Turnover of endogenous ceramide in cultured normal and Farber fibroblasts

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Abstract De novo synthesis and turnover of endogenous ceramide in cultured skin fibroblasts from patients affected with Farber lipogranulomatosis were studied by biosynthetical labeling of cellular sphingolipids with [^{14}C]serine. The cellular uptake of [^{14}C]serine and incorporation into de novo synthesized ceramide was similar in normal and Farber fibroblasts, with a half life of newly synthesized ceramide of 2.7 h in normal and diseased cells. Newly synthesized ceramide was found to be channeled directly into biosynthesis of complex sphingolipids rather than contributing to the pool of accumulated ceramide in Farber fibroblasts. The degradation of ceramide generated by the catabolism of complex sphingolipids in Farber cells was greatly delayed compared with control fibroblasts, with differences in the amount of radiolabeled cellular ceramide becoming evident after 6 h chase time. Individual Farber cell lines differed from each other in the amount of accumulated ceramide; however, no correlation was found between ceramide accumulation and residual acid ceramidase activity as determined in vitro. In addition, the amount of radiolabeled sphingomyelin was significantly increased in Farber fibroblasts suggesting a delayed degradation of this compound in this ceramide storage disorder. We propose biosynthetical labeling of endogenous ceramide with [^{14}C]serine, in addition to other established methods, as a highly sensitive and reliable method for the diagnosis of Farber disease, allowing semiquantitative measurement of ceramide accumulation in cultured skin fibroblasts of patients affected with Farber lipogranulomatosis—**van Echten-Deckert, G., A. Klein, T. Linke, T. Heinemann, J. Weisgerber, and K. Sandhoff.** Turnover of endogenous ceramide in cultured normal and Farber fibroblasts. *J. Lipid Res.* 1997. **38:** 2569–2579.

Supplementary key words Farber lipogranulomatosis • acid ceramidase • [^{14}C]serine labeling • skin fibroblasts

Farber disease (FD; lipogranulomatosis) is a rare autosomal recessively inherited lipid storage disorder caused by a deficiency of lysosomal acid ceramidase (N-acylsphinogosine deacylase; EC 3.5.1.23), the cDNA of which has been cloned recently (1). The enzyme catalyzes the hydrolysis of ceramide (Cer) to sphingosine and free fatty acid which is the final step in lysosomal degradation of all sphingolipids (SL) (2). Cer is the precursor of SL which are complex components of the plasma membrane of eukaryotic cells, where they form cell type specific patterns. Clinical symptoms of FD include painful swelling of joints, subcutaneous nodules, a hoarse cry, hepatosplenomegaly, and nervous system dysfunctions of markedly variable degree (3). Predominant biochemical parameters in FD are an accumulation of Cer in tissues (2) and elevated levels of Cer in urine (4), whereby the amount of Cer accumulated in tissues was shown to correlate with the age of death of the patients (3, 5).

Intracellular Cer levels are maintained by a balance between degradation and replacement by newly synthesized molecules. The de novo biosynthetic pathway of Cer begins with the serine palmitoyltransferase-catalyzed condensation of serine and palmitoyl-CoA to form 3-ketosphinganine, this first step in Cer synthesis being rate-limiting (6). Further sequential enzymatic modifications lead to dihydroceramide (DHCer) which is converted into Cer (7). While Cer is the precursor for complex SL, it is also formed by their degradation, mainly by degradative hydrolysis of sphingomyelin (SM) which is the major membrane sphingolipid. The changes in

Abbreviations: FD, Farber disease; SM, sphingomyelin; SL, sphingolipids; TLC, thin-layer chromatography; Cer, ceramide, N-acylsphingosine; DHCer, dihydroceramide; GlcCer, glucosylceramide, Glcβ1-1Cer; LacCer, lactosylceramide, Galβ1-4Glcβ1-1Cer; GbOse3Cer, Galα1-4Galβ1-4Glcβ1-1Cer; GbOse4Cer, globoside, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; GM3, Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; GM2, GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; GM1, Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; GD3, NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer; GD1a; Neu Acα2-3Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer.

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cellular metabolism caused by elevated Cer levels in FD are not well characterized yet. In addition to its central role in SL biosynthesis and membrane formation, evidence emerged during the past years that Cer plays an important role as an intracellular signaling molecule for a variety of different processes such as neurite growth (8), monocyte differentiation (9), fibroblast proliferation (10), and apoptosis (11–13). A number of direct targets for the signaling action of Cer have been identified including a Cer-activated protein phosphatase (14), Cer-activated serine/threonine protein kinase (CAP kinase) (15, 16), and the protein kinase C isoform ζ (17).

Based on the biological functions and the elevated levels of Cer in FD, studies on the turnover of this biomodulating molecule may contribute to a more detailed view of the cellular events taking place in FD. It is not clear, for example, whether accumulation of Cer in FD affects Cer de novo biosynthesis, whether the biosynthesis of complex SL is affected, and whether newly synthesized Cer contributes to the pool of accumulated Cer. A possible way to get more insight into the pathway of SL metabolism is provided by the use of cultured cells of patients with sphingolipid storage disorders. For Farber lipogranulomatosis, many approaches have been undertaken to demonstrate Cer accumulation in cultured cells of affected patients by feeding either radiolabeled exogenous Cer (18, 19) or various complex SL from which Cer is released by their degradation after cellular uptake (20–27). However, as these methods were devised for pragmatic diagnosis of FD, they allow us to examine the impaired degradation of Cer in Farber lipogranulomatosis but do not provide conclusive information about the biosynthesis and turnover of endogenous Cer in this disease.

Here we report on the metabolism of endogenous Cer biosynthetically labeled with [^{14}C]serine in skin fibroblasts affected with FD. De novo biosynthesis of Cer was found to be similar in normal and Farber fibroblasts. [^{14}C]serine labeling of Cer is shown to allow semiquantitative measurement of Cer accumulated in Farber fibroblasts. Although requiring special laboratory skills, we propose this method as a reliable assay for the diagnosis of FD in addition to other established methods, providing a high diagnostic sensitivity.

