ENHANCED SYNTHESIS OF HEXACOSANOIC ACID IN THE CULTURED FIBROBLASTS FROM PATIENTS WITH ADRENOLEUKODYSTROPHY

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ABSTRACT

We previously demonstrated the increased activity of fatty acid elongation in the microsomal fraction of adrenoleukodystrophy (ALD) fibroblasts. In the present study, detailed pathways for the synthesis of hexacosanoic acid (C26:0) were delineated using deuterium labeling and mass fragmentography. Replacement rate or fractional turnover rate of C26:0 in ALD fibroblasts increased by 67% of the control, while the rate of replacement from C16:0 to C24:0 decreased in ALD. This may indicate an activation of fatty acid synthesis specific for C26:0. When two elongation activities using endogenously formed precursors and exogenous or preexisting fatty acids were separately measured in ALD, elongation products starting from exogenous fatty acids increased in C16:0, C18:0, C20:0, C22:0 and C24:0, but the corresponding fraction in C26:0 decreased as compared to the control. These results suggest that the synthesis of C26:0 may take place in a compartment different from those for the synthesis of fatty acids whose chains are shorter than C26:0, and that the activity of C26:0 synthesis is enhanced *in vivo* in ALD.

Adrenoleukodystrophy (ALD) is an X-linked disorder that involves mainly the adrenal cortex and the cerebral white matter (12). Igarashi *et al.* (4, 5) first reported the substantial increase in very long-chain fatty acids in cholesterol esters, cerebroside, and gangliosides in cerebral white matter and adrenal gland. Schaumburg *et al.* (13) and Powell *et al.* (10) demonstrated characteristic cytoplasmic inclusions, which were subsequently shown to consist of cholesterol esters with very long-chain fatty acids (4, 8, 11). The increase in very long-chain fatty acids was also shown in myelin cholesterol esters (3), and in myelin cerebroside and sulfatides (9) isolated from

the ALD patient brains. The abnormality of very long-chain fatty acids was found in sphingomyelin (19) and glycerophospholipids (1, 2) of erythrocyte membranes, and of skin fibroblasts from patients with ALD (6, 18), suggesting the generalized abnormality in the metabolism of very long-chain fatty acids in ALD.

In order to understand the abnormality of the fatty acid metabolism, several approaches were carried out in the literature. Two possible mechanisms have been proposed to explain the accumulation of very long-chain fatty acids. The first is impaired degradation of very long-chain fatty acids in ALD (14, 15). Kishimoto *et al.* (7) demonstrated in a terminal case

of ALD that deuterated hexacosanoic acid mixed with food was incorporated into the cholesterol esters in white matter. Singh *et al.* (15) presented evidence for the decreased oxidation of long-chain fatty acids in the fibroblasts and adrenal homogenates obtained from one ALD patient. Kishimoto's group, therefore, speculates that the primary defect involves the degradation of very long-chain fatty acids derived from food intake (7, 15). On the other hand, Tsuji *et al.* (18) reported that a substantial amount of hexacosanoic acid was synthesized from stearic acid by ALD fibroblasts. Tsuji *et al.* (17) further demonstrated the increased activities of fatty acid elongation in the microsomal fraction of ALD fibroblasts, suggesting an involvement of accelerated fatty acid elongation. In this study, we present evidence for the enhanced synthesis of hexacosanoic acid by elongation *in vivo* in cultured fibroblasts. In order to determine separately the elongation products using exogenous fatty acids and newly synthesized fatty acids as substrates, deuterium labeling and mass fragmentography (16) were employed.

MATERIALS AND METHODS

Culture of Fibroblasts

Skin fibroblasts were obtained from patients with ALD and patients without any neurological diseases. The diagnosis was made on the basis of clinical signs and the increased hexacosanoic acid in sphingomyelin isolated from erythrocyte membranes, plasma and cultured skin fibroblasts (18, 19). The skin fibroblasts were seeded at 5×10^5 cells per 75-cm² Falcon flask containing 15 ml of Eagle's MEM (Nissui Seiyaku, Tokyo) in the presence of 10% (v/v) fetal calf serum (GIBCO 200-6140, Gland Island, New York) and 2 mM glutamine. After 1-day culture, the medium was exchanged with 15 ml of Eagle's MEM containing 10% (v/v) fetal calf serum, 2 mM glutamine and 20% deuterium oxide. The culture was continued under 5% CO₂ for 4 days.

