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Studies of Lipids, Lipoproteins, and Apolipoproteins in Menkes' Disease

P. R. BLACKETT, D. M. LEE, D. L. DONALDSON, J. D. FESMIRE, W. Y. CHAN, J. H. HOLCOMBE, AND O. M. RENNERT

Department of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73129 [P. R. B., D. L. D., W. Y. C., J. H. H., O. M. R.] and Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104 [D. M. L., J. D. F.]

Summary

Three patients with Menkes' disease, an inherited disorder of copper transport, were studied to determine whether the copper deficiency was associated with a lipoprotein disorder. Hypocuprinemia was documented in all three cases. Two patients had severe copper and ceruloplasmin deficiencies, whereas the third patient had a less severe deficiency. Hypertriglyceridemia was observed in the first patient, and elevations in triglyceride, cholesterol, apolipoprotein B (ApoB), and apolipoprotein C-III (ApoC-III) occurred predominantly in the very low density lipoprotein fraction (VLDL). This patient had normal lipoprotein lipase activity but mild glucose intolerance. The second patient had a borderline high cholesterol level with normal plasma triglycerides and apolipoproteins, whereas the third patient appeared to have normal total cholesterol but slightly higher triglycerides with elevated plasma apolipoprotein E (ApoE). No striking differences were observed in the chemical composition of all lipoprotein subfractions between patients and controls except that the neutral lipid content of VLDL was higher in patients than in controls. The ApoB was initially normal in molecular weight but degraded faster than the controls during storage. The appearance of the major low density lipoprotein (LDL) fraction of the first two patients was opaque white, in contrast to clear yellow in the third patient and in the age- and diet-matched controls. This abnormal appearance of LDL in these patients was associated with low plasma levels of β-carotene and ceruloplasmin. These findings suggest that decreased serum copper levels may be associated with lipid and lipoprotein abnormalities and may enhance lipid peroxidation of LDL accounting for the color change. The increase in neutral lipids and the damaging effects on lipoprotein-transported substances may lead to atherosclerosis.

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Reprint requests may be addressed to P. R. Blackett, M.D., University of Oklahoma, Health Sciences Center, OCMH-2B251, P.O. Box 26901, Oklahoma City, OK 73190.
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Abbreviations

EEG, electroencephalogram
P/S ratio, polyunsaturated to saturated fatty acid ratio
VLDL, very low density lipoproteins
LDL, low density lipoproteins
HDL, high density lipoproteins
VHDL, very high density lipoproteins
Apo, apolipoprotein
GSH, glutathione
SDS, sodium dodecyl sulfate

Copper is an essential dietary component (13) and is known to be present in many plant and animal tissues (13). Clinical evidence of deficiency was first observed in animals and many of the clinical manifestations have been related to low tissue activities of copper enzymes such as tyrosinase, lysyl oxidase, lactase, ascorbic acid oxidase, cytochrome oxidase, uricase, monoamine oxidase, and dopamine-β-hydroxylase (31). Clinical effects of copper deficiency are manifested as widespread derangements in tissues such as skin, bone, connective tissue, red blood cells and the central nervous system. In addition, increased tortuosity of arterial wall tissues may be attributed to defective

inter- and intramolecular cross-linking of elastin and collagen molecules (28, 35).

Following the suggestion that copper deficiency and a low ratio of serum copper to zinc may lead to increased mortality due to coronary atherosclerosis (16), Allen and Klevay (1) studied plasma cholesterol concentrations in copper-deficient rats. The plasma cholesterol concentration of the rats was 230% that of pair fed controls, and the difference was apparent even when cholesterol-free diets were fed to both groups. Increased incorporation of [^{3}H]mevalonate into total plasma lipids, cholesterol, and cholesterol esters was subsequently demonstrated in the deficient rats, suggesting that increased cholesterol synthesis was occurring (1). In view of these findings, we have studied the effects of copper deficiency on human plasma lipoproteins in three cases of Menkes' disease (9, 26), a sex-linked recessively inherited disorder of copper transport (2, 9, 37).

CASE REPORTS

Case 1. R. S., a 3160-g baby boy, was born at full term to a 16-year-old prima gravida. At 5 days of age, he was noted to be feeding poorly, and during the first 2 months he was said to be fussy and periodically constipated. At the age of 2 months, he was admitted to the hospital with a right-sided seizure. Seizures became frequent and were uncontrolled by phenobarbital, 20 mg. b.i.d. This was followed shortly by the inability to swallow, necessitating nasogastric feeding. At approximately 8 months, the seizures became myoclonic. Blindness developed prior to death at 16 months due to cardiac arrest. The patient's mother was deaf. According to the family history, the mother's sister was unable to walk, and three maternal brothers and one sister had died in infancy due to unknown causes. The maternal grandfather was known to have had diabetes mellitus.