MATERIALS AND METHODS

Materials

L-[3^{14}C]serine (54 mCi/mmol) was purchased from Amersham-Buchler (Braunschweig, Germany). L-Serine and palmitoyl-CoA were from Sigma (Deisenhofen, Germany). Culture medium (Dulbecco's modified Eagle's medium (DMEM) and minimal essential medium (MEM) containing Glutamax^{R}), fetal calf serum (FCS), and trypsin were obtained from Gibco BRL (Grand Island, NY). FCS was heat-inactivated before use. The plastic culture dishes were from Costar (Bodenheim, Germany). LiChroprep^{R} RP-18 and precoated thin-layer silica gel 60 as well as cellulose plates were purchased from Merck (Darmstadt, Germany). Ultima Gold was from Packard (Groningen, Netherlands). X-ray film AR 5 was supplied by Kodak. All other chemicals and organic solvents were of the highest purity available.

Cell culture

Six different cultured fibroblast lines from patients positively diagnosed for FD (F1–F6) were used for the study. The Farber cell line F1 was from Dr. A.H. Fensom, London, U.K.; F2 was from Dr. M.T. Zabot at the Hospital Debrousse, Lyon, France; F3 (GM 02351; 19) and F4 (GM 05752; 20) were from the Human Genetic Mutant Cell Line Repository, Camden, NJ; F5 was from Dr. A. Reuser at the Erasmus University, Rotterdam, The Netherlands; F6 was from Prof. Dr. H.W. Moser, Baltimore, MD; F7 and the carriers P1 and P2 were from Dr. E. Vamos at the Brugmann University Hospital, Bruessel, Belgium. Normal control fibroblasts were from the Johanniter Children's Hospital, Sankt Augustin, Germany. Cells were cultivated in DMEM supplemented with 10% heat-inactivated FCS. The metabolic studies were performed with confluent cells in 21-cm^2 plastic culture dishes.

Labeling of cultured cells

Culture medium was changed to MEM supplemented with 5% FCS 1 h before the labeling experiments. Cells were then labeled for different time periods (from 10 min to 24 h) with [^{14}C]serine (1 μCi per ml medium; 18.5 μM) in 2 ml MEM containing 0.3% heat-inactivated fetal calf serum. Chase periods were started by replacing the medium with MEM containing non-labeled L-serine (185 μM) and 0.6% FCS. After the respective time periods, ranging from 1 h up to 120 h, the cells were washed with PBS (phosphate-buffered saline), detached from dishes by incubation with 0.25% trypsin for 15 min at 37°C, and collected by centrifugation (2 min; 2000 rpm; 4°C).

Isolation of cellular sphingolipids

The cell pellets obtained from one confluent grown 21-cm^2 plastic culture dish were resuspended in 400 μl water. Aliquots for protein determination were saved. Then total lipids were extracted with 7 ml chloroform–

methanol–water–pyridine 60:160:6:1 (by vol) for 24 h at 50°C. Phospholipids were degraded by mild alkaline hydrolysis with 2 ml methanolic sodium hydroxide (50 mM) for 2 h at 37°C. After neutralization with acetic acid, the lipids were desalted by reversed-phase chromatography (28) and aliquots were measured for radioactivity by scintillation counting. The SL were applied to TLC plates and chromatographed with chloroform–methanol–0.22% aqueous $CaCl_2$ 60:35:8 (by volume). For borate plates the solvent was chloroform–methanol 90:10 (by volume). All SL were identified from their R_f values. Radioactive spots were evaluated by the bio-imaging analyzer Fujix Bas 1000 using software TINA 2.08 (Raytest, Straubenhardt, Germany) and visualized by autoradiography. Alternatively, chloroform–methanol–water 80:10:1 (by vol) was used as a solvent system. Radioactive spots corresponding to Cer were quantified as described above.

In vitro ceramidase assay

Acid ceramidase of fibroblasts was assayed with the synthetic substrate N-lauroylsphingosine in the presence of detergents as previously described (29). Cultured fibroblasts from 175-cm^2 plastic flasks were harvested after incubation with trypsin. After lysis of the cells in an aqueous solution of Triton X-100 (0.5% w/v) the homogenate was centrifuged (2 min, 10.000 *g*, 4°C) and the clear supernatant was adjusted to 250 mM sucrose and 20 mM phosphate buffer, pH 7.2, in a final volume of 0.5 ml. Incubation mixtures contained N-lauroylsphingosine (150 μM), sodium acetate-buffer (250 mM, pH 4.2), Triton-X 100 (0.5%, w/v), Tween 20 (0.2% w/v), sodium cholate (0.8%, w/v), Nonidet P-40 (0.2%, w/v), EDTA (1 mM), and up to 100 μg of cellular protein in a final volume of 100 μl. After incubation for 30 min at 37°C, the reaction was stopped with methanol (300 μl) and a mixture of C14- and C16-sphinganine (500 pmol each) was added as an internal standard. The mixture was dried under a stream of nitrogen, submitted to alkaline hydrolysis, and long chain bases were determined as described by Merrill et al. (30).

Determination of radiolabeled serine in the culture medium

Aliquots of culture medium, saved after the indicated times of labeling, were applied onto cellulose TLC plates using buthanol–acetic acid–water 120:30:50 (by vol) as a solvent system. [^{14}C]serine was visualized by autoradiography and identified on the basis of the R_f value of authentic serine. Radioactive spots were evaluated by the bio-imaging analyzer Fujix Bas 1000 using software TINA 2.08 (Raytest, Staubenhardt, Germany).

Protein determination

Cellular proteins were estimated according to Bradford (31) using bovine serum albumin as a standard.

