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HIGH CONCENTRATION OF HEXACOSANOATE IN CULTURED SKIN FIBROBLAST LIPIDS FROM ADRENOLEUKODYSTROPHY PATIENTS

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SUMMARY. Cultured skin fibroblasts from nine patients with the clinical diagnosis of adrenoleukodystrophy (ALD) and 16 control individuals were studied for fatty acid compositions. Total lipids from 7 of the 9 ALD samples showed a high content of hexacosanoic acid (26:0) and a higher ratio of 26:0 to 22:0 acids (0.78-0.92) than the controls (0.03 to 0.09). The samples obtained from two patients with atypical manifestations had a ratio similar to control values. A similar abnormality was demonstrated in the sphingomyelin fatty acids. Diagnosis of ALD and study of its disease mechanism may thus be feasible in skin fibroblast cultures.

INTRODUCTION: ALD is an X-linked disorder associated with progressive demyelination of the cerebral white matter, adrenal insufficiency, and characteristic cytoplasmic inclusions in these organs. While earlier biochemical studies had not shown specific changes (1-5), our laboratories recently demonstrated a specific excess of very long chain fatty acids in the cholesterol ester fractions of the brain and adrenal gland and in the brain gangliosides (6,7). These findings were later confirmed (8). Although ALD is considered to be one of the relatively commonly occurring inherited disorders which affect the central nervous system (9), the absence of specific morphological or biochemical changes in readily accessible tissues makes it difficult for us to distinguish ALD from other progressive neurological diseases (10).

Recently we studied the uptake of [1-^{14}C]lignoceric acid (24:0) and

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[1-^{14}C]hexacosanoic acid (26:0) in cultured skin fibroblasts and found that some of ALD fibroblasts had a higher uptake of both fatty acids than controls (11). Further study on the fatty acid incorporation showed the most prominent uptake of 24:0 into the sphingolipids, sphingomyelin and hematoside (G_{M3}-ganglioside)*. This observation, together with the need to search for diagnostic procedures in accessible tissues, prompted us to investigate the fatty acid composition of sphingolipids in these fibroblasts.

MATERIALS AND METHODS: Patients: We studied cultured skin fibroblasts from 9 patients in whom the clinical diagnosis of ALD had been made on the basis of neurological signs and symptoms which conformed to previously described criteria (12). In cases 2†, 3, and 6, the diagnosis was confirmed by postmortem study (13,14). In case 8, adrenal function was borderline, and ACTH stimulation test was not performed (14); all other patients had severe adrenal insufficiency. In cases 5 and 9, neurological impairment was relatively mild and not clearly progressive. All other patients showed progressive neurological disability (14). As controls, we used fibroblasts from 10 normal persons, aged 2 months to 43 years, from both sexes. As disease controls, we used fibroblasts from one patient each with Fabry, Farber and Sandhoff disease and from 3 patients with progressive bud undiagnosed neurological disease.

Materials: Methyl heptacosanoate (27:0) was purchased from Analabs, North Haven, Conn. Methanolic HCl was obtained from Supelco, Bellefonte, Pa., titrated, and was adjusted to 3 percent by the addition of methanol. All solvents, glass redistilled, were purchased from Burdik and Jackson, Muskegon, Mich. Silica gel G TLC plates were obtained from Analtech, Newark, Del. These materials were used without purification.

Fibroblasts: The fibroblast cell lines were grown in 75 cm^2 Falcon flasks maintained at 37°C in Eagle's minimum essential medium plus Earle's salts and 13 percent fetal calf serum in a 5 percent CO_2 atmosphere as described by Taylor et al. (15). The cells were harvested 3 to 4 days after confluence.

Extraction of Lipids: Cells were treated with 0.2 percent trypsin and centrifuged. The packed cells were suspended in a known volume of water and disrupted by sonication to form a completely homogeneous suspension. An aliquot of this water suspension was taken for protein analysis (16), and the rest was extracted with 10 volumes of chloroform-methanol (1:1). The residue precipitated was removed by centrifugation and washed again with the same solvent. The extracts were combined and washed according to Folch et al. (17). The lower phase was evaporated to dryness.