At the initial physical examination at the age of 2 months, the infant's height was at the 75th percentile and his weight at the 25th percentile. The anterior fontanel was open, and the frontal regions appeared to be slightly flattened. Slight thinning of the ear cartilage was noted and the pinna was large. The hair was fine and brittle, with loss of coloration in the distal portions of the shafts. The upper lip had a Cupid's bow appearance. Gross neurological testing suggested that the baby was cortically blind. Recurrent hypothermia necessitated the use of a radiant warmer. An EEG revealed multiple cortical dysfunction. An electroretinogram indicated dysfunction of the rods and cones. The Denver Developmental Screening Test revealed severe psychomotor retardation. Plasma copper and zinc, urine copper, alkaline phosphatase (a zinc-dependent enzyme), and ceruloplasmin (a copper transport protein) were studied at 2 months old and results are listed in Table 1. Serum copper and ceruloplasmin were extremely low. Serum zinc was also below normal initially but reached a normal level at 5 months old after treatment with zinc infusion designed to induce metallothionein synthesis. His 24-hr intake of formula provided 1300–1400 cal at 9–10 months. At 1 year of age, his intake was 50–70 cal/kg body weight. His weight was at the 50th percentile at 9 months, and at the 90th percentile at 12 months, corresponding with the 50th percentile for his height. His infant formula had a polyunsaturated to saturated (P/S) fatty acid ratio of 0.8.

Table 1. *Serum copper, zinc, ceruloplasmin, and alkaline phosphatase values in Menkes' patients*

Tests	Case 1 (R. S.)*	Case 2 (K. S.)†	Case 3 (M. S.)‡	Normal
Copper (μg/dl)	12.5	5.09	65	80–120
Urine copper (μg/24 h)	5.1			16–114
Zinc (μg/dl)	33,66.7§	92.7	133.4	63–147
Alkaline phosphatase (37°C) (IU/liter)	247	267		100–350 (6m)
Ceruloplasmin (IU/liter)	<2.5	<2.5	11	20–50

* Values at 2 months old.

† Values for 2 weeks to 2 months old.

‡ At 1 year old.

§ At 4 and 5 months old, respectively.

Case 2: K. S. This 2740-g baby boy was born to a gravida 3 para 2 mother at 38 weeks of gestation. The Apgar scores at 1 and 5 min were 6 and 7, following which some respiratory distress was noted. An x-ray revealed that he had a right lower lobe pneumonia. There was some hyperbilirubinemia on day 3. Following treatment with bilirubin reduction lights, the total bilirubin dropped from 13.0 to 7.8 mg/dl.

Physical examination revealed a normal appearing baby boy with kinky and sparse scalp hair. Pili torti were noted on microscopy. An EEG was normal at 3 weeks. Marked developmental delay was noted in all areas with the exception of gross motor control. At the age of 2 months, he was generally hypertonic; he developed seizure activity characterized by deviations of the eyes, and clenching of the fists. These seizures subsequently recurred and were poorly controlled with phenobarbital. Blood tests performed for trace metals prior to 2 months of age are shown in Table 1. He had extremely low serum copper and ceruloplasmin but normal zinc content.

Positive guaiac test results suggested some gastrointestinal blood loss, and subsequent development of anemia necessitated a blood transfusion. At 2 weeks of age, he had seven feedings amounting to 104 cal/kg/24 h (the formula had a P/S ratio of 0.8). Feeding was satisfactory in spite of poor head control and developmental delay. At 10 months of age, his weight was 7.8 kg and height, 75.2 cm. His height remained close to the 50th percentile throughout the 1st year and his weight between the 5th and 10th percentiles.

On physical examination he was noted to have a Cupid's bow appearance of the upper lip and decreased subcutaneous tissue. The lower extremities were spastic. He was unable to follow objects from the midline, to vocalize, or to smile responsively and there was no response to auditory stimuli. The baby showed progressive neurological deterioration, and died following a cardiac arrest at 14 months.