Presentation of data

Cell labeling experiments were performed in at least two different experiments in which separated cultured fibroblasts were used. For each experiment at least double determinations were performed. Deviations from the mean never exceeded ± 15% within the same experiment. There were, however, considerable differences between the absolute cpm values of separately cultured cells and, therefore, results are given as % of the values of the respective control cells. Standard deviations for the cpm values relative to controls never exceeded ± 20% among separately cultured fibroblasts. Samples were placed on TLC plates and calculated on the basis of confluent dishes. Comparable protein values per dish were obtained after 3 days of confluency.

Acid ceramidase determination was performed for each cell line in two different experiments in which separately cultured fibroblasts were used. Each assay was performed in triplicate. Deviations from the mean never exceeded ± 10% within the same experiment. The statistical scatter of the six values, obtained for each cell line, never exceeded ± 16%.

RESULTS

De novo ceramide biosynthesis is not significantly affected in Farber cultured fibroblasts compared with control cells

Fibroblasts obtained from normal controls and from patients with FD were incubated with [^{14}C]serine for increasing time periods. Total radioactivity taken up by control and Farber fibroblasts increased linearly with time. After a 10-min pulse, 1.2% of total radioactivity added to the medium was taken up by the cells, increasing continuously up to 9.4 ± 2.4% after 1.5 h pulse time. A maximum of cellular uptake was reached after 24 h labeling time when 17.2 ± 1.7% of initial radioactivity was cell associated. As illustrated in **Fig. 1** (insert), about 10% of the radiolabeled serine initially supplied was still present in the medium after 24 h of labeling time. The half life of radioactive intact free serine in the medium was 4.2 h.

The incorporation of [^{14}C]serine into cellular SL was studied in normal fibroblasts in order to determine the shortest pulse period sufficient for maximum labeling of Cer. As depicted in Fig. 1 and **Fig. 2** (control cells), [^{14}C]serine labeling of Cer was rapid, reaching a maxi-

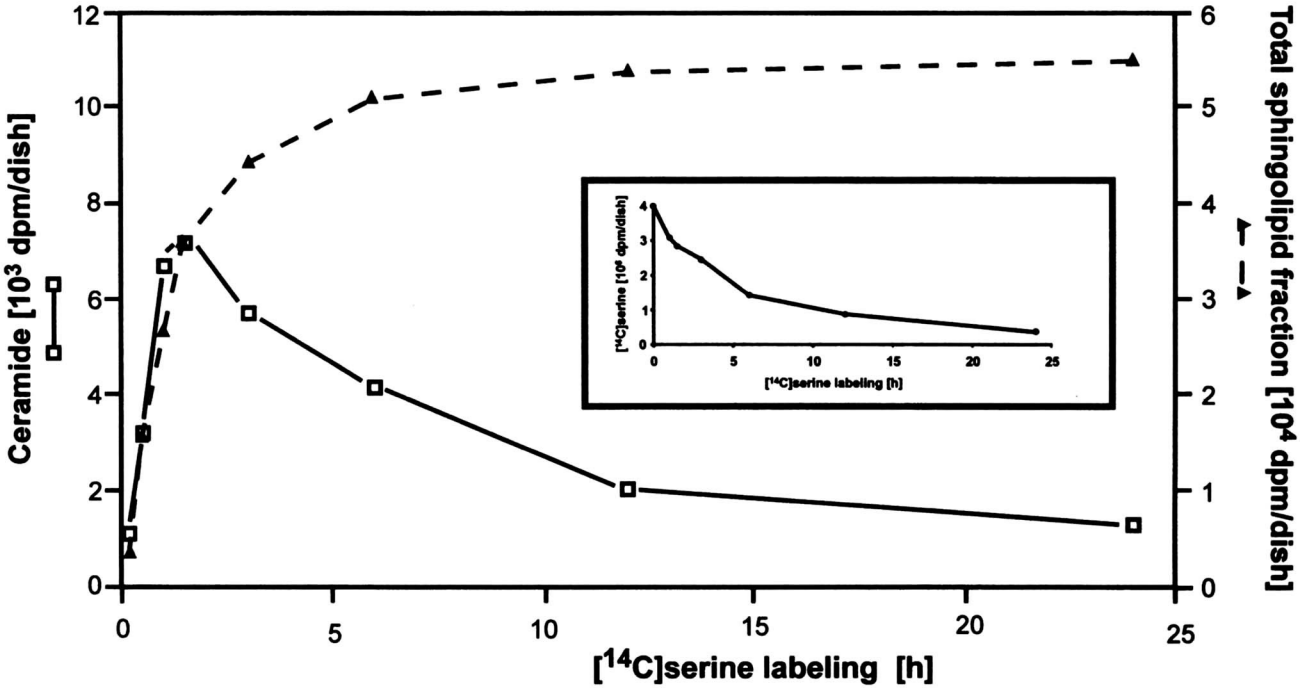

Fig. 1. Time course of [^{14}C]serine incorporation into ceramide and total sphingolipid fraction of cultured normal skin fibroblasts. Fibroblasts from normal controls were labeled with [^{14}C]serine (1 μCi/ml) for the indicated time periods. Then the cells were harvested, the sphingolipid fraction was isolated, separated by TLC using chloroform–methanol–water 80:10:1 (by vol) as solvent system and identified as described in Materials and Methods. Maximum labeling of Cer was reached after 1.5 h. Insert: Cell-conditioned medium was saved at every time point and radiolabeled serine was determined as described in Material and Methods.

