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The in situ degradation of ceramide, a potential lipid mediator, is not completely impaired in Farber disease

Thierry Levade, Marie-Caroline Tempesta and Robert Salvayre

Laboratoire de Biochimie, 'Maladies Métaboliques', CJF INSERM 9206, Institut Louis Bugnard, CHU Rangueil, Toulouse, France

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The time course of degradation of a radiolabelled natural ceramide has been studied in intact, living lymphoid cells and skin fibroblasts from normal individuals and from patients affected with Farber disease, an inborn disorder of ceramide metabolism due to deficient activity of lysosomal ceramidase. The hydrolysis of ceramide in lysosomes was selectively followed by examining the turnover of an LDL-associated radioactive sphingomyelin. This permitted to estimate accurately the effective lysosomal ceramidase activity and to demonstrate: (i) a very active catabolism of ceramide in normal cells; and (ii) the absence of a complete block of ceramide degradation in Farber cells. The possible implication of ceramide as a lipid mediator of the pathogenesis of Farber disease is discussed

Ceramide; Lysosome; Farber disease; Ceramidase; Sphingomyelin; Lipid targetting

1. INTRODUCTION

Ceramide (*N*-acylsphingosine) is a key intermediate in the synthesis and degradation of sphingomyelin (SPM), gangliosides and other glycolipids, i.e. membrane, lipoprotein and myelin constituents [1,2]. Little is known about the physiological role of ceramide. Due to its high concentration in the stratum corneum [3,4], it may account for the preservation of water impermeability of the skin [5]. Recently, ceramide and its metabolic products have been suggested as very potent biological effectors [6–8]. In particular, the pivotal participation of ceramide in an intracellular signal transduction pathway which implicates SPM degradation has been proposed in various cellular events as differentiation [9,10], proliferation [11] or apoptosis [12].

The hydrolysis of ceramide is catalyzed by ceramidases (*N*-acylsphingosine deacylases; EC 3.5.1.23). Three ceramidases have been distinguished based on their pH optima, substrate specificity, tissue distribution and subcellular localization [13–20]. Of these, only the lysosomal ceramidase exhibits a deficient activity in Farber disease [21]. This rare, inherited lipid storage disorder, also called lipogranulomatosis, is associated with accumulation of ceramide in the patient tissues and is clinically characterized by painful swelling of joints, subcutaneous nodules and a hoarse cry [22].

As for other sphingolipidoses, the mechanisms underlying the pathophysiology of Farber disease are not yet clarified. One may postulate that, if ceramide has indeed key regulatory second messenger functions, the absence of its degradation might lead to fatal lesions. Since the effective lysosomal ceramidase activity in intact living cells has never been reported, the aim of the present study was to estimate accurately this activity in order to appreciate the possible role of the lysosomal storage of ceramide as a lipid mediator of the pathogenesis of Farber disease. Intact cells were incubated with the immediate radiolabelled precursor of ceramide, SPM which was associated to human LDL to ensure specific targetting of the sphingolipid to the lysosomes and selective determination of the lysosomal ceramidase activity.

2. MATERIALS AND METHODS

2.1. *Chemicals and radiolabelled lipids*

[^{3}H]Sphingomyelin ([^{3}H]SPM, 400 mCi/mmol) was obtained from CEA (Gif-sur-Yvette, France) by catalytic tritiation of bovine brain SPM (Sigma), the sphingolipid was purified by preparative TLC, using chloroform/methanol/water (100:42:6, v/v) as developing solvent. The purity of the radioactive SPM was further checked by analytical TLC and corresponded to 97–99% Acidic hydrolysis showed that the SPM was radiolabelled on both the sphingoid base and fatty acid moieties, in which the radiolabel represented 45 and 55%, respectively. [9,10-^{3}H]Oleic acid (10 Ci/mmol) was from DuPont NEN (Les Ulis, France). Standard radiolabelled ceramide was synthesized by condensing [^{3}H]oleic acid and sphingosine (Sigma) according to previously described procedures [23,24]. Silica gel 60 TLC plates were from Merck (Darmstadt, Germany) All solvents and other reagents obtained from Merck or SDS (Peypin, France) were of analytical grade.