Case 3: M. S. This 3100-g baby boy was born at term to a 28-year-old prima gravida. The infant first presented at age 10 months with floppiness which was noticed at 4 months of age. There were no other serious problems in the neonatal period. Developmental milestones were delayed and he was considered to be functioning at a 4-month level when examined at age 10 months. He failed to sit unaided or to crawl, and there was no vocalization; however, he followed objects well and smiled appropriately. Since 4 months of age, he had tended to become obese, with a truncal and buccal distribution.

On physical examination, moderate obesity was noted. He had a cherubic face and a characteristic Cupid's bow appearance of the upper lip. The hair was sparse, light brown in color, and irregular in length. Hair shafts tended to break off at the base. Pili torti were observed by microscopy. Neurological evaluation revealed generalized hypertonia with brisk reflexes.

Laboratory tests revealed that he was not anemic or leukopenic and that there was no electrolyte imbalance. The serum copper was 65 μg/dl (normal, 80–120 μg/ml), and ceruloplasmin was 11 IU/liter (normal, 20–50 IU/liter) (Table 1). His serum zinc was in the normal range. No clear-cut abnormalities were observed in the EEG and electrocardiogram. Skull x-rays demonstrated wormian bones along the lambdoidal suture (a normal variant). Slight demineralization of the long bones of the lower extremities, early coxa valga, and tubulation of the long bones were noted. The electroretinogram revealed normal cone function but moderately abnormal rod function. The visually evoked response indicated depressed macular function. Following discharge from hospital, subsequent reports on progress were unavailable.

MATERIALS AND METHODS

Glucose tolerance. Intravenous glucose tolerance tests were performed by injecting glucose (1 g/kg) intravenously over 2 min and subsequently taking blood samples at 0, 5, 10, 20, 30, 40, 50, and 60 min. The disappearance rate (K value) was calculated by dividing 0.693 by the half-time and multiplying by 100 (25).

Apolipoproteins. Apolipoproteins were determined by electroimmunoassay utilizing methods described previously (4–8).

Lipoprotein lipase activity. Plasma samples were taken before and 20 min after intravenous injection of 100 units/kg of heparin (Upjohn Co., Kalamazoo, MI). Lipoprotein lipase was separated from triglyceride lipase activity by heparin-Sepharose affinity chromatography and both total lipases and triglyceride lipase activities were determined using [^{14}C]triolein as substrate (36).

Fractionation of lipoproteins. Blood samples were drawn into tubes containing sufficient EDTA to provide a final concentration of 0.1%. After removal of red blood cells, a preservative mixture was added immediately to the plasma, yielding final concentrations of 500 units/ml penicillin G, 50 μg/ml streptomycin sulfate, 0.13% ε-aminocaproic acid, 0.2% EDTA, and 0.05% reduced GSH (20). Nitrogen was blown gently on the surface of the plasma (20). Six ml of NaCl (d = 1.075 g/ml) containing 0.05% EDTA and 0.05% GSH were placed in a cellulose nitrate centrifuge tube. Five milliliters of plasma were then layered on top. Finally, 2 ml of 1.006 g/ml NaCl containing 0.05% EDTA and 0.05% GSH were layered on top of the plasma before centrifugation at 39,000 rpm for 43 h in a swinging bucket rotor (SW 40 Ti). Control plasma samples were centrifuged simultaneously. During centrifugation, a density gradient was formed and the lipoproteins were separated into bands at their equilibrium density regions (Fig. 1). The top fraction (layer 1, d < 1.010 g/ml), containing VLDL, was opaque. Layer 3 (d = 1.032–1.043 g/ml), the major LDL fraction, was about 6 mm in height at the midpoint of the tube and bright orange-colored as for normal adult plasma. The bottom layer (d > 1.074 g/ml), 2.5 cm in height and yellow-colored, contained HDL, VHDL, and plasma proteins. The zones between layers 1 and 3 and between layers 3 and 5 were clear and were labeled layers 2 and 4, respectively. The five lipoprotein fractions were collected separately with disposable pipettes from the surface.