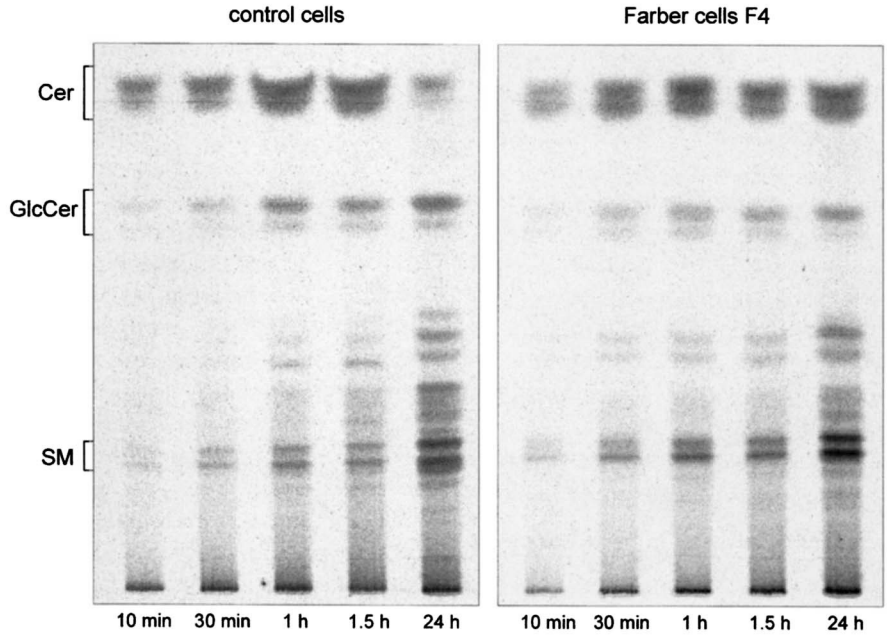

Fig. 2. Biosynthetic labeling of sphingolipids with [^{14}C]serine in cultured normal and Farber fibroblasts. Fibroblasts from a normal control cell line and from Farber cell line F4 were incubated with [^{14}C]serine (1 μCi/ml). After the indicated pulse time cells were harvested and lipids were extracted. The desalted lipid extracts were separated by TLC using the solvent system chloroform–methanol–aqueous 0.22% $CaCl_2$ 60:35:8 (by vol). The R_f values of authentic ceramide (Cer), sphingomyelin (SM), and glucosylceramide (GlcCer) are indicated. Similar results were obtained for Farber cell lines F2 and F6.

mum at 1.5 h pulse time followed by a continuous decrease up to 24 h labeling time when about 20% of the peak Cer labeling was detectable. In contrast, radiolabeled total SL continued increasing up to 6 h labeling time, when an equilibrium was reached that showed only a slight further increase up to 24 h. In addition to Cer, SM and GlcCer were also detectable after a 10-min pulse and their levels increased with ongoing time, as shown in Fig. 2.

Pulse experiments were also performed in Farber cell lines F2, F6, and F4, the results of the latter are shown in Fig. 2. In both Farber cell lines no significant differences in the profile of labeled SL could be detected compared with normal cells up to 1.5 h. No statistically significant differences were detectable in labeling of Cer within 1.5 h (Fig. 2), suggesting that de novo biosynthesis of Cer is not affected in Farber fibroblasts. When the labeling time was extended to 24 h, however, the amount of labeled Cer in Farber cells was found to be significantly elevated compared with normal cells, representing the impaired ultimate degradation of Cer in these cells. In contrast, after 1.5 h pulse time, labeled SM was found to be slightly but significantly elevated above the level of control cells with marked differences between individual Farber cell lines. The amount of labeled SM remained increased compared with normal cells up to 24 h labeling time.

Recent results suggest that DHCer rather than Cer is a direct precursor of complex SL (32). To confirm that the labeled double band comigrating with authentic Cer represents mainly Cer and not DHCer, the respective bands were scraped off TLC plates after 1.5 h pulse

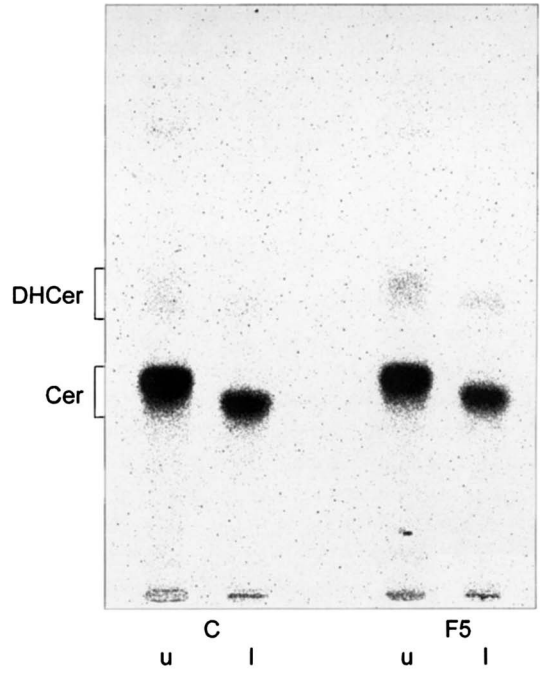

Fig. 3. Separation of biosynthetically labeled dihydroceramide and ceramide from human fibroblasts. Fibroblasts of controls (C) and Farber cell line F5 (F5) were pulsed with [^{14}C]serine (1 μCi/ml) for 1.5 h. Then cells were harvested and lipids were extracted, desalted, and ceramide was separated by TLC developed in chloroform–methanol–water 80:10:1 (by volume). The upper (u) and lower (l) portions of the double band, respectively, comigrating with authentic ceramide, were scraped off the TLC plate, reextracted, and applied onto a borate plate developed in chloroform–methanol 90:1 (by volume). R_f values of authentic dihydroceramide (DHCer) and ceramide (Cer) are indicated.