RPMI 1640 medium, penicillin, streptomycin, L-glutamine, trypsin-

Correspondence address: T. Levade, Laboratoire de Biochimie, 'Maladies Métaboliques', Institut Louis Bugnard, Bât. L3, CHU Rangueil, 1 Avenue Jean Poulhès, F-31054 Toulouse Cédex, France. Fax: (33) 6132-2953.

Abbreviations: SPM, sphingomyelin; LDL, low density lipoprotein; TLC, thin-layer chromatography.

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EDTA and fetal calf serum were from Gibco BRL (Cergy-Pontoise, France). The serum substitute, Ultroser HY was from IBF (Villeneuve-la-Garenne, France).

2.2. *Cell cultures*

Long-term human lymphoid cell lines were established by Epstein-Barr virus-transformation of peripheral blood B lymphocytes [25,26] from normal individuals and from patients affected with Niemann-Pick disease Type A (lines EIG and MS-325) and Farber disease (lines Moz and GM 5748). Human skin fibroblasts derived from normal individuals and from patients affected with Niemann-Pick disease Type A (line GM 370) and Farber disease (lines Fra and GM 5752). The lymphoid cell line GM 5748 and the fibroblast cell line GM 5752 derived from the same patient with Farber disease; both leukocytes and fibroblasts of this patient showed undetectable acid ceramidase activity [27]. The cell line MS-325 was kindly provided by Drs. E.H Schuchman and R.J. Desnick (Division of Medical Genetics, The Mount Sinai Medical Center, New York), the line Fra was from Drs. R. Gatti and P. Durand (Istituto G. Gaslini, Genova, Italy), the line Moz from Dr. J.M. Richardet (Hôpital Trousseau, Paris, France) and the GM 370, GM 5748 and GM 5752 lines were from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Identical culture conditions were used for lymphoid cells and fibroblasts. The cells were routinely grown in a humidified 5% CO_2 atmosphere at 37°C in RPMI 1640 medium containing L-glutamine (2 mmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and heat-inactivated fetal calf serum (10%) as previously reported [26]. Cultured skin fibroblasts were used just after reaching confluency.

2.3. *Preparation of serum-enriched medium containing [^{3}H]sphingomyelin*

A chloroform/methanol (2:1 v/v) solution of the radioactive SPM was evaporated under nitrogen. The residue was dissolved in a small volume of absolute ethanol and mixed with sterile culture medium containing 10% fetal calf serum [26]. This medium was warmed at 37°C for about 15 min. The final concentration of radiolabelled SPM in the incubation medium averaged 4×10^6 dpm/ml (i.e. 4.5 nmol/ml) and that of ethanol did not exceed 0.7% (by vol.).

2.4 *Preparation of LDL-associated [^{3}H]sphingomyelin*

This method will be described in detail elsewhere (T Levade, D. Graber and R. Salvayre, manuscript in preparation). Briefly, an ethanolic solution of [^{3}H]SPM (about 700×10^6 dpm) was mixed with 10 ml of filtered pooled fresh human sera and incubated overnight at 37°C. The subsequently labelled LDL were isolated by discontinuous density gradient ultracentrifugation in a vertical rotor [28], extensively dialyzed and filtered through a 0.2 μm pore diameter membrane filter. The incorporation of SPM into LDL averaged 12,500 dpm/μg apolipoprotein B. More than 95% of the radioactive SPM was associated with LDL as measured after agarose gel (Hydragel Sebia) electrophoresis. The physical and biological properties of these [^{3}H]SPM-labelled LDL were similar to their native counterparts (T. Levade, D. Graber and R. Salvayre, in preparation).

2.5. *Incubation of intact cells with [^{3}H]sphingomyelin*

Before the experiments were initiated, the cells were grown for 2–3 days in RPMI 1640 medium containing L-glutamine, antibiotics and 2% Ultroser HY, a serum substitute. Cells were then incubated at 37°C either with fetal calf serum-enriched medium containing [^{3}H]SPM (data not shown) or with medium containing 2% Ultroser HY and LDL-associated [^{3}H]SPM (the final concentration of LDL in the incubation medium approximated 50 μg apolipoprotein B/ml). There was no difference in the [^{3}H]SPM uptake levels between normal and Farber cells. After either 3 or 24 h pulse, the medium was removed and the cells were washed twice (lymphoid cells were sedimented by slow-speed centrifugation) in RPMI medium containing 10% fetal calf serum. Fresh medium supplemented with 10% fetal calf serum was added to the cells and incubation continued for the indicated times (from 0 to 96 h). At the end of incubation, cells were washed three times with PBS containing bovine serum albumin (2 mg/ml) and then twice with PBS alone [26]. The cell pellets were stored at −20°C.