Chemical analyses. The lipoproteins were dialyzed against 0.15 M NaCl + 0.05% EDTA, pH 7.0, under N_2 prior to chemical analyses. Protein was determined by the method of Lowry *et al.* (24) modified by Lees and Paxman (22) except for the fractions containing albumin or other proteins as in layers 4 and 5. The protein contents of the latter were calculated by the summation of all known apolipoproteins determined by electroimmunoassay. Phospholipid analyses were carried out according to a micromethod of Gerlach and Deuticke (10). Triglyceride and total cholesterol of lipoproteins and of whole plasma were determined by autoanalysis according to the methods of Kessler and Lederer (15) and of Rush *et al.* (33), respectively. Free cholesterol was assayed according to the method of Sperry and Webb (34). Cholesteryl ester was calculated from the difference between total

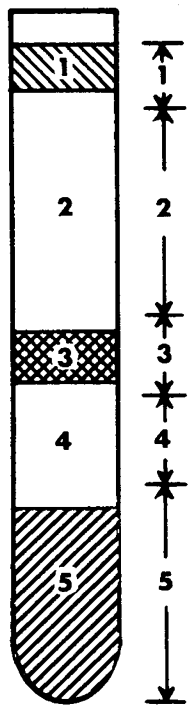

Fig. 1. Lipoprotein banding pattern in a cellulose nitrate tube after density gradient centrifugation at 39,000 rpm in a swinging bucket rotor (SW 40 Ti) for 43 h at 5°C. *Layer 1*, d < 1.010 g/ml, opaque, containing VLDL; *layer 2*, d 1.010–1.028 g/ml, clear; *layer 3*, d 1.032–1.043 g/ml, orange for normal adults, yellow for normal infants, and white for severe Menkes' disease; *layer 4*, d 1.054–1.063 g/ml, clear; *layer 5*, d > 1.074 g/ml, yellow, containing HDL, VHDL, and plasma proteins. The layer numbers and depths are indicated to the *right* of the tube. The colored layers were collected 2 mm into the adjacent clear zone.

Table 2. *Lipid profiles of whole plasma from controls and patients with Menkes' disease*

Subject	Age	State*	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Free cholesterol (mg/dl)	Cholesterol ester† (mg/dl)
Control (T. O.)	5 mo	N	106	51	ND‡	ND
Control (H. H.)	6 mo	N	148	96	ND	ND
Control (H. C.)	13 mo	N	179	90	ND	ND
Control (M. B.)	18 mo	F	153	76	58	160
Case 1 (R. S.)	13 mo	N	162	177	48	191
Case 1 (R. S.)	13 mo	F	160	149	51	183
Case 2 (K. S.)	6 mo	F	178	66	47	220
Case 3 (M. S.)	12 mo	F	130	105	28	171
Normal§	1–3 yr	F	138.2 ± 15.4¶	81.37 ± 28.5	ND	ND
Mother of Case 2		F	196	90	ND	ND

* N, nonfasting plasma sample (1–3 h after meal); F, fasting plasma sample (6–7 h after meal for infants; 10 h for 1–3-yr-old normal children, and 14 h for the mother).

† Calculated as 1.68 (total minus free cholesterol) for cholesteryl linoleate.

‡ ND, not determined.

§ n = 7.

Mean ± SD.

Table 3. *Lipid distribution in density fractions of controls and patients with Menkes' disease*

Lipoprotein fraction	Subject	Age (mo)	State*	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Free cholesterol (mg/dl)	Cholesterol ester (mg/dl)	Total cholesterol/ triglycerides
Layer 1, $d < 1.010$ g/ml	Case 1 (R. S.) (1)	13	N	18.2	128.8	11.4	11.3	0.14
	Case 1 (R. S.) (2)	13	F	16.8	96.0	11.0	9.7	0.19
	Case 2 (K. S.)	6	F	4.7	26.6	2.7	3.4	0.18
	Control (H. C.)	13	N	3.1	63.1	2.5	1.1	0.05
	Control (M. B.)	18	F	11.0	37.1	5.5	9.3	0.29
Layer 2, d 1.010–1.028 g/ml	Case 1 (R. S.) (1)	13	N	9.7	15.3	5.6	7.0	0.63
	Case 1 (R. C.) (2)	13	F	18.5	24.2	8.8	16.3	0.76
	Case 2 (K. S.)	6	F	14.8	14.0	5.5	15.7	1.06
	Control (H. C.)	13	N	8.8	15.8	6.1	4.4	0.56
	Control (M. B.)	18	F	14.0	15.6	5.5	14.4	0.90
Layer 3, d 1.032–1.043 g/ml	Case 1 (R. S.) (1)	13	N	76.8	10.3	21.9	92.1	7.5
	Case 1 (R. S.) (2)	13	F	86.6	11.0	26.8	99.8	7.9
	Case 2 (K. S.)	6	F	70.3	10.5	21.5	81.9	6.7
	Control (H. C.)	13	N	109.0	19.6	29.0	134.9	5.6
	Control (M. B.)	18	F	79.5	13.7	35.7	73.5	5.8
Layer 4, d 1.054–1.063 g/ml	Case 1 (R. S.) (1)	13	N	25.5	6.5	9.8	26.3	3.9
	Case 1 (R. S.) (2)	13	F	26.0	6.7	8.3	29.6	3.9
	Control (H. C.)	13	N	29.4	5.5	8.2	35.6	5.4
	Control (M. B.)	18	F	15.0	5.0	3.5	19.3	3.0
Layer 5, $d > 1.074$ g/ml	Case 1 (R. S.) (1)	13	N	22.5	6.5	6.5	26.8	3.5
	Case 1 (R. S.) (2)	13	F	27.8	6.2	9.5	30.6	4.5
	Case 2 (K. S.)	6	F	32.0	5.1	7.8	40.6	6.3
	Control (H. C.)	13	N	28.4	5.0	8.8	33.1	5.7
	Control (M. B.)	18	F	34.0	13.8	6.0	47.0	2.5

