Fatty Acid Composition of Phospholipids in Human Serum Lipoprotein Fractions

Akira YAMAMOTO, M D, Katsunori ISHIKAWA, M D, Yuji MATSUZAWA, M D,
Susumu MISUGI, M D, Hiroshi SUDA, M D and Taku YAMAMURA, M D

Running title: Phospholipid Fatty Acids in Hyperlipemia

The major fatty acids of sphingomyelin in human serum are palmitic (16:0) and nervonic (24:1) acids. There was a marked difference in distribution of fatty acids between lipoprotein fractions in normal subjects. The concentration of 24:1 and the other long chain fatty acids with carbon chain length no less than 22 was significantly higher in a fraction containing high density lipoproteins (HDL) than in another fraction containing low and very low density lipoproteins (LDL). The LDL fraction was rich in 16:0. The difference in fatty acid composition of sphingomyelin between these two lipoprotein fractions was not marked in cases of hyperlipidemia, mostly because of a decrease in 24:1 in HDL fraction. There was a considerable increase in concentration of behenic acid (22:0) in HDL fraction in some cases of hyperlipidemia. LDL fraction seems to be more strict in selecting molecular species of sphingomyelin than HDL. The fatty acid composition of phosphatidylcholine (lecithin) was essentially the same for HDL and LDL fractions.

Key Words: Hyperlipidemia, Lipoproteins (high density), Lipoproteins (low density), Fatty acids (in serum lipoproteins), Sphingomyelin (in human serum), Lipids (in serum lipoproteins), Lecithin (in serum lipoproteins)

Lecithin and sphingomyelin are two major components of phospholipid in serum. These phospholipids are distributed unevenly among different lipoprotein classes. High density lipoproteins (HDL) are rich in lecithin and low density lipoproteins (LDL) contain a larger amount of sphingomyelin than HDL^{1,2)}. There is a close association between the concentration of sphingomyelin and cholesterol in serum almost independent of the distribution pattern of lipoprotein classes³⁾.

Sphingomyelin is a complex lipid which shows a specific distribution to the surface membrane of tissue cells^{4,5)}. Aortic tissue

shows a steady increase in sphingomyelin by aging⁶⁻⁸⁾. One of the characteristics of this surface membrane lipid is the presence of saturated or monoenoic fatty acids with carbon chain length of 22-24 in high concentration⁹⁻¹¹⁾.

It is the purpose of the present investigation to measure the distribution pattern of molecular species of sphingomyelin among different lipoprotein fractions in normal subjects and its change in hyperlipidemia. The selection of molecular species of complex lipids is one of the decisive factors for the function of a biological membrane^{12,13)}. Discussion will be made on the role of synthesis of sphingomyelin with long chain fatty acids as a regulatory factor for the pathogenesis of hyperlipidemia.

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Reprints request to: Akira Yamamoto, The Second Department of Internal Medicine, Osaka University Medical School, 1-50, 1-chome Fukushima, Fukushima-ku, Osaka 553, Japan.

MATERIALS AND METHODS

Blood samples were obtained after overnight fasting from normal subjects of both sexes aged between 30 and 45 and from cases of hyperlipidemia with mild coronary heart disease and allied disorders. Serum was separated after the blood was left standing at room temperature for two hours. Lipoproteins were separated from 3 ml of serum into (1) high density plus very high density lipoproteins (HDL fraction) and (2) low density plus very low density lipoproteins (LDL fraction) by using heparin-manganese ion precipitation method described by Burstein¹⁴⁾. The procedure was carried out after diluting the serum with an equal volume of saline. In case the lipid concentration was high and the precipitation of LDL fraction appeared to be incomplete, the sample was further diluted with an increasing volume of saline. Heparin and manganese ion were added in proportion to the volume of the sample. The separation of the two fractions was judged to be complete semiquantitatively on agarose gel and cellulose acetate membrane electrophoresis with lipid staining and densitometry. The recovery of cholesterol from the supernatant of the reaction mixture containing HDL and lipid free protein fractions (obtained by ultracentrifugation at $d > 1.063$) was 98-99%. Fresh serum was always used in this experiment, because a considerable amount of HDL-cholesterol is precipitated with heparin after serum is frozen or kept at 4°C for a long time before use. Srinivasan et al¹⁵⁾ suggested that HDL interact with heparin in the presence of Mn^{++} , leading to the precipitation of 25% of HDL. Their experiment was done by using an isolated HDL fraction from pooled serum. Burstein and Samaille have mentioned that HDL precipitates when the fraction is isolated and dialyzed against buffered distilled water¹⁴⁾. Quantitative recovery of HDL-cholesterol was reported by Wilson and Spiger¹⁶⁾ and recently confirmed by Ishikawa et al¹⁷⁾.