2.6. *Lipid extraction and analyses*

Cell pellets were suspended in 0.5 ml distilled water and sonicated for 3×15 s (Soniprep MSE sonicator). An aliquot was taken for protein determination [29], another for estimating the total cell-associated radioactivity by liquid scintillation counting (Packard Tricarb 4530 spectrometer), and the remaining was extracted with 2.5 ml of chloroform/methanol (2:1 v/v), vortex mixed and centrifuged at $1000 \times g$ for 15 min [30]. The lower phase was evaporated under nitrogen and the lipids were resolved by analytical TLC developed in chloroform/methanol/water (100:42:6 v/v) up to the 2/3 of the plate and then in chloroform/methanol/acetic acid (96:4:1 v/v). The distribution of the radioactivity on the plate was analyzed using a Berthold LB 2832 radiochromatoscan. Unlabelled and radioactive lipid standards were used to identify the various [^{3}H]SPM metabolic products

3. RESULTS AND DISCUSSION

Previous studies have examined on intact Farber fibroblasts the metabolic fate of the ceramide itself [31,32] or the ceramide derived from sulfatide hydrolysis [33]. However, the effective residual activity of lysosomal ceramidase has not been determined because no pulse-chase experiments were performed [33] or because the ceramide was probably degraded by non-lysosomal ceramidase(s) due to deficient targetting of the lipid substrate to the lysosomes [31,32]. In one of these studies [32], the turnover of a ceramide incorporated into reconstituted LDL was followed by a pulse-chase experiment. But, presumably due to abnormal processing of the delipidated and relipidated lipoprotein, even normal cells could not degrade the ceramide during the 24 h pulse and to a small extent (30%) during the chase period. We therefore undertook the re-estimation of the lysosomal ceramidase activity in Farber cells. As LDL are delivered to lysosomes [34], they represent a useful vehicle for transport of lipids to these organelles. Since ceramide is not a normal component of LDL, we decided to follow the metabolism of SPM, the natural sphingolipid constituent of lipoproteins which is also the immediate precursor of ceramide.

The first approach we used was to introduce directly radiolabelled SPM into a culture medium containing fetal calf serum. This mode of administration has indeed been shown to essentially result in LDL receptor-mediated endocytosis and lysosomal hydrolysis of SPM [26]. Unexpectedly, two different lymphoid cell lines and two different fibroblast cell lines from Farber disease patients showed a considerable ability to metabolize the exogenously supplied sphingolipid (data not shown). Although the levels of undegraded ceramide (expressed as percentage of the total SPM metabolites) in Farber cells remained higher than in normal cells, they represented less than 20–30% after 4 days chase. We hypothesized that this high residual ceramidase activity in Farber cells might be related to the participation of non-lysosomal ceramidase(s) because of incomplete target-

ting of the SPM to the lysosomes. This was supported by the finding that, during the same chase period, about 10–15% of the SPM administered in the presence of serum was hydrolyzed in cells from patients with Niemann-Pick disease Type A, which are completely deficient in lysosomal sphingomyelinase activity [26] (data not shown). Therefore, the procedure of administration of SPM was modified in order to avoid non-lysosomal degradation.

We recently developed a convenient method to stably associate radiolabelled SPM to purified human LDL and showed that this SPM was selectively internalized through the LDL receptor-mediated pathway and processed into the lysosomes of intact cells (T. Levade, D. Graber and R. Salvayre, in preparation). Hence, we investigated the metabolism of LDL-associated [^{3}H]SPM in Farber cells. The fact that this SPM was specifically hydrolyzed into the lysosomal compartment was ascertained by the virtual absence of degradation in cells derived from the most severe form of Niemann-Pick disease, i.e. Type A (see inset of Fig. 2).