* N, nonfasting (1–3 h after meal); F, fasting (6–7 h after meal).

cholesterol and free cholesterol and expressed as cholesteryl linoleate.

Polyacrylamide gel electrophoresis. To analyze the size of ApoB in lipoprotein subfractions, 3.3% polyacrylamide gel electrophoresis containing SDS was carried out according to the method described previously (20). Intact lipoproteins containing 10–15 μg ApoB were applied after incubation with SDS-containing buffer in the presence of 0.25% dithiothreitol at 100°C for 2 min under N_2.

RESULTS

Table 2 shows the lipid profile of whole plasma of the three patients with Menkes' disease compared to control values from babies of the same age on a similar formula. The formula for control babies also had a P/S ratio of 0.8. Because of the difficulty of obtaining fasting blood samples from small control babies, we have studied the difference between fasting (6–7 h) and nonfasting (1–3 h after meal) in case 1. Values from a group of 1–3-year-old normal fasted children and the mother of patient 2 are also included as references. It was found, from the results of case 1, that there was no difference in total cholesterol and free cholesterol between fasting (7 h) and nonfasting (3 h) plasma. The nonfasting plasma triglyceride value was 16% higher than the fasting value. Thus, an extrapolated fasting (7 h) triglyceride value was obtained from the nonfasting (1–3 h after meal) control subjects. The triglyceride levels for three fasting control subjects (T. O., H. H., and H. C.) were approximately 43, 81, and 76 mg/dl, respectively. Case 3 appeared to have normal total cholesterol but a slightly higher triglyceride level. Case 2 showed a normal plasma triglyceride and a borderline high cholesterol level. Case 1 had elevated triglyceride but normal cholesterol levels. Chylomicrons were not observed in plasma of any of the patients.

The appearance of lipoprotein layer 3 separated by density gradient centrifugation from the plasma of cases 1 and 2 differed markedly from that of the age- and diet-matched controls and case 3. An opaque white band was observed in both cases 1 and 2 but was clear yellow in the controls and in case 3. In addition, layer 1 from case 1 was strongly milky in appearance; only a slight opacity was present in layer 1 from cases 2 and 3 and from control plasma. The milkiness of layer 1 from patient 1 correlated well with the elevated triglyceride and cholesterol contents (Table 3). Increased triglyceride was also present in layer 2 of case 1 in the fasting state. Patients and controls showed no marked differences in lipid levels in any other lipoprotein fractions (Table 3).

Chemical compositions of the subfractions are shown in Table 4. Wide variation was observed in the composition of layer 1 among the three patients. This reflects the heterogeneity of layer 1 and probably dependence on the degree of hypertriglyceridemia present. Layer 1 of all the patients appeared to have a lower content of polar components and a higher content of neutral lipids, suggesting the presence of more of the larger triglyceride-rich particles in the patients. The chemical composition of layer 2 from patient M. S. resembled a subfraction isolated at 1.009–1.019 g/ml from normal fasting adults (19), except in this case, the neutral lipid content was even higher. A lipoprotein fraction with similar composition has been reported to be elevated in Type III hyperlipoproteinemic patients (30). Although the white appearance of layer 3 for patients R. S. and K. S. contrasted with the yellow color of that of M. S. and normal controls, the chemical composition showed no dramatic difference among them. The composition of M. S., and particularly the higher cholesterol ester content in layer 3, more closely resembled that

of an adult's (18). The chemical compositions of layer 5 from patient R. S. and from normal controls were very much alike, suggesting that the patient's HDL was normal. The chemical composition of layer 4 from normal controls was not available for comparison due to insufficient quantity of sample for analysis.