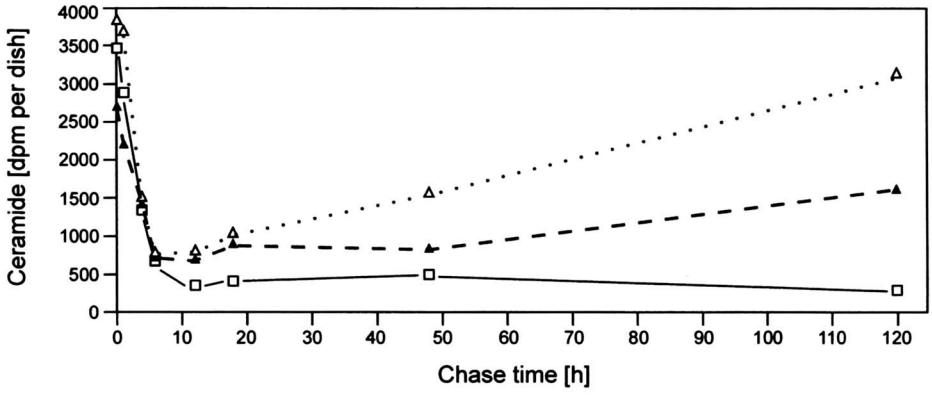

Fig. 4. Turnover of [^{14}C]serine-labeled ceramide in cultured normal and Farber fibroblasts. Normal control fibroblasts (□) and Farber cell lines F2 (△) and F6 (▲) were pulsed with [^{14}C]serine (1 μCi/ml) for 1.5 h. The medium was then changed and the cells were chased for 1, 4, 6, 12, 18, 24, 48, and 120 h, respectively, in a medium containing unlabeled serine. After harvesting the cells, the sphingolipids were isolated, separated by TLC, and the amount of radiolabeled ceramide was evaluated as described in the legend to Fig. 1. The first data time point represents the ceramide-incorporated radioactivity at 1.5 h pulse. All determinations were run in duplicate and deviations from the mean never exceeded ±20%.

and were reapplied onto borate TLC plates. As demonstrated in **Fig. 3** more than 90% of labeled compound comigrated with a Cer standard while only trace amounts comigrated with authentic DHCer, suggesting that the double band in both, normal and Farber fibroblasts, represents mainly Cer.

Endogenous newly synthesized ceramide is predominantly utilized for synthesis of complex SL in FD

To determine the half life of biosynthetically labeled Cer and to follow the time course of Cer accumulation in FD, normal fibroblasts as well as Farber cell lines F2 and F6 were pulsed with [^{14}C]serine for a time period of 1.5 h, which was found as being sufficient for maximum labeling of Cer in normal cells, and were then chased with a 10-fold amount of unlabeled serine for various time periods from 1 h up to 120 h. Results are given in **Fig. 4**. During the first 6 h of chase the amount of labeled Cer decreased rapidly and equally in both, Farber and normal fibroblasts. The half life for newly synthesized labeled Cer was 2.7 h in both, control and Farber fibroblasts. This decrease was most likely caused by the utilization of newly synthesized Cer for the biosynthesis of complex SL as the radioactive labeling of SM and complex glycosphingolipids increased substantially up to 6 h of chase in both, normal and Farber fibroblasts, as shown for cell line F4 in **Fig. 5**. Only after a chase time longer than 6 h did the fate of the radioactive labeled Cer differ significantly in normal and Farber fibroblasts. In normal cells the amount of labeled Cer decreased continuously within 12 h chase time when it reached an equilibrium showing only very slow further decrease with ongoing time. In contrast, in both Farber cell lines, the level of labeled Cer after 6 h chase remained constant up to 12 h and then increased continuously up to 120 h, reaching 40% and 80% of the 1.5 h pulse level, respectively. These results suggest that newly synthesized Cer is predominantly utilized for synthesis of complex SL from which it is again released and ultimately stored in the lysosomal compartment. The early rapid decrease of labeled Cer and the increase after 12 h in Farber cells also indicates the newly synthesized Cer does not directly contribute to the pool of accumulated Cer in this disease.

Various Farber cell lines differ in the amount of ceramide accumulation which does not correlate to residual acid ceramidase activity

To evaluate the magnitude of differences in Cer accumulation in various Farber cell lines, normal cells as well as Farber fibroblasts (F1–F6) were pulsed with [^{14}C]serine for 1.5 h followed by a chase up to 120 h. As given in **Table 1**, after 120 h of chase all of the Farber cell lines showed severalfold increased levels of labeled Cer compared with control cells. Significant differences in the accumulation of Cer between the individual Farber cell lines were observed. These results demonstrate that Cer labeling with [^{14}C]serine allows a semiquantitative determination of Cer accumulation in Farber fibroblasts.

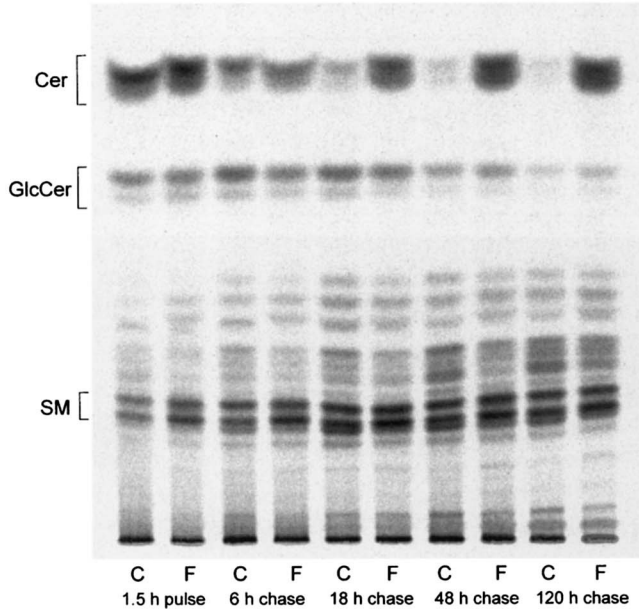

Fig. 5. Turnover of [^{14}C]serine-labeled sphingolipids in cultured control and Farber fibroblasts. Fibroblasts from normal controls (C) and Farber cell line F4 (F) were incubated in the presence of [^{14}C]serine (1 μCi/ml) for 1.5 h. The medium was then changed and the cells were chased for the indicated times in a medium containing unlabeled serine. Cells were harvested after 1.5 h pulse and the different chase times are indicated, respectively. Sphingolipids were isolated, separated by TLC, and identified by autoradiography as described in Materials and Methods. R_f values of authentic ceramide (Cer), glucosylceramide (GlcCer), and sphingomyelin (SM) are indicated.