As illustrated in Fig. 1, normal lymphoid cells pulsed with LDL-associated [^{3}H]SPM exhibited a very active metabolism of the ceramide produced via SPM hydrolysis. Radiolabelled glycerophospholipids, neutral lipids and sphingolipids including SPM and glycolipids were found on TLC and resulted from the cellular re-utilization of the fatty acid and sphingoid base liberated by ceramidase. Although the absolute amounts of radiolabelled ceramide in Farber lymphoid cells remained higher than in normal cells (Fig. 1, middle), they decreased after 24 h incubation. This was accompanied by a clear increase in the amounts of the various metabolic products (Fig. 1, right). The degradation of ceramide

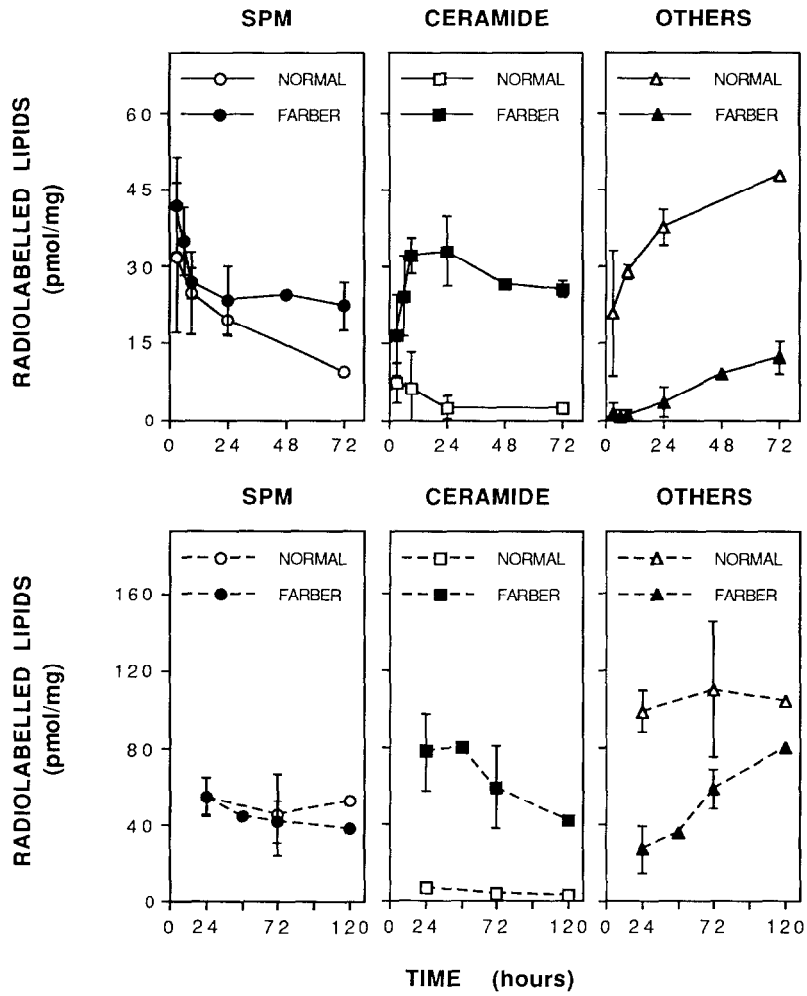

Fig. 1. Incorporation of radioactivity into different lipids of cultured normal and Farber lymphoid cells pulsed with LDL-associated [^{3}H]SPM. Lymphoid cell lines derived from 5 different normal subjects and from a patient with Farber disease (GM 5748) were incubated in medium supplemented with 2% Ultroser HY and LDL-associated [^{3}H]SPM (~ 53 μg apolipoprotein B/ml; ~ 12500 dpm/μg). After either 3 h (—; upper panel) or 24 h (- - -; lower panel) pulse, cells were sedimented, washed twice with medium containing 10% fetal calf serum and chased in this medium for the indicated times. The cell-associated radiolabelled lipids (SPM, ceramide and other metabolites) were extracted, separated by TLC and quantified as described in Materials and Methods. The data correspond to the mean ± S.D. of up to 4 and 8 separate experiments for normal and Farber cells, respectively and are expressed as pmol/mg cell protein.