Table 5 shows the apolipoprotein profile of the Menkes' patients in comparison with age- and diet-matched controls as determined by electroimmunoassay of whole plasma. When comparing apolipoprotein values between fasting and nonfasting plasma of patient 1, it was found that ApoE content 3 h after feeding was more than twice as high as in fasting (7 h) plasma, while ApoA-I was not significantly different. Diurnal studies carried out on five adults showed that there was no statistical change in plasma ApoB at 2, 4, and 6 h after meal, compared to 0 h (before the meal) (P. R. Blackett, unpublished results). Also, plasma ApoC-III in four subjects and ApoA-I in three subjects did not show changes after meals. This would indicate similarity of apolipoprotein values between the fasting and nonfasting states except for ApoE. Indeed, in control infants, the fasting ApoE was 8.2 ± 1.4 mg/dl while the nonfasting ApoE was 13.6 ± 0.4 mg/dl. This difference between fed and fasting plasma ApoE was also observed in case 2, for which the nonfasting ApoE was 15.8 mg/dl and the fasting ApoE was 9.3 ± 1.3 mg/dl. Both values were within the normal ranges. Case 3 had an elevated fasting ApoE. Among the three Menkes' cases, all other apolipoproteins showed normal levels except that patient 1 had elevated plasma ApoB (133.2 ± 20.9 mg/dl) and ApoC-III (10.4 ± 1.0 mg/dl) when compared either to young controls or to 1–3-year-old normal subjects. The ApoB elevation was then demonstrated to reside in lipoprotein layers 1 and 2 (Table 6). The ApoC-III elevation was localized primarily in layer 1 (Table 6). These ApoB and C-III elevations were associated with the elevation of triglyceride and cholesterol in the same fractions (Table 3).

Intravenous glucose tolerance tests showed that in case 1, K = 0.82; in case 2, K = 1.98%/min. Thus, from the disappearance rate or K value, only case 1 demonstrated borderline glucose intolerance.

Lipase studies on case 1 plasma showed the lipoprotein lipase activity to be 14.94 ± 3.62 units/ml/h. When hepatic triglyceride lipase was separated by heparin-Sepharose affinity chromatography, its activity was found to be 10.94 ± 4.63 units/ml/h. Therefore, both enzymes were in the normal range (36).

Since the appearance of the major LDL fraction from patients 1 and 2 was abnormal, we have analyzed the ApoB size by 3.3% polyacrylamide gel electrophoresis containing SDS. The results showed that only a single band with apparent molecular weight of 360,000 was found for ApoB from all fractions for patients and controls, as well as for adults, when samples were fresh.

Table 4. *Chemical compositions (%) of the lipoprotein subfractions from Menkes' patients and normal controls*

Fractions	Proteins	Phospho-lipids	Free choles-terol	Choles-terol esters	Triglycer-ides
Patients					
Layer 1 (R. S.)	18.0	20.2	5.8	5.0	50.9
Layer 1 (K. S.)	20.8	27.2	4.3	5.4	42.2
Layer 1 (M. S.)	20.8	15.8	2.7	18.0	42.7
Layer 2 (M. S.)	19.5	17.1	6.1	32.9	24.4
Layer 3 (R. S.)	25.6	27.9	9.0	33.7	3.8
Layer 3 (K. S.)	23.1	32.1	8.4	32.2	4.1
Layer 3 (M. S.)	24.9	23.2	7.8	40.9	3.2
Layer 4 (R. S.)	32.1*	33.2	6.4	22.9	5.2
Layer 5 (R. S.)	45.7*	32.2	4.6	14.6	3.0
Controls					
Layer 1 (M. B.)	25.7	25.9	5.1	8.6	34.7
Layer 3 (H. C.)	25.9	30.6	5.9	32.0	4.6
Layer 5 (H. C.)	47.0*	32.7	3.8	14.3	2.2

* Due to the presence of albumin or other proteins, the protein content was based on the sum of all known apolipoproteins determined by electroimmunoassay.