To evaluate whether the magnitude of Cer storage reflects the residual activity of acid ceramidase in the respective Farber cell lines, acid ceramidase activity in normal and Farber fibroblasts was determined. As shown in Table 1, the average activity of acid ceramidase in control fibroblasts was 1.10 nmol/mg × h, while in Farber cells from different patients (F1–F7) the enzyme activity ranged from nondetectable levels (F6) to 0.39 nmol/mg × h (F2). No correlation could be observed between the percentage of Cer storage and residual acid ceramidase activity as determined in vitro in the presence of detergents.

In order to further examine the relationship between Cer accumulation and residual acid ceramidase activity and to validate the accuracy of [^{14}C]serine Cer labeling for the diagnosis of FD, the Cer storage in fi-

TABLE 1. Comparison of the amount of labeled ceramide, the in vitro acid ceramidase activity and the Farber phenotype

	F1	F2	F3	F4	F5	F6	F7	P1	P2	C
Sex	M	M	F	F	F	F	F	M	F	M/F
Age of clinical onset	6 months	10 months	10 months	3 weeks	6 months	2 months	2 months	–	–	–
Lifetime	>3 years	2.2 years	>9 years	6 months	>6 years	>4.5 years	2 years	–	–	–
Acid ceramidase [nmol/h × mg]	0.13	0.39	0.18	0.007	0.26	n.d.	0.10	0.55	0.59	1.1
Labeled ceramide [% of control]	794	676	303	468	506	424	500	125	133	100
Hoarseness	+	+	+	+	+	?	+	–	–	–
Skin nodules	+	+	+	+	+	+	+	–	–	–
Soft tissue swelling	–	–	–	+	+	+	+	–	–	–
Joint contractures	+	+	+	+	+	+	+	–	–	–
Hepatosplengomegaly	–	–	–	+	+	–	+	–	–	–
Neurological features	Ataxia and spasticity of lower limbs Action and intentional tremor Absences Myoclonic jerks Loss of speech EEG: focal bioccipital spikes, diffuse spike and wave complexes	NCV decreased Psychomotoric retardation Muscular hypotonia EEG: normal	NCV normal EEG: normal	EEG: normal	No focal signs NCV decreased EEG: generalized changes		No focal signs			
Others			Muscular wasting	Generalized lymph-adenopathy			Muscular wasting			

Fibroblasts were pulsed with [^{14}C]serine for 1.5 h; then the medium was changed and the cells were chased for 120 h. Cells were then harvested and sphingolipids were isolated, separated, and the levels of ceramide were quantified by radio-scanning as described in Materials and Methods. The residual activities of acid ceramidase were assayed in the fibroblast extract prepared as described in Materials and Methods. Control fibroblasts (C) were from 3–5-year-old healthy donors. F1, F2, F3 (42), F4 (43), F5, F6, F7 (44), fibroblasts from Farber patients; P1, P2, fibroblasts from the heterozygous parents of patient F7; n.d. not detectable.

broblasts of heterozygous carriers of FD was determined and was correlated with the respective activity of acid ceramidase (Table 1). In fibroblasts of both heterozygous parents of Farber patient F7, which were clinically not affected, metabolic labeling of endogenous Cer revealed only a slight increase of about 130% of that of control cells. In contrast, the amount of labeled Cer in the fibroblasts of the affected offspring (F7) was about 500% compared with control cells. In both heterozygous carriers acid ceramidase activity was found to be reduced by about 50% when compared with normal control cells.

In our study, neither the amount of accumulated ceramide nor residual acid ceramidase activity in Farber diseased fibroblasts was correlated to the lifetime or to particular clinical features of the respective patients, as given in Table 1.

These results suggest that factors other than the amount of accumulated Cer or the residual acid ceramidase activity may influence, at least in part, the clinical picture of the affected patients.

Decrease of labeled complex sphingolipids is delayed in Farber fibroblasts compared with normal cells

To examine whether the increased [^{14}C]serine labeling of complex SL observed up to 24 h in the pulse experiments in Farber fibroblasts remains elevated for longer time periods, the level of SM and GlcCer of normal and Farber cells biosynthetically labeled by a 1.5 h pulse were studied after 24 h and 120 h chase, respectively. **Table 2** shows the differences in [^{14}C]serine incorporation into Cer, SM, and GlcCer between control cells and various Farber cell lines (F1–F6). In normal fibroblasts only a small percentage of the pulse level of labeled Cer was detectable after 24 h chase which further decreased up to 120 h chase. In contrast, in Farber cells at 24 h, a substantial percentage of Cer remained [^{14}C]serine labeled which in all Farber cell lines further increased up to 120 h. While differences in the turnover of GlcCer were less pronounced between normal and Farber cells, SM labeling was significantly increased in Farber cells compared with normal fibro-

TABLE 2. Relative labeling of ceramide, glucosylceramide and sphingomyelin in control and Farber fibroblasts

Cell Line	Chase time	Incorporation of [^{14}C]Serine into SL		
		Cer	SM	GlcCer
	h		*% of pulse*	
C1	24	17	128	85
	120	4	108	17
C2	24	10	(−)	58
	120	4	77	15
C3	24	6	113	60
	120	3	71	23
F1	24	44	157	58
	120	76	93	21
F2	24	35	174	65
	120	77	81	16
F3	24	52	215	99
	120	77	110	30
F4	24	44	202	80
	120	53	93	24
F5	24	47	141	48
	120	62	83	21
F6	24	28	206	62
	120	40	94	16