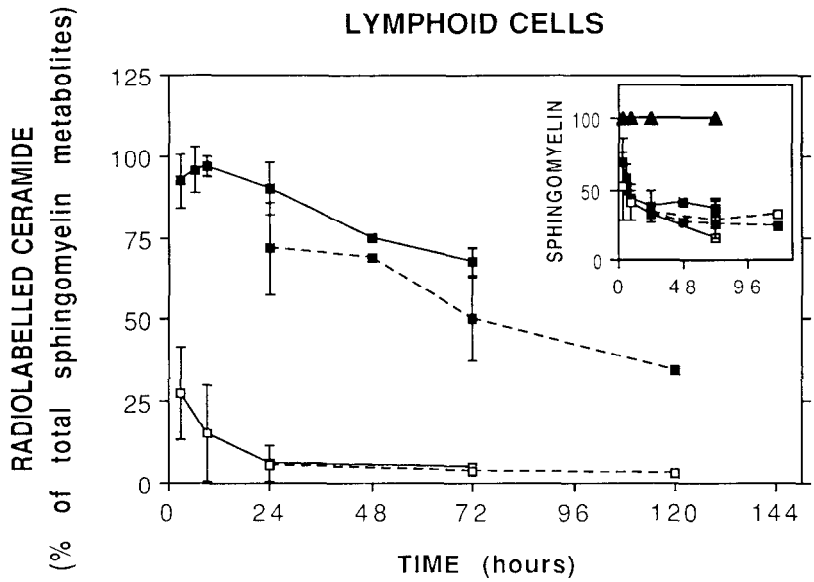

Fig. 2. Time course of ceramide degradation in normal and Farber cultured lymphoid cells pulsed with LDL-associated [^{3}H]SPM. Lymphoid cell lines derived from 5 different normal subjects (□), a patient with Farber disease (GM 5748, ■) and a patient with Niemann-Pick disease Type A (▲) were incubated exactly as described in the legend to Fig. 1. After 3 h (——) or 24 h (- - -) pulse, cells were chased and processed as indicated in the legend to Fig. 1. The amount of undegraded ceramide is expressed as the percentage of the total radiolabelled lipids minus SPM [33]. The inset shows the time course of degradation of [^{3}H]SPM (expressed as percentage of total cell-associated lipids) in the different cell lines.

was also calculated by the ratio of undegraded ceramide to the total lipid products of SPM hydrolysis, that is by subtracting the SPM in order to consider the ceramide as the starting substrate [33]. After a 3 h pulse, lymphoid cells from Farber disease exhibited a ceramide level of about 92% of the total SPM metabolic products (Fig. 2). This value further increased to 97% during the following 3–6 h of chase due to the continuation of SPM hydrolysis (see Fig. 1, left). After the rate of SPM degradation levelled off, i.e. after 9–24 h (see also Fig. 2, inset), a continuous decrease of ceramide was noted in Farber cells. When cells were pulsed for 24 h, a similar time course of degradation was observed. Thus, after a 5 days incubation, undegraded ceramide in Farber lymphoid cells represented only 34% of the SPM metabolites (Fig. 2).

As Farber disease is characterized by a striking involvement of subcutaneous tissues [22], the metabolism of LDL-associated [^{3}H]SPM was also examined in cultured skin fibroblasts. Fig. 3 shows that fibroblasts exhibited almost the same metabolic pattern as lymphoid cells. After a short increase, the absolute amounts of radiolabelled ceramide in Farber fibroblasts showed a progressive decrease (Fig. 3, middle) while its metabolic products increased (Fig. 3, right). Fig. 4 shows that, under conditions of selective lysosomal hydrolysis of SPM (see the pattern for Niemann-Pick disease cells in the inset of Fig. 4), the kinetics of ceramide degradation in Farber fibroblasts were superimposable on those obtained in Farber lymphoid cells.

From the decay curves of the ceramide derived from LDL-associated [^{3}H]SPM, we calculated the approximate half-times and initial velocities of lysosomal ceramide degradation. These parameters still remain underestimated since both SPM and ceramide degradation occurred very soon after uptake of the LDL-associated substrate (half of the receptor-bound LDL particles are internalized every 3 min [34]). Nevertheless, as shown in Table I, the half-time of ceramide in Farber cells was 27–39 times more elevated than in normal cells. However, the ceramidase activity in Farber cells was not completely deficient since a residual activity of about 4% was found. These calculations were done for cells derived from the same Farber patient. According to our results when using serum-enriched medium containing SPM (data not shown), one can reasonably speculate that the residual ceramidase activity is even higher in the two other Farber patients we studied.