Lipids were extracted with 15 volumes of chloroform-methanol 2:1 (v/v) and washed by the method of Folch et al¹⁸⁾. Cholesterol was determined by the method of Zurkowski¹⁹⁾ and phospholipids by modified Chen's method^{20,21)} for each lipoprotein fraction. The isolation of lecithin was performed on silicic acid thin layer chromatography (TLC)^{22,23)}. An aliquot of the sample containing 160 μ g of total phospholipids was applied on one plate, and 2-4 plates were used for each sample. The plates were developed with chloroform-methanol-28% aqueous ammonia-water 120:80:10:5 into the first dimension and with chloroform-acetone-methanol-acetic acid-water 100:40:30:20:12 into the second dimension. For analysis of sphingomyelin, samples were first subjected to mild alkaline hydrolysis according to the method of Dawson et al²⁴⁾. The unsaponifiable matter containing sphingomyelin was concentrated and applied on silicic acid TLC. Plates were developed with chloroform-acetone-methanol-acetic acid-water 100:40:30:20:12 (single dimension). Spots of TLC were visualized with rhodamine-6 G, scraped and transferred into a culture tube with a teflon-coated screw cap. Samples were methanolized with 2-3 ml of 14% (w/v) boron trifluoride in methanol at 100° for 30 min (for lecithin) or 90 min (for sphingomyelin). Treatment of samples were carefully done under an atmosphere of nitrogen. The recovery of phospholipids from spots on TLC was 95-100% by phosphorus assay and the recovery of sphingolipids by sphingosine assay was 93-97%. The recovery of a polyunsaturated fatty acid (C 22:6, ω -3) after methanolysis by this procedure was 97%, although a longer treatment caused a considerable loss of unsaturated fatty acids (20% for oleic acid, C 18:1, after 5 hours). Methyl esters of fatty acids were analyzed on GLC. Samples from sphingomyelin which were essentially free of polyunsaturated fatty acids were analyzed on a column of 3% SE-30 on Gaschrom Q at 220°. The recovery of monoenoic fatty acids was complete and the analyses at 210°-230° gave

Table 1. Serum concentrations of cholesterol and phospholipids and their distribution between high and low density lipoprotein fractions in normal and hyperlipidemic subjects

Group	Case	Age and Sex	Cholesterol (mg/100 ml serum)				Phospholipids (mg/100 ml serum)				Triglycerides (mg/100 ml serum)
			Whole Serum	Fractions HDL	Fractions LDL	Ratio LDL/HDL	Whole Serum	Fractions HDL	Fractions LDL	Ratio LDL/HDL	
Normal Subjects	C-1	42 m	224	78	146	1.88	231	130	101	0.78	85
	2	33 m	220	45	175	3.89	195	78	117	1.50	96
	3	38 m	201	60	141	2.34	206	108	98	0.91	57
	4	37 m	228	61	167	2.72	185	82	103	1.26	114
	5	29 m	174	55	119	2.15	200	116	84	0.72	57
	6	39 f	215	76	139	1.83	267	153	114	0.75	150
	7	42 f	188	42	146	3.48	178	87	91	1.05	67
	8	34 f	259	85	174	2.05	249	141	108	0.77	78
	Average C 1~8		214	63	151	2.54	214	112	102	0.97	88
	(+standard deviation)		(±26)	(±16)	(±20)	(±0.77)	(±32)	(±28)	(±11)	(±0.28)	(±32)
Hyperlipidemia (Type II b)	H-1	54 m	243	34	209	6.15	253	83	170	2.05	223
	2	42 m	249	34	215	6.32	202	63	169	2.68	231
	3	49 f	285	64	221	3.48	423	185	238	1.29	169
	4	46 f	221	25	196	7.84	208	60	148	2.47	162
	5	67 f	229	32	197	6.16	231	72	159	2.21	221
	6	51 m	329	43	286	6.65	320	88	232	2.63	527
	7	39 m	275	35	240	6.86	244	63	181	2.87	363
	Average H 1~7		262*	38**	223***	6.21***	269	88	185***	2.31***	271**
	(+standard deviation)		(±38)	(±13)	(±31)	(±1.34)	(±78)	(±44)	(±35)	(±0.53)	(±130)
Hyperlipidemia (Type II a)	H-8	32 f	366	40	326	8.15	259	67	192	2.87	121
	9	16 f	1003	21	982	46.8	619	36	583	16.2	401
Miscellaneous	H-10	61 m	182	42	140	3.33	231	102	129	1.26	255
	11	46 f	382	52	330	6.35	274	75	199	2.65	125
	12	60 f	232	28	204	7.28	197	72	125	1.74	164