Table 5. *Apolipoprotein profiles in whole plasma of normal controls and patients with Menkes' disease*

Subject	Age	State*	Apolipoproteins (mg/dl) A-I	A-II	B	C-III	D	E
Control (T. O.)	5 mo	F	146.6	72.3	50.8	4.7	3.0	8.9
Control (S. S.)	6 mo	F	125.4	64.3	50.2	7.1	2.8	6.3
Control (H. H.)	6 mo	N	129.5	57.6†	95.7	4.7	3.1	13.2
Control (H. C.)	13 mo	N	145.1	ND†	121.0	ND	ND	14.0
Control (M. B.)	18 mo	F	98.1	56.3	99.8	7.2	4.4	9.5
Average	10 mo	F + N	128.9 ± 17.5‡	62.6 ± 6.4	79.4 ± 30.3	5.9 ± 1.2	3.3 ± 0.6	8.2 ± 1.4 (F) 13.6 ± 0.4 (N)
Case 1 (R. S.)	7 mo	N	ND	76.4	128.0	11.7	ND	12.0
Case 1 (R. S.)	9 mo	N	119.7	ND	161.0	9.4	ND	11.8
Case 1 (R. S.)	13 mo	N	108.7	ND	ND	ND	ND	17.8
Case 1 (R. S.)	13 mo	F	100.2	51.7	110.6	10.1	3.8	7.0
Average (R. S.)	11 mo	F + N	109.5 ± 8.0	64.1 ± 12.4	133.2 ± 20.9	10.4 ± 1.0	3.8	13.9 ± 2.8 (N)
Case 2 (K. S.)	0.8 mo	N	114.9	ND	47.7	5.6	ND	15.8
Case 2 (K. S.)	2 mo	F	131.2	ND	85.5	7.0	ND	8.0
Case 2 (K. S.)	6 mo	F	97.4	52.8	110.9	6.2	4.8	10.5
Average (K. S.)	3 mo	F + N	114.5 ± 13.8	52.8	81.4 ± 26.0	6.3 ± 0.6	4.8	15.8 (n) 9.3 ± 1.3 (F)
Case 3 (M. S.)	12 mo	F	120.4	55.7	69.5	7.1	2.1	15.1
Normal §	1–3 yr	F	106.0 ± 11.1	ND	78.3 ± 13.9	5.8 ± 1.6	ND	8.8 ± 2.0
Mother of case 2		F	120.1	67.8	112.2	7.8	8.3	10.0

* F, fasting (6–7 h after meal for infants, 10 h for 1–3-yr-old normal subjects, 14 h for the mother); N, nonfasting (1–3 h after meal).
† ND, not determined.
‡ Mean ± SD.
§ n = 8.

Table 6. *Apolipoprotein B and C-III concentrations in triglyceride-rich lipoprotein fractions of Menkes' patients*

Density fraction	Subject	State*	Apolipoproteins (mg/dl) B	C-III
Layer 1	Control (M. B.)	F	6.3	1.3
	Control (H. C.)	N	2.7	1.2
	Case 1 (R. S.)	F	13.8	3.6
	Case 2 (K. S.)	F	7.7	0.9
Layer 2	Control (M. B.)	F	7.2	0.6
	Control (H. C.)	N	8.2	0.7
	Case 1 (R. S.)	F	15.8	1.1
	Case 2 (K. S.)	F	8.6	0.6

* F, fasting (6–7 h after meal); N, nonfasting (3 h after meal).

However, upon storage of LDL, the ApoB from patients degraded much faster than that from controls. At the time when the degraded ApoB from patients showed 7 to 8 faster moving bands, only three bands were observed in controls.

DISCUSSION

This study shows that lipoprotein disorders are variably present in the patients with Menkes' disease. This is the first evidence of a possible relationship of copper metabolism to plasma lipoproteins and apolipoproteins in a clinical disorder of copper transport. However, we did not demonstrate a direct relationship between copper deficiency and hypercholesterolemia as shown in experimental animals (1) or copper deficiency and reduction of lipoprotein lipase (17), possibly because the difference in severity of the clinical manifestations, and different degrees of copper deficiency, caused some variation both in the lipid levels and in glucose tolerance. None of the patients had a high plasma zinc level. Thus, the observed abnormalities in lipoproteins associated with low copper/zinc ratio could be attributed more likely to relative copper deficiency, but not to high zinc levels.