The effective levels of in situ lysosomal ceramidase activity in Farber disease are in the same range as those found in cells from the late-onset types of other sphingolipidoses, e.g. juvenile and adult GM2-gangliosidoses [35], metachromatic leukodystrophy [35], or Niemann-Pick disease Type B (D. Graber, R. Salvayre and T. Levade, in preparation).

Thus, our data support the conclusion that the hydrolysis of ceramide by lysosomal ceramidase is not completely blocked in intact Farber cells. The results could also be interpreted by an efflux of ceramide out of the lysosomes and a subsequent non-lysosomal deg-

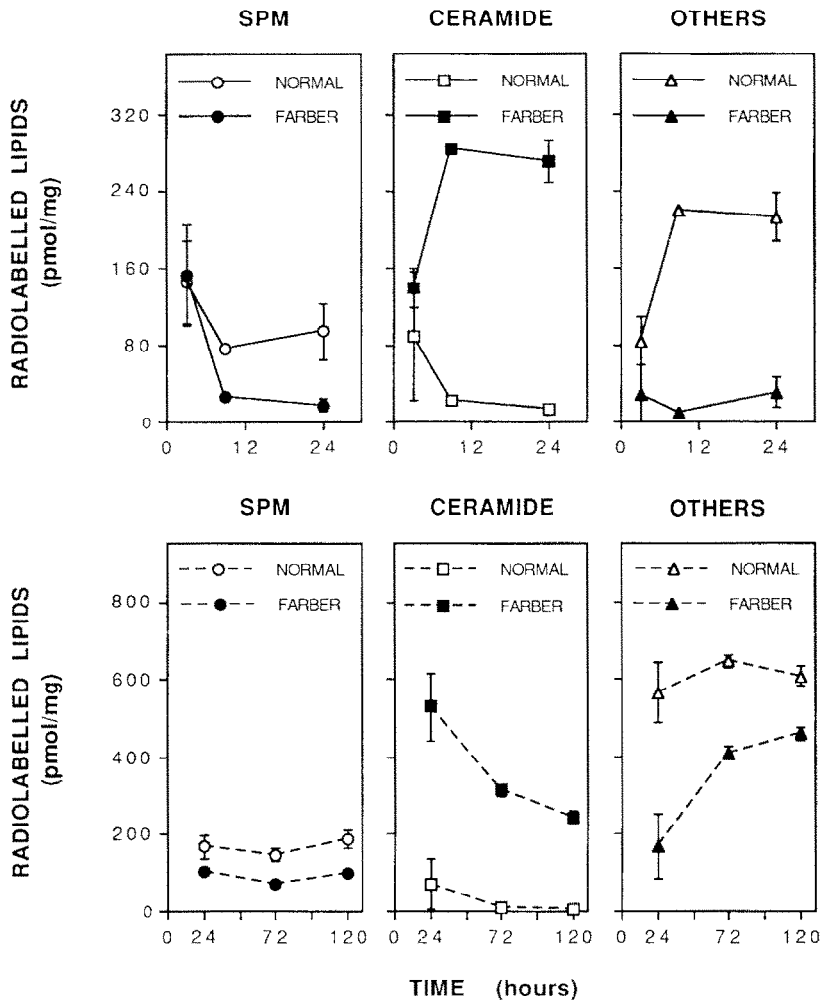

Fig. 3. Incorporation of radioactivity into different lipids of cultured normal and Farber fibroblasts pulsed with LDL-associated [^{3}H]SPM. Skin fibroblasts from 7 different normal subjects and a patient with Farber disease (GM 5752) were incubated with LDL-associated [^{3}H]SPM as described in the legend to Fig. 1. After either 3 h (—, upper panel) or 24 h (- - -; lower panel) pulse, cells were chased and cell-associated radiolabelled lipids (SPM, ceramide and other metabolites) were extracted, separated and quantified as described in Materials and Methods. The data are expressed as pmol/mg cell protein and correspond to the mean ± S.D of up to 9 and 3 separate experiments for normal and Farber fibroblasts, respectively.

radation. However, in contrast to short chain ceramides which are cell-permeable [9–12], it is not known whether natural, hydrophobic ceramides can get easily across a membrane. In addition, electron microscopy studies have indeed demonstrated ceramide accumulation in the lysosomes of cultured Farber fibroblasts overloaded with natural ceramides [36].