*P < 0.02, **P < 0.01, ***P < 0.001 against the normal subjects, HDL: High density lipoprotein fraction
LDL: Low density lipoprotein fraction
(see text)

the same results. The analysis of samples from lecithin was performed on a column of 10% polyethylene-glycol succinate on Chromosorb W at 190°. The response of polyunsaturated fatty acids was satisfactory (98% at 184° and 96.6% at 196° for 1 µg of C 22:6 compared with the response of C 18:1).

RESULTS

1) Serum concentration of cholesterol and phospholipids and their distribution between lipoprotein fractions

Concentration of total cholesterol, phospholipids and triglycerides in whole serum and the distribution of cholesterol and phospholipids between HDL and LDL fractions were shown in Table 1. The distribution ratio of serum cholesterol, LDL/HDL, was 2.54 (±0.77) for normal subjects. Among 9 cases with coronary heart disease and allied disorders followed in this study, seven had type IIb hyperlipidemia and the other two had type IIa hyperlipidemia with xanthomatosis. The mean value of serum cholesterol concentration of type IIb patients was 262 mg/100

ml. Although the value was not so significantly high compared with the value for normal subjects ($0.01 < P < 0.02$), the amount of cholesterol in HDL fraction from 100 ml blood serum (HDL-cholesterol) was significantly decreased and the amount of cholesterol in LDL fraction (LDL-cholesterol) increased, so that the distribution ratio of cholesterol, LDL/HDL, showed a marked elevation in this group of patients (6.21 ± 1.34): ($P < 0.001$ vs normal control).

Three miscellaneous cases, one with hyperlipidemia and hypothyroidism under treatment with thyroid extract, another with alcoholic hyperlipemia at remission stage, and the third with sequelae of cerebral hemorrhage, were also used in this study. Although the last case was normolipidemic, the distribution ratio of cholesterol, LDL/HDL, was markedly increased (case H-12).

2) Fatty acid composition of lecithin in HDL and LDL fractions

There was essentially no difference in fatty acid composition of lecithin between HDL and LDL, except slight differences in concentration of oleate (18:1), linoleate (18:2) and arachidonate (20:4) (Table 2).

Table 2. Fatty acid composition of phosphatidylcholine in high and low density lipoprotein fractions in blood serum

Fatty acids* ¹	Case C - 1		Case C - 2		Case C - 4	
	HDL* ²	LDL* ³	HDL	LDL	HDL	LDL
14:0	0.4%	0.1	0.3	0.3	0.3	0.3
16:0	34.5	33.7	32.4	32.9	35.7	35.7
1	1.3	1.5	1.2	0.9	0.7	0.7
18:0	17.7	16.9	15.6	16.0	16.3	16.4
1	15.0	15.2	11.8	12.3	12.4	13.3
2	23.4	24.8	24.3	25.2	19.6	19.8
20:3 (w - 6)	0.7	0.5	1.4	1.3	1.3	1.4
4 (w - 6)	5.4	4.1	6.7	4.7	9.0	8.3
5 (w - 3)	0.3	0.8	1.6	1.7	0.6	0.5
22:3 (w - 9)	0.3	0.4	2.1	1.6	1.9	1.7

*¹ Carbon chain length:degree of unsaturation

*² High density lipoprotein fraction (HDL + VHDL)

*³ Low density lipoprotein fraction (LDL + VLDEL)

Lecithin in HDL-fraction was slightly richer in polyunsaturated fatty acid (20:4) than lecithin in LDL-fraction.