Confluent cells were pulsed for 1.5 h with [^{14}C]serine and then chased in the presence of unlabeled serine for 24 and 120 h. Sphingolipids were extracted, isolated and quantified as described in Materials and Methods. The pulse levels (dpm/dish) of labeled Cer (ceramide), SM (sphingomyelin), and GlcCer (glucosylceramide) of controls were 5250 ± 1150, 2250 ± 540, 1230 ± 240, respectively and of Farber cells 5950 ± 710, 3910 ± 1215, 1910 ± 170, respectively. C1-3, control fibroblasts; F1-6, fibroblasts from Farber patients.

blasts after 24 h chase, showing a decrease up to 120 h chase time when the level still remained above that of control cells. While in normal cells the overall total amount of sphingolipid-associated radioactivity decreased substantially between 24 h and 120 h, reaching about 65%–70% of the pulse level after 120 h, the decrease was less pronounced in Farber cells. Instead, a slow shift of radioactivity from the SM fraction to Cer was observed between 24 h and 120 h chase. These results suggest that most likely a delayed degradation of labeled SM or a reduction of influx rate of Cer overloaded endosomes into the lysosomal compartment prevented Farber fibroblasts from a similar decrease in [^{14}C]serine-labeled complex SL as was observed in normal fibroblasts.

DISCUSSION

Loading of cultured cells with radiolabeled metabolites of the sphingolipid pathway is an approach widely used for studies on Cer metabolism and the diagnosis of Farber lipogranulomatosis. Intermediates used for studying the metabolism of Cer in FD include radiolabeled Cer (18), cerebroside sulfate (sulfatide) (21–23), glucosylceramide (20), and SM (22, 24–27). However, results obtained from these studies vary significantly, which may be attributed to several possible reasons, including *i*) differences in the cellular uptake of different water-insoluble lipid compounds (18); *ii*) the possibility of an incomplete targeting of the radiolabeled lipids to the lysosomal compartment leading to subsequent degradation, as was suggested for SM by a non-lysosomal degradation pathway (33) and for Cer by non-lysosomal neutral and alkaline ceramidases that are not deficient in FD (18, 26, 27); and *iii*) a lipid overloading of the lysosomes by exogenous lipids that may impair normal lysosomal functions.

In the present study we used [^{14}C]serine for metabolic labeling of SL for examining the turnover of Cer in cultured skin fibroblasts from patients affected with Farber lipogranulomatosis. Serine is substrate for serine palmitoyltransferase, catalyzing the condensation of serine with palmitoyl-CoA to form 3-ketosphinganine, which is the committed step in the de novo biosynthesis of Cer and complex SL (6). Thus, loading cells with radiolabeled serine allows us to circumvent the various drawbacks associated with the use of exogenous SL metabolites listed above. In addition, [^{14}C]serine labeling allows us to examine the rate of de novo biosynthesis of Cer in FD. While the turnover of exogenous Cer in Farber lipogranulomatosis was intensively studied (18,19), no data are available in the literature as to whether the impaired turnover of Cer in Farber lipogranulomatosis causes alterations in the rate of de novo biosynthesis of Cer. The results of our study demonstrate that the magnitude and time course of both, [^{14}C]serine uptake as well as initial [^{14}C]serine incorporation into Cer, do not differ significantly between Farber and normal fibroblasts. In both fibroblasts a rapid labeling of Cer was observed and a maximum was reached after 1.5 h pulse time. In normal and diseased cells, the level of labeled Cer decreased rapidly with a halflife of 2.7 h when chased with unlabeled serine for 6 h, while incorporation of radioactivity into complex SL increased during that time.

De novo biosynthesis of Cer is thought to occur very fast in vivo as free long-chain bases are not detected as intermediates in sphingolipid biosynthesis (6). The first intermediate in de novo Cer biosynthesis, 3-ketosphinganine, is rapidly reduced to sphinganine and then converted into N-acylsphinganine (DHCer) by Cer synthase (7). It was suggested that DHCer rather than Cer might be the direct precursor of complex SL and that most of the cellular Cer is derived from degradation of complex SL (32). However, the enzyme catalyzing the introduction of the 4,5-double bond, con-

verting DHCer to Cer, has been characterized recently in rat liver microsomes in vitro, indicating the de novo synthesized Cer is indeed an intermediate in the biosynthesis of complex SL (34). The results from our study support the concept that Cer formed from DHCer is the predominant precursor of complex SL in human skin fibroblasts. When cells were chased after 1.5 h pulse, the decrease of labeled Cer and the coincident increase of [^{14}C]serine incorporation into complex SL indicates that in fibroblasts most, if not all, of de novo synthesized Cer is utilized as precursor for complex SL.

The initial rapid increase of labeled Cer and the steady decrease after 1.5 h in normal fibroblasts up to 12 h labeling time suggests a burst-like incorporation of [^{14}C]serine into Cer. It was demonstrated in a recent study in J774 cells, that changing of cells in culture to fresh medium induces a transient burst of de novo sphingolipid biosynthesis as determined by an increase of sphinganine, with a maximum occurring within 60 min and returning to normal levels within 4 h (32). As assessed by Cer labeling, in our pulse experiments a similar but somewhat delayed time course was observed for Cer in skin fibroblasts upon changing to a pulse medium containing [^{14}C]serine. Besides an ongoing incorporation of labeled de novo synthesized Cer into complex SL, the decrease in labeled Cer after 1.5 h pulse is most likely attributed to an easing away of the burst and returning of de novo SL biosynthesis to normal levels, as there is no depletion of [^{14}C]serine in the medium at that time. The further decrease up to 24 h labeling time in normal cells might, in part, reflect the efficient ultimate lysosomal degradation of Cer, possibly combined with a growing depletion of labeled serine available in the culture medium.