Lipid Preparation

The total lipids were extracted from the cells harvested, and applied to preparative thin-layer chromatography (TLC, Silica gel 60, Kanto Chemicals, Tokyo). The plates were

developed with chloroform/methanol/acetic acid/water (25:15:4:2), and the band corresponding to sphingomyelin was scraped out. Sphingomyelin was extracted from the silica gel, and subjected to methanolysis (100°C, 3 h) with 3% hydrochloric acid in methanol to release fatty acids as their methyl esters. The fatty acid methyl esters were separated by high performance liquid chromatography using a reversed phase C18 column (4.6 mm i.d. $\times$ 15 cm). Palmitate plus stearate, C20:0, C22:0, C24:0 and C26:0 were isolated by eluting with *n*-hexane/ethanol/acetonitrile (10:20:70) as monitored at 205 nm. Non-deuterated fatty acids were obtained from rat liver sphingomyelin by the same method as mentioned above.

Mass Fragmentography

Fatty acid methyl esters were injected into a gas chromatograph equipped with a capillary column (OV-101 or CBP20-M25-025, 0.25 mm i.d. $\times$ 25 m) which was connected to a mass spectrometer (Shimadzu-LKB 9000B, Shimadzu Seisakusho, Kyoto). The mass spectrometer was operated under an electron impact condition by using a computer-assisted mass fragmentography program, which was a modified method of single-ion monitoring so as to monitor ten different ions. Deuterium contents were measured from the intensities of isotope peaks corresponding to the molecular ions. The deuterium contents and distribution patterns of deuterated fatty acid methyl esters were calculated according to the method reported previously (16).

RESULTS AND DISCUSSION

The method of deuterium labeling followed by mass spectrometric determination of deuterated molecules provides detailed information about *de novo* synthesis and chain elongation occurring *in vivo* (16). We have employed this method to assess the fatty acid elongation activities in the cultured skin fibroblasts obtained from ALD patients. In the previous study (16) mice were given 30% deuterium oxide without any side effects. The culture system in this study contained 20% deuterium oxide. This concentration of deuterium oxide did not affect the cell number seriously after

4-days of culture. The cell contents in deuterium oxide medium was $85 \pm 2\%$ of those in ordinary water medium.

The contents of total lipids and sphingomyelin in the cultured fibroblasts and the percentage of C26:0 in sphingomyelin fatty acid moieties are listed in Table 1. The contents of the total lipid and sphingomyelin in ALD fibroblasts were not different from those in control fibroblasts. The level of C26:0 and the ratio of C26:0 to C22:0 increased six times and seven times, respectively, in ALD as compared to control. Hexacosanoic acid was

already shown to increase in sphingomyelin of fibroblasts from ALD patients (6). The fatty acids were obtained as their methyl esters from the sphingomyelin fraction of the fibroblasts cultured in a medium containing deuterium oxide. Hexacosanoate was isolated by high performance liquid chromatography so as to determine the deuterium contents accurately by mass fragmentography in such a minor component. Deuterium labeling patterns were delineated by computer-assisted mass fragmentography as shown in Fig. 1, in which a non-deuterated (fraction I) and two

Table 1 Lipid Contents in the Cultured Fibroblasts from ALD Patients and Normal Control Subjects, and the Percentage of Hexacosanoate in Sphingomyelin

	Total lipid (TL)	Sphingomyelin		C26 fatty acids in sphingomyelin		
	$\mu\text{g}/\text{mg}$ protein	$\mu\text{g}/\text{mg}$ protein	% in TL	% of C26:0	% of C26:0 plus C26:1	C26:0/C22:0
Control (n=3)	263 ± 14	14.3 ± 3.6	4.8 ± 1.1	0.13 ± 0.04	1.86 ± 0.18	0.020 ± 0.006
ALD (n=3)	291 ± 32	11.0 ± 0.9	3.8 ± 0.6	$0.76 \pm 0.20^*$	3.39 ± 1.24	$0.151 \pm 0.043^*$