3) Fatty acid composition of sphingomyelin in lipoprotein fractions from normal subjects

Palmitate (16:0) and nervonate (24:1) were major fatty acids of sphingomyelin in blood serum. The sum of the concentration of these two fatty acids attained to more than half of the total fatty acid in sphingomyelin (Table 3).

The concentration of 24:1 was higher in HDL than in LDL fraction, while the con-

Table 3. Fatty acid composition of sphingomyelin in serum high and low density lipoprotein fractions in normal subjects

Fatty acids (Carbon chain length (degree of unsaturation))	Normal subjects (C 1-8)		
	HDL* ¹	LDL* ²	difference between HDL and LDL
16:0	23.8 ± 2.7*	32.3 ± 3.7	P < 0.001
18:0	7.3 ± 1.7	8.6 ± 1.4	
1	3.4 ± 1.9	1.6 ± 0.8	
20:0	4.2 ± 0.4	4.1 ± 0.5	
1	0.5 ± 0.2	0.4 ± 0.3	P < 0.02
21:0	0.6 ± 0.2	0.5 ± 0.1	
22:0	10.4 ± 1.8	9.6 ± 1.6	
1	3.1 ± 2.2	2.3 ± 2.0	
23:0	3.7 ± 0.9	3.5 ± 0.5	P < 0.001
1	1.0 ± 0.6	0.7 ± 0.3	
24:0	7.2 ± 1.9	6.6 ± 2.0	
1	34.9 ± 3.4	29.4 ± 4.4	
sum of 22:0~24:1	60.2 ± 2.2	52.1 ± 4.1	P < 0.001
16:0/24:1	0.69 ± 0.13	1.14 ± 0.30	P < 0.005
16:0/24:1 ratio between LDL/HDL	1.65 ± 0.30		

*¹High density lipoprotein fraction (HDL + VHDL).

*²Low density lipoprotein fraction (LDL + VLDEL).

*³Per cent of total fatty acids (± standard deviation).

centration of 16:0 was higher in LDL than in HDL fraction. In general, fatty acids with longer carbon chain were concentrated in HDL much more than in LDL fraction, so that the concentration of the long chain fatty acids with carbon chain length no less than 22 in HDL fraction was significantly higher than that in LDL fraction.

4) Fatty acid composition of sphingomyelin in hyperlipidemia

In patients with hyperlipidemia of Type IIa and IIb, the difference in fatty acid composition of sphingomyelin between HDL and LDL fractions was less marked than in normal subjects (Table 4, Fig. 1). There was a tendency for the average chain length becoming shorter in HDL fraction, while it becoming slightly longer in LDL fraction in hyperlipidemics compared with normal subjects.

Fig. 1. Fatty acid composition of sphingomyelin in serum lipoprotein fractions from normal and hyperlipidemic subjects.

Table 4. Fatty acid composition of sphingomyelin in serum high and low density lipoprotein fractions in hyperlipidemics