Total Lipid Fatty Acid Analysis: A portion of the cell total lipids equivalent to 0.5-1.0 mg cell protein was mixed with 1.4 μg of 27:0 methyl ester as an internal standard, and heated with 1.5 ml of 3 percent methanolic HCl at 75° for 16 hours. The methanolysate was extracted twice with 3 ml of hexane. The combined hexane extracts which contained fatty acid methyl esters,

*Y. Kishimoto, K. Suzuki, A. Moser, and H. Moser: unpublished results.
†The case numbers correspond to those given in Table 1 and Figures 1 and 3.

cholesterol and its by-products, cholesta-3, 5-diene and cholesterol methyl ether (18), were evaporated to dryness. The residue was then applied to a TLC plate in a streak of 1.5-2 cm width and the plate was developed with hexane-ether (7:3). Methyl esters of 16:0 and 27:0 were cochromatographed as references After the plate was sprayed with bromothymol blue solution and dried, the area corresponding to the methyl ester references was scraped. The scraping included methyl esters and cholesterol methyl ether and these lipids were eluted twice with 3 ml of hexane. The hexane extract was subjected to GLC. A Packard model 417 gas chromatograph with a 3 mm x 2 m glass column packed with 3 percent SE-30 on Chromosorb W DMCS was used. The temperature was programmed from 160° to 240° at 1° per minute. Fatty acid composition was calculated by the triangulation method. The amounts of methyl esters were calculated from their peak areas by comparison with that of the internal standard.

Sphingomyelin Fatty Acid Analysis: A portion of the crude lipids, as described above, were subjected to mild alkaline methanolysis (19) and the product was separated by TLC with chloroform-methanol-water (65:25:4). The TLC plate was sprayed with bromothymol blue solution and two spots of sphingomyelin were scraped together. Sphingomyelin was then eluted with chloroform-methanol (2:1) and the eluant was washed by Folch's procedure (15). The washed eluant was then evaporated to dryness and the residue was methanolyzed. Fatty acid methyl esters obtained were analyzed by GLC as described above.

RESULTS: The fatty acid content of total lipids in skin fibroblasts varied between 100 to 200 μg per mg protein in various controls with a few exceptions, and between 40 to 80 μg in ALD patients with two exceptions (Figure 1). Typical GLC tracings of total lipid fatty acid methyl esters are shown in Figure 2. The longer chain fatty acids (C_{22} to C_{26}) accounted for only 3-5% of the total fatty acids in normal control fibroblasts (Figure 2A). ALD fibroblasts except those from cases 8 and 9 gave an abnormally high concentration of longer chain fatty acids, especially 26:0, as illustrated in Figures 1 and 2B. Cases 8 and 9, and the disease controls showed essentially the same fatty acid patterns as the normal controls. The difference of fatty acid compositions between ALD and control tissues is much more clearly demonstrated when a ratio 26:0/22:0 is compared (Figure 3). The ratio 24:0/22:0 was also higher in ALD cases 1 to 7; it ranged from 5.4 to 10.1 in these cases, 2.1 to 4.5 in controls, and 3.5 and 2.1 in ALD cases 8 and 9, respectively.

Since the fatty acids of control esters in ALD fibroblasts were found to be normal*, the higher concentration of longer chain fatty acids probably

*Y. Kishimoto, A. Mulunsky, M. Igarashi, H. Schaumburg and K. Suzuki; unpublished results.

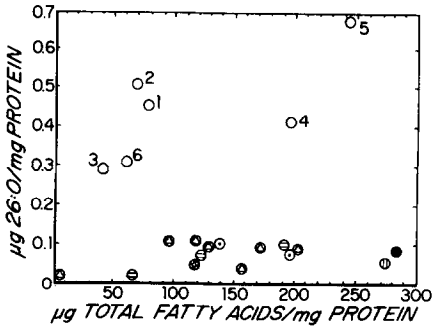

Figure 1: Concentrations of 26:0 and total fatty acids in skin fibroblasts: the number next to a clear circle denotes the assigned patient case number. ● is homozygote of Farber disease. ⊙ is heterozygote of Farber disease. ⊗ is Sandhoff disease. ◍ is Fabry disease. ⊖ is uncharacterized neurological diseases. △ is normal controls.

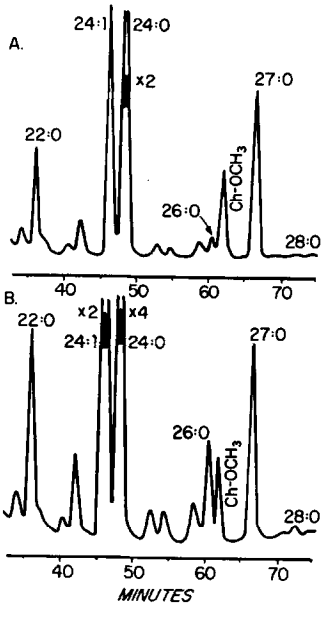

Figure 2: A portion of the GLC tracing of the total lipid fatty acid esters from skin fibroblasts: A is the control and B is ALD (case 4). See text for preparation of samples and GLC conditions. Ch-OCH_3 is cholesterol methyl ether.