Patient 3 had a normal plasma cholesterol and slightly higher triglyceride levels. His fasting ApoE level was elevated. Layer 3 of the LDL fraction had a normal yellow color in contrast to the white color found in that from patients 1 and 2. This correlates with the finding that his serum copper and ceruloplasmin were not as severely deficient as in cases 1 and 2. Patient 2 had normal triglyceride and borderline high cholesterol values. His lipid and apolipoprotein distributions in the density fractions appeared to be normal. In contrast, patient 1 had elevated plasma triglycerides, ApoB, and ApoC-III. These elevations were mainly in VLDL. Since the lipoprotein lipase activity was normal and chylomicrons were absent, a defect in triglyceride clearance can be excluded. Therefore, enhanced hepatic lipoprotein production would be one explanation for the hyperlipidemia. Since, patient 1 was abandoned by his parents, studies on the family were not done, so we cannot rule out the possibility that his hypertriglyceridemia may be primarily inherited, rather than due to copper deficiency. However, lipid levels of the parents of the other two patients were normal. Thus, the borderline elevations in cholesterol of patient 2 and in triglyceride and ApoE of patient 3 are associated with copper deficiency. The plasma lipid ranges of our control infants (Table 2) compared favorably with the values reported by the Lipid Research Clinic's Program for the same age group. The mean cholesterol in this study was somewhat higher (167 ± 24 mg/dl; $n = 7$) and the triglycerides lower (51 ± 10 mg/dl) than we observed using the same methodology. Chemical composition of lipoprotein subfractions from Menkes' patients did not demonstrate outstanding differences from those of normal controls; however, there was a higher neutral lipid content in the lighter density region, suggesting a larger number of triglyceride-rich particles.

In vitro studies indicate that copper as a cupric ammonia complex solubilizes ApoB (14) suggesting the possibility that copper might be an intrinsic part of ApoB *in vivo*. This remains as yet totally unproven, and furthermore, the ApoB from Menkes' disease patients showed no immunochemical differences from that of control patients when conventional polyclonal anti-ApoB was employed. The ApoB in Menkes' disease is of normal size, but it degrades faster than that from controls. If the degradation of ApoB is caused by oxidation or lipid peroxidation of LDL, it suggests that the LDL of patients are more susceptible to oxidation or lipid peroxidation than those of normal subjects. Further studies suggest that the latter may be the case or that the combination of both lipid peroxidation and high protease activity may be the case.

LDL isolated from fresh plasma of normal adults is orange in color. It may turn opaque white after storage, which we attribute to either lipid peroxidation or bacterial contamination. The white color of fresh LDL in severe cases of Menkes' disease led us to suspect that an abnormality may exist in the substances carried by LDL since ApoB and the chemical composition of layer 3 itself were not abnormal. Analyses of serum vitamin A and β-carotene in case 1 showed 51 μg/dl for the former (normal, 20–80 μg/dl) and 13 μg/dl for the latter (normal, 80–400 μg/dl), whereas case 3 showed normal β-carotene (102 μg/dl) content. Thus, the abnormally low content of carotene explains the absence of orange or yellow color in this patient's LDL. Since carotene and ceruloplasmin function as antioxidants in plasma (12), their low contents in plasma may not be sufficient to protect LDL from lipid peroxidation and resulted in the change of orange color to an opaque white. In this respect, patient 3 had a much higher content of ceruloplasmin than patients 1 and 2, had a normal carotene level, and exhibited a normal LDL color. Since lipid peroxidation has been suggested as the initial event for pathogenesis of human atherosclerosis (38), the relationship between copper deficiency and atherosclerosis may be explained by the deficiency of ceruloplasmin leading to higher susceptibility to lipid peroxidation.

At present, we have not yet determined whether the absorption of carotene is related to intestinal transport of copper; however, the low carotene level may suggest that fat absorption is impaired in severe cases of copper deficiency. The caloric intake for all three Menkes' patients was normal, yet the cases all had retarded growth, a known clinical finding in Menkes' disease (26). In a syndrome of infant malnutrition and diarrhea, many of the infants studied were found to have copper deficiency (11). However, the reverse has not been demonstrated: that is, that copper deficiency causes malabsorption. The mechanism for the association of copper deficiency with growth retardation is still unknown.

Progressive obesity in patient 1 may have caused secondary glucose intolerance which could in turn give rise to excess hepatic triglyceride synthesis (32). Because of the younger age of patient 2, this situation may not have yet come into effect.

It is possible that extreme copper deficiency may be necessary before a significant increase in cholesterol occurs as was observed both in rats (23, 29) and monkeys (27). Although our data did not demonstrate unequivocally the abnormalities in lipoproteins and apolipoproteins for all three Menkes' patients, it appears that the effect of ceruloplasmin deficiency may play an important role in the lack of protection for LDL, ApoB, and carotene from oxidation and the result may lead to atherosclerosis. Further studies on the relationship between ceruloplasmin, the major copper transport protein, and its protection against lipid peroxidation and atherosclerosis are warranted.

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