Fatty acids* ¹	Hyperlipidemics (H-1~9)		Miscellaneous cases					
	HDL* ²	LDL* ³	Case H-10		Case H-11		Case H-12	
			HDL	LDL	HDL	LDL	HDL	LDL
16:0	25.0 ± 3.9	30.9 ± 4.2	25.0	39.2	22.5	32.3	26.8	42.1
18:0	8.6 ± 2.2	8.6 ± 2.1	5.5	4.6	5.6	7.6	6.8	10.4
1	4.0 ± 1.4	1.1 ± 0.8	5.1	1.0	3.3	0.6	0.6	0.9
20:0	3.5 ± 0.9	4.1 ± 0.6	3.4	3.4	3.0	4.8	2.4	3.0
1	0.8 ± 0.5	0.5 ± 0.7	1.0	0.2	0.5	0.3	-	0.3
21:0	0.8 ± 0.3	0.6 ± 0.3	0.6	0.2	0.5	0.4	1.5	0.3
22:0	12.1 ± 4.9	10.6 ± 2.7	15.3	14.1	15.4	13.8	23.0	6.7
1	5.1 ± 2.6	4.1 ± 1.9	1.5	1.0	3.7	2.2	6.5	2.1
23:0	3.1 ± 0.9	2.8 ± 0.6	3.9	4.1	4.1	4.1	1.4	1.2
1	0.6 ± 0.7	0.6 ± 0.5	0.6	0.2	0.6	0.2	0.1	0.3
24:0	5.8 ± 1.9	6.0 ± 1.8	14.3	12.1	11.4	8.0	0.7	2.5
1	30.7 ± 3.4	30.3 ± 3.0	23.8	19.9	29.4	25.7	30.2	30.2
Sum of 22:0-24:1	57.4 ± 4.9	54.4 ± 2.6	59.4	51.4	64.6	54.0	61.9	43.0
16:0/24:1 Ratio bet- (ween LDL/HDL)	0.83 ± 0.18 (1.27 ± 0.16)	1.03 ± 0.18	1.05	1.97 (1.88)	0.77	1.26 (1.64)	0.89	1.39 (1.56)

*¹ Carbon chain length:degree of unsaturation*² High density lipoprotein fraction (HDL + VHDL)*³ Low density lipoprotein fraction (LDL + VLDL)*⁴ Percent of total fatty acids (± standard deviation)*⁵ $P < 0.005$ against normal subjects

The major change in distribution of fatty acids in hyperlipidemics appeared in HDL fraction as a decrease in 24:1. Compared with a marked difference between HDL and LDL fractions observed in normal subjects, there was almost no difference in the total amount of fatty acids with chain length 22-24 in hyperlipidemics between the two fractions.

In some of the cases of hyperlipidemia (case 6, 7 and 9), there was a marked increase in concentration of behenic acid (22:0) especially in HDL fraction. All of these cases had the serum triglyceride concentration higher than 300 mg/100 ml. The variation in concentration of 22:0 in LDL fraction was not so significant as in HDL fraction.

5) Changes in fatty acid composition of sphingomyelin in hyperlipidemics by treatment with hypocholesterolemic agents

Treatment of hyperlipidemics with a derivative of nicotinic acid, nicomol (2, 2,

Fig. 2. Effects of nicomol and estrogen on fatty acid composition of sphingomyelin in HDL and LDL-fractions.

— treatment with nicomol
 --- treatment with estrogen

6, 6-tetrakis (nicotinyloxymethyl) - cyclohexanol), or estrogens increased the concentration of 24:1 with a decrease of 16:0. The ratio of 16:0 to 24:1 was significantly decreased, so that the distribution pattern of fatty acids in lipoprotein fractions was normalized (Fig. 2). The change took place mainly in HDL and there was no effect of nicomol on the fatty acid composition of sphingomyelin in LDL fraction. But, estrogens had also an effect on LDL fraction, decreasing the concentration of 24:1 and increasing the concentration of 16:0.

6) The change in concentration of 22:0 in relation to the change in serum triglyceride level

A long term administration of nicomol often causes a significant increase in concentration of triglyceride in blood serum and enhances alcohol-induced hyperlipidemia. As the triglyceride level going up by treatment with nicomol, the concentration of 22:0 was also increased. Including the three cases mentioned before, in which the

triglyceride concentration was originally high, all the cases with triglyceride level higher than 300 mg/100ml had a concentration of 22:0 significantly higher than the control value (Fig. 3).

Three miscellaneous cases showed an increase of 22:0 in spite of the normal triglyceride level. A case with alcoholic hyperlipidemia (case H-10) and another with sequelae of cerebral hemorrhage (case H-12) were even normocholesterolemic, but the ratio of LDL to HDL-cholesterol was higher than the control value. The other case was under treatment with thyroid hormone. In case H-12, the administration of estrogen resulted in a marked decrease in concentration of 22:0.