In contrast to normal cells, the level of labeled Cer in Farber fibroblasts remained constant after 6 h chase and rose again after 12 h chase, indicating the impaired ultimate degradation of Cer. These results indicate that the bulk of labeled Cer had reached the lysosomes after about 6 h chase. The time course of a rapid decrease of labeled Cer after 1.5 h pulse and an increase after about 6 h chase in Farber cells indicate that newly synthesized Cer does not directly contribute to the pool of accumulated Cer but rather appears to be channeled into the biosynthesis of complex SL, before again being released in the lysosomal and/or late endosomal pathway. It is noteworthy that it took more than 6 h for the Cer incorporated pulse peak label to appear in the accumulation compartment. In previous studies it was shown that in skin fibroblasts incubated with LDL-associated [^{3}H]sphingomyelin, differences in Cer levels between skin fibroblasts of normal subjects and Farber patients were detectable after 3 h (3, 26). In these studies, in contrast to ours, the bulk of radiolabeled SM was instantaneously available for degradation into Cer after cellular uptake. Therefore, the time difference most likely reflects the turnover rate of complex SL, which was demonstrated previously as being relatively slow (35).

As was shown recently (3, 5) and confirmed in our study, Cer accumulation is different in various Farber cell lines. However, in our study we could not find a correlation between the amount of accumulated Cer and acid ceramidase activity. The in vitro determination of the residual acid ceramidase activity is widely used and has been proven to be a valuable method for diagnosis of FD (36). However, the use of detergents in in vitro assays is known to affect enzyme specificity and stability and, therefore, our results on acid ceramidase activity in vitro may not accurately reflect the in vivo situation. A detergent-free acid ceramidase assay is not available as of yet. Moreover, it has been shown that sphingolipid activator protein D (*sap*-D) stimulates the lysosomal degradation of Cer in vivo (37). These findings indicate that the determination of the amount of accumulated Cer should be considered as part of the diagnosis of FD.

Previous studies showed a correlation between the magnitude of ceramide accumulation in skin fibroblasts and the clinical course of the disease as assessed by the age of death of affected patients (3, 5). In our study neither the amount of accumulated ceramide nor the residual acid ceramidase activity in skin fibroblasts was found to be correlated to the life time or clinical features of the affected patients. As the clinical picture varies remarkably among patients, these results suggest that in addition to ceramide accumulation, other metabolic factors might influence the clinical course of affected patients. Interestingly, in the Farber cell lines, the amount of SM was found to be elevated compared with normal cells as soon as after 1.5 h pulse. After 24 h chase time, labeled SM was elevated in all Farber cell lines and remained increased up to 120 h chase. This observation probably should not be interpreted as a recycling of the abundant lysosome-stored radiolabeled Cer into the biosynthesis of complex SL as physiological Cer is most likely unable to escape the lysosome at significant rates to be re-used for SL biosynthesis. When Farber fibroblasts, lacking any residual activity of acid ceramidase, were incubated with labeled sulfatide of different chain length, only the short chain Cer but not Cer of physiological chain length, released from the respective sulfatide, was found to be incorporated into SM (38). More likely, the increased amount of SM in Farber cells observed in our study might be interpreted as an expanded pool of SM in Farber diseased cells due to a delayed degradation of SM. In an in vitro assay, acid sphingomyelinase activity was found not to

be reduced in Farber fibroblasts compared with normal controls (K. Ferlinz, H. Moczall, and K. Sandhoff, unpublished results). However, it remains questionable whether these results represent the in vivo situation as Cer at a concentration of 500 μM was found to reduce acid sphingomyelinase activity substantially in vitro (H. Moczall and K. Sandhoff, unpublished results). The high levels of Cer in FD might reduce the kinetic of SM degradation, which might explain the increased level of labeled SM observed in Farber fibroblasts. Further studies are necessary to examine whether the clinical course of patients affected with FD is, at least in part, related to a delay in the turnover of complex SL in this disease.

The action of Cer as a potent biomodulator raises the intriguing question whether elevated Cer levels in FD may result in a permanent stimulation of the sphingomyelin–ceramide-mediated signaling pathway, leading with time to deleterious effects on the cell function (26). It is noteworthy that in our study elevated levels of Cer as well as SM in Farber cells did not appear to result in significant damage to the cells even up to 120 h chase, as assessed by light microscopy. It was proposed recently that, in FD, the residual activity of acid ceramidase may prevent cells from early fatal lesions (26). Also, the lysosomal Cer may not qualify for a biomodulating action because the molecular species of this Cer might be distinct from the signaling Cer (39). Most likely the ceramide sequestered within the lysosome is not physically available as a signaling molecule. Moreover, recent studies suggest the existence of different intracellular pools of Cer. When sphingomyelinase activity was increased by stable transfection of Molt-4 leukemia cells with the *B. cereus* sphingomyelinase gene driven by an inducible promoter, Cer levels rose up to 240% of control levels, causing apoptosis after 2 h (39). In contrast, while incubation of cells with exogenous *B. cereus* sphingomyelinase also increased Cer levels, apoptosis was not induced (39). The part of SM not accessible for exogenous sphingomyelinase appears to localize to the inner leaflet of the plasma membrane or to a closely related compartment (40). It was shown in a recent study that Cer production is highly compartmentalized at the cell surface in caveolae, which may be essential for a delivery of Cer to specific sites of biomodulating action within the cell (41). Therefore, the Cer accumulated in Farber cells or tissues might be without biomodulating effect on its cellular targets due to its localization in the lysosomes (26).

The results of our study show that [^{14}C]serine labeling of cultured skin fibroblasts provides a simple and reliable method for semiquantitative determination of Cer accumulation in FD. Moreover, this method allows us to determine the magnitude of turnover delay of complex SL in this disease. Although requiring special laboratory skills, this method because of its high sensitivity provides a valuable tool in the diagnosis of FD in addition to other established assays.

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