Values are presented as means $\pm$ SD. * $P < 0.05$ vs. control (normal *t*-Test)

Fig. 1 A typical distribution pattern of C26:0 labeled with deuterium. Open bars show the observed distribution of differently labeled molecules. The vertically hatched bar shows a fraction of exogenous or preexisting molecules (fraction I). Dotted bars represent fatty acid molecules formed by chain-elongation starting from non-deuterated molecules (fraction II). Closed bars are ascribed to fatty acids newly formed by elongation of deuterated precursors or *de novo* synthesis (fraction III). Distributions of fraction II and III were calculated by using the equation of Bernoulli distribution with the aid of computer to fit the actually observed distribution pattern of C26:0 molecules (see reference 16).

Fig. 2 Percent replacement rates by newly formed molecules of fatty acids in sphingomyelin in control (□) and ALD patients (▨). Values are means of two samples in control, and are expressed as means $\pm$ SD ($n=3$) in ALD patients.

Fig. 3 Contribution of chain elongation activity using non-deuterated precursors (dotted column) (fraction II in Fig. 1) to the total fatty acid synthesis (fractions II + III). C, control; A, ALD patients

differently labeled groups (fractions II and III) appeared to overlap. These fractions were clearly separated by employing a fitting technique using Bernoulli distribution equation according to the method previously reported (16). The total percentage of fractions II and III reveals the replacement rate of the sphingomyelin pool by newly formed molecules. The fractions II and III are supposed to be assigned to hexacosanoates synthesized by chain elongation of non-deuterated and deuterated precursors, respectively.

Replacement rate may be equivalent to fractional turnover rate, and it gives real turnover

rate if the pool size of molecules concerned is known. Fig. 2 shows the replacement rates by newly formed molecules of the fatty acids in sphingomyelin. The rate of replacement from C16:0 to C24:0 decreased in ALD fibroblasts as compared to the control, explaining the retarded turnover rates of these fatty acids in ALD. On the contrary, the replacement rate of C26:0 increased by 67% of the control. This indicates an increase in fatty acid synthetic activity specific for C26:0 in ALD. This enhanced synthesis of C26:0 may account for the increased pool size of C26:0 sphingomyelin species that is calculated, from the data in

Table 1, to be more than 4-fold in ALD (84 ng and 19 ng/mg protein in ALD and control, respectively). On the other hand, Singh *et al.* reported the defect in oxidation of very-long chain fatty acids (C24:0 and longer) in patients with ALD (14). Thus impaired degradation of very long-chain fatty acids may also contribute to the increase in C26:0 lipid species.

Newly formed fatty acids labeled with deuterium are separated into two fractions, II and III, as stated above. As shown in Fig. 3, the fraction III corresponding to the products formed by elongation using endogenously formed substrates or by *de novo* synthesis appeared predominant in C16:0, while the fraction II formed by elongation using exogenous or preexisting fatty acids is abundant in C18:0, C20:0, C22:0 and C24:0. Tsuji *et al.* (17) have found an increase in microsomal elongation activity in ALD by 50–80% that of control by an experiment using exogenous substrates. Elongation products starting from exogenous fatty acids increased in fact in ALD sphingomyelin species of C16:0, C18:0, C20:0, C22:0 and C24:0, but the corresponding fraction of C26:0 was rather lower in ALD than in control (Fig. 3). In other words, the contribution of the elongation using newly formed precursors to the C26:0 synthesis was larger in ALD than in control, 65% and 55%, respectively. Based on the present results, it is suggested that the synthesis of C26:0 may take place in a compartment different from those for the synthesis of fatty acid chains shorter than C26:0, and that the activity of C26:0 synthesis is enhanced *in vivo* in ALD.

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