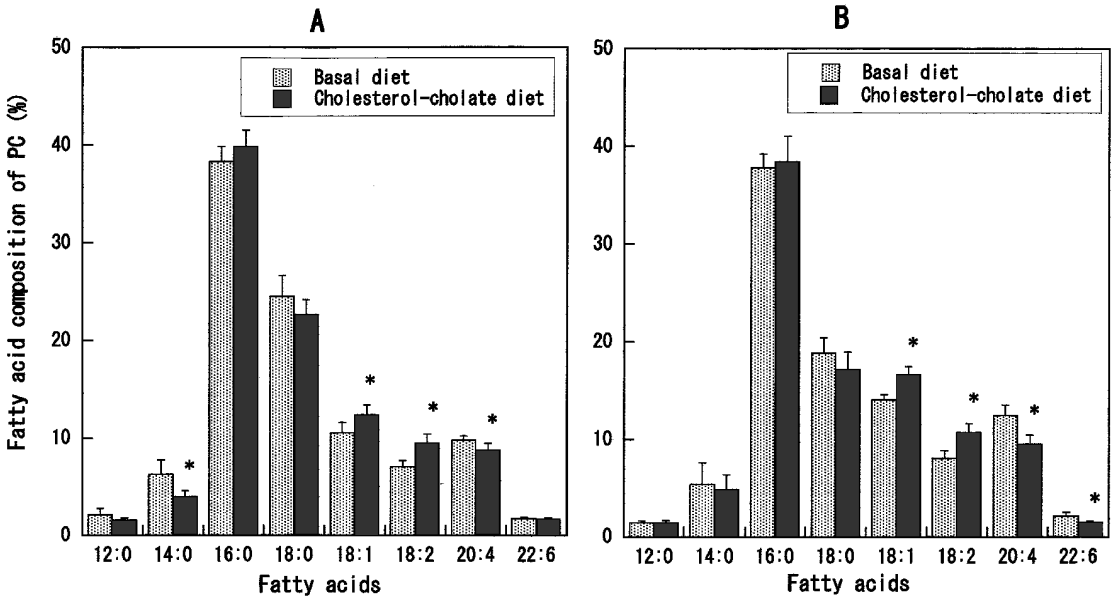

Fig. 4. Changes in Fatty Acid Composition of Phosphatidylcholine of Erythrocyte Membranes after Feeding Rats with Diets for Two (A) and Six Weeks (B).
Each bar represents mean ± SD. *Abbreviations*: PC, phosphatidylcholine. * indicates $p<0.05$ by Student's *t*-test.

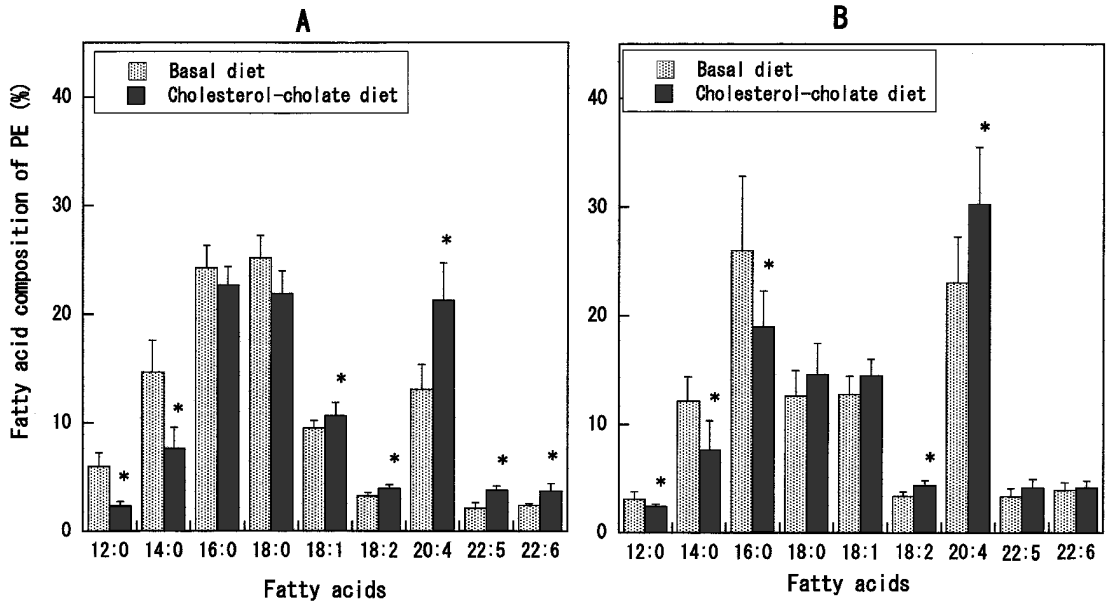

Fig. 5. Changes in Fatty Acid Composition of Phosphatidylethanolamine of Erythrocyte Membranes after Feeding Rats with Diets for Two (A) and Six Weeks (B).
Each bar represents the mean ± SD. *Abbreviastions*: PE, phosphatidylethnolamine. * indicates $p<0.05$ by Student's *t*-test.