The high residual activity of lysosomal ceramidase in lymphoid cells might explain the absence or mild involvement of the bone marrow and reticuloendothelial system in Farber disease [22]. The involvement of subcutaneous tissues in the patients may be accounted for by the production of large quantities of ceramide by the skin [3–5] that could not be accomodated by the residual ceramidase activity present in fibroblasts.

Our data also provide some indication about the possible role of ceramide as a lipid mediator [6–12]. If

Table I

Approximate half-time and initial rates of degradation of ceramide in intact normal and Farber cells

Cell type	Half-time (hours)		Initial velocity (pmol/h.mg)	
	Normal	Farber	Normal	Farber
Lymphoid cells	2.1	82.8	15.4	0.6
Fibroblasts	2.7	73.1	95.9	3 4

The half-time of ceramide was calculated from the exponential curves of the ceramide degradation derived from the data in Figs. 2 and 4 (for Farber cells, the values of ceramide concentration at 3 and 6 h were omitted and mean values were considered for the 3 h pulse and 24 h pulse experiments). The initial velocities were calculated based on a mean uptake of LDL-associated [^{3}H]SPM of 110 and 550 pmol/mg cell protein for lymphoid cells and fibroblasts, respectively and assuming a 70% degradation of SPM to ceramide (see insets of Figs. 2 and 4).

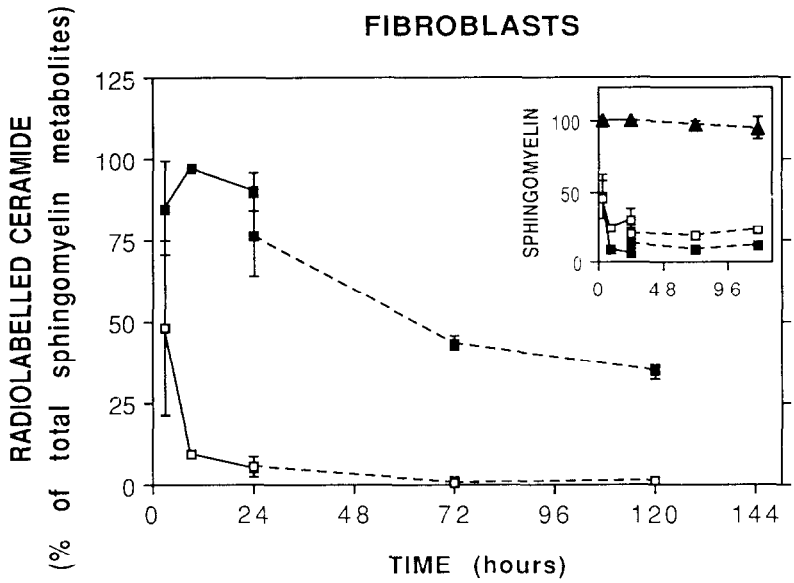

Fig. 4. Time course of ceramide degradation in normal and Farber cultured fibroblasts pulsed with LDL-associated [^{3}H]SPM. Skin fibroblasts from 7 different normal subjects (□), a patient with Farber disease (GM 5752, ■) and a patient with Niemann-Pick disease Type A (▲) were incubated for 3 h (——) or 24 h (- - -) with LDL-associated [^{3}H]SPM, and then chased as described in the legend to Fig. 3. The data are presented as in Fig. 2.

ceramide is a potent second messenger, a relative increase of the ceramide levels may lead with time to deleterious effects on the cell functions. However, the presence of a still active lysosomal ceramidase in Farber disease as found in this study may protect from early fatal lesions. Alternatively, the ceramide found in Farber cells or tissues could be without effect on its cellular targets because it remains localized in the lysosomes. This would implicate that the acid, lysosomal sphingomyelinase is not the enzymatic system which has been proposed by some authors [37,38] to generate ceramide molecules as bioeffectors. Finally, it cannot yet be completely excluded that ceramide may, in part, escape intralysosomal degradation and leave the lysosomes.

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