reflects the abnormality in sphingolipids. The TLC examination of mild-alkaline-methanolyzed total lipids from ALD fibroblasts revealed no significant abnormality in sphingolipid compositions when compared with normal controls. Sphingomyelin was the predominant sphingolipid and this was followed by hemato-

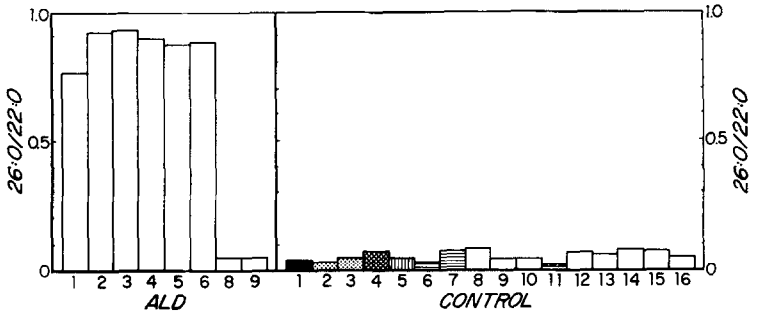

Figure 3: 26:0/22:0 Ratio in skin fibroblast total lipids. The left half shows the ratios in ALD fibroblasts and the right half shows the controls. The bar with solid shading indicates homozygote of Farber disease. The dots are heterozygotes of Farber disease. The cross-hatching is Sandhoff disease. The vertical lines are Fabry disease. The horizontal lines are unknown diseases The open bars are normal controls.

side which was much less concentrated. The examination of sphingomyelin fatty acids from ALD fibroblasts demonstrated that the ratios of 24:0/22:0 and 26:0/22:0 were in fact significantly higher than the controls; the ratios of cases 8 and 9 were again similar to the controls (Table 1).

DISCUSSION: Excess of very long chain fatty acids has been demonstrated in the cultured fibroblasts of the three autopsy proven cases of ALD (patients 2,3, and 6) and in 4 patients in whom this diagnosis was made on the basis of clinical findings (patients 1,4,5 and 7). Two other patients in whom the diagnosis also was based on clinical findings (patients 8 and 9) failed to show this change. It is possible that these cases belong to a different disease category, since in case 8, the documentation of adrenal insufficiency was incomplete (14) and since in case 9, the neurological disorder has been unusually mild and non-progressive. The uptake of radioactive 24:0 and 26:0 by these patient fibroblasts was also in control levels (11).

The basic metabolic defect in ALD remains undefined. The fact that the abnormality in fatty acid composition was most striking in the cholesterol ester fraction (6) had at first suggested a primary defect in cholesterol ester metabolism. However, cholesterol ester hydrolase activity was not

Table 1 Fatty Acid Composition of Sphingomyelin From Skin Fibroblasts of ALD Patients and Control

Case No.	16:0 %	17:0 %	18:1 %	18:0 %	20:0 %	22:0 %	23:0 %	24:1 %	24:0 %	25:0 %	26:0 %	Others*	24:0/22:0 Ratio	26:0/22:0 Ratio
ALD														
1	55.1	1.9	1.8	3.8	0.2	2.3	0.8	13.8	16.4	0.2	0.7	3.0	7.1	0.30
3	49.4	1.4	0.7	2.9	0.1	2.0	0.6	22.0	19.0	0.2	0.8	0.9	8.6	0.40
5	57.9	1.2	2.0	7.0	0.3	1.9	0.9	8.6	16.9	0.3	1.1	1.9	9.9	0.55
7	40.2	0.6	0.8	1.9	0.2	2.1	0.9	29.5	21.0	0.5	1.4	0.9	10.0	0.67
8	66.3	1.8	1.7	3.9	0.3	2.3	0.5	13.5	9.0	0.07	0.07	0.6	3.9	0.03
9	62.9	1.4	0.4	2.8	0.2	3.5	0.5	15.6	12.2	0.08	0.09	0.3	3.5	0.02
Control Average	75.1	1.5	1.2	5.7	0.2	2.1	0.5	5.5	7.6	0.09	0.09	0.4	3.6	0.04

*Others include 14:0, 16:1, 22:1, 23:1, 25:1, 26:1

defective in ALD brains (11). Our present finding of an abnormality in the very long chain fatty acids of the sphingolipids of cultured skin fibroblasts, coupled with the previous demonstration that these fatty acids also accumulated in ALD brain gangliosides (6,7), make it more likely that the primary metabolic defect involves a perturbation of very long chain fatty acid metabolism. Study of cultured amniotic fluid cells is in progress in the hope that it will aid prenatal diagnosis.

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