pared to the basal diet (Fig. 2). Furthermore, arachidonic acid decreased in PC and all unsaturated fatty acids increased in PE (Fig. 4, 5). Interestingly, oleic acid and linoleic acid increased in both PC and PE. The detection of PL with 200–210 nm of UV depends primarily on the double bond in fatty acids.[15,17] Therefore, the changes in membrane PL detected by the HPLC method reflect mainly the changes in polyunsaturated fatty acids. Actually, the unsaturation index of fatty acids decreased in PC and increased in PE after feeding with the cholesterol-cholate diet. The diet containing 0.5% cholesterol (cholesterol diet) caused similar changes in serum lipoproteins and in erythrocyte lipids to cholesterol-cholate diet (Fig. 1, 2), which indicate that high dietary cholesterol but not dietary cholate is responsible for these changes in erythrocyte membrane.

In this study, the diet containing cholesterol and sodium cholate induced a decrease in serum HDL-chol and an increase in serum LDL chol (Fig. 1).

Srivastava *et al.* reported that a high fat diet (containing 20% coconut oil and 0.5% cholesterol) supplemented with 1% cholic acid induced low plasma levels of HDL in mice, as compared to a high fat diet without cholic acid, suggesting that cholic acid may be responsible for the decrease in plasma HDL.[19,20] and they reported that dietary cholic acid decreased plasma levels of mouse and human lipoprotein A-1 by a transcriptional mechanism and increased plasma levels of apolipoprotein B in mice by posttranscriptional mechanisms.[19,20] However, the cholic acid supplement (1%) was much larger than that in this study (0.15%), and this study on rats showed that the serum level of HDL-chol decreased to the same extent in both the cholesterol diet group and the cholesterol-cholate diet group (Fig. 1).

Bernasconi *et al.* reported that feeding rats with a high cholesterol diet containing 1% cholesterol and 0.5% cholic acid increased the cholesterol content in liver microsomes.[10] The high cholesterol diet decreased stearic acid and arachidonic acid, and increased oleic acid and linoleic acid in liver microsomal PC, however, the fatty acid composition of the PE was not reported.[10] The changes in fatty acids were considered to be due mainly to the increase of cholesterol and changes of desaturase in the liver microsomes.[10,11] The increases in oleic acid and linoleic acid, and the decreases in stearic acid and arachidonic acid in liver microsomal PC appeared to coincided with changes in erythrocyte membrane PC.

Erythrocyte membrane cholesterol decreased in both the cholesterol diet group and the cholesterol-cholate diet group (Fig. 2). Most mammalian somatic cells, including erythrocytes, are unable to catabolize cholesterol and need to export cholesterol in order to maintain cholesterol homeostasis.[21,22] Reportedly, at least two independent mechanisms have been identified to account for cholesterol homeostasis.[21] One is a nonspecific diffusion-mediated cholesterol efflux from the surface of cells. Cholesterol molecules desorbed from cells can be trapped by various extracellular acceptors including lipoproteins and albumin, and extracellular cholesterol esterification mainly on HDL may provide a driving force for the net removal of cell cholesterol by maintaining a cholesterol gradient between the lipoprotein surface and cell membrane. The other mechanism is an apolipoprotein-mediated process to generate new HDL by removing cellular cholesterol and phospholipids. However, it is considered that the apolipoprotein-mediated process does not work with erythrocytes.[21]

Bile acid may activate plasma phospholipid transfer protein (PLTP) gene expression.[23,24] PLTP is present in plasma as a free or HDL-bound protein, and is considered to play an important role in reverse cholesterol transfer mediated by HDL. PLTP facilitates PL transfer between LDL and HDL, modulates HDL particle size and lipid composition, generates a pre-beta HDL as the earliest acceptor of cellular cholesterol in reverse cholesterol transport, and interacts with cholesterol-loaded cells and the efflux of excess cholesterol.[23–26] Scavenger receptor class B type I protein (SR-BI), a receptor for HDL, mediates the bidirectional flux of cholesterol between cells and HDL.[27–35] SR-BI is also involved in transport of PL and Toc between cells and HDL.[30–33] High cholesterol diets may affect expression of SR-BI.[28,30,35] Reportedly, there was an inverse correlation between SR-BI expression and HDL-chol level in transgenic mice,[28] and erythrocytes from mice lacking the SR-BI gene showed marked increases in the membrane cholesterol.[34] It may be possible that the diets containing high cholesterol activate SR-BI gene expression in rats. High dietary cholesterol may also affect expression of other genes related to the regulation of intracellular and extracellular cholesterol levels,[36–38] but the possible relationships between changes in cholesterol, PL, and α-Toc in the erythrocyte membrane to the expressions of genes, including PLTP and SR-BI, are not clear.

The diets supplemented with cholesterol induced changes in rat erythrocyte membranes, including a decrease in cholesterol, an increase in α-Toc, and changes in fatty acids in PC and PE. It may be noteworthy that the amounts of both cholesterol and sodium cholate used to supplement the diets in this study were smaller than other reported high cholesterol diets fed in order to induce experimental hypercholesterolemia.[6–11,19,20]

Acknowledgments

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