DISCUSSION

Fractions of lipoproteins in blood serum have different phospholipid composition. HDL is rich in lecithin and LDL contains a larger amount of sphingomyelin than HDL^{1,2)}. The molar ratio of lecithin to sphingomyelin is about 7 for HDL and 2 for LDL. There is a close association between sphingomyelin and cholesterol and a linear relationship was obtained for whole serum which was shown by a formula $[\text{Cholesterol}] = 31 + 4.3 [\text{Sphingomyelin}]$ ³⁾.

Complex lipids show a characteristic distribution for each fraction of subcellular particles⁵⁾. Cardiolipin, for example, is specific for mitochondria, and cerebroside for myelin in nervous system. Sphingomyelin is a complex lipid which is specific to the surface membrane, and shares the presence in this structure with phosphatidylserine and cholesterol^{4,5,9)}. Red cell membrane, blood vessels and nervous tissue are typical examples for the kind of tissues predominantly composed of the surface membrane and consequently relatively rich in these lipids⁵⁾. Lipids in serum lipoproteins seem to be slightly peculiar in this sense because it is rich in sphingomyelin and cholesterol, but contains no phosphatidylserine. However, it is not so far to say that the coating membrane of plasma lipoproteins is a

Fig. 3. Relationship between serum triglyceride level and the concentration of behenate (22:0) in sphingomyelin in HDL fraction.

kind of remnant of the surface membrane.

One of the peculiar characteristics of sphingolipids is the content of long chain fatty acids with chain length more than $20^{9-11,25-28}$. Among these fatty acids, nervonic acid (24:1) is the commonest component. In nervous tissue it is shown that this fatty acid increased its concentration continuously during the course of development²⁵⁻²⁸. It was suggested that long chain fatty acids are needed for stabilizing the membrane structure²⁹. In cerebrosides in the adult brain, long chain fatty acids with carbon chain length 20-26 occupy about 90% of total fatty acids^{10,26-28}. A question arises why palmitate (16:0) or stearate (18:0) are also present in large amounts in sphingomyelin showing a hiatus between 16:0 (or 18:0) and 24:1.

Only a few information is available concerning the fatty acid composition of sphingomyelin in lipoprotein fractions of human blood serum³⁰⁻³². According to the present results, there was a marked difference in fatty acid composition of sphingomyelin between HDL and LDL fractions. In normal subjects, HDL contains a larger amount of long chain fatty acids than LDL. In contrast to sphingomyelin, there was essentially no difference in fatty acid composition of lecithin. Lecithin in serum moves very easily between HDL and LDL³³⁻³⁵ and also shows an easy exchange with lecithin in tissue such as liver³⁶. But, it was reported that the exchange of sphingomyelin between HDL and LDL as well as between tissue and plasma is not so fast nor complete as the movement of lecithin between lipoprotein classes or between tissue and plasma^{25,36}. Such a difference must be related to the selection of molecular species for membrane structure.

Compared with normal subjects, the difference in fatty acid composition of sphingomyelin between HDL fractions was not marked in hyperlipidemics. The change was mainly due to the decrease in concentration of long chain fatty acids in HDL fraction. This could be explained in the following way. In normal subjects, the

synthesis of 24:1 was relatively in excess compared with the rate of synthesis of cholesterol. LDL seems to be very strict for selecting the molecular species of sphingomyelin. The surplus of the long chain fatty acid was incorporated into HDL so that the variation in fatty acid composition of sphingomyelin was larger in HDL fraction than in LDL. In hyperlipidemia, there was an increased demand for long chain fatty acids as a component of LDL, and consequently, the incorporation of 24:1 into HDL was relatively decreased. The increase in 22:0 must be to compensate for the deficiency in 24:1. The abnormally high values of 22:0 in our miscellaneous cases, especially in cases H-10 and 12, pointed out a covered profile of hyperlipidemia which could not be traced by total serum cholesterol level. It is suggested that the rate of synthesis of sphingomyelin with a long chain fatty acid is a complemental factor for the development of hyperlipidemia.

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