Impairment of exogenous sphingomyelin degradation in cultured fibroblasts from familial hypercholesterolemia

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The degradation of exogenous sphingomyelin was investigated in cultured fibroblasts from normal subjects and subjects with familial hypercholesterolemia, either in whole medium or in lipoprotein-deficient medium. When introduced in whole medium, sphingomyelin degradation was significantly decreased (about 1.5-fold) in heterozygotes, and dramatically (about 4-fold) in homozygotes from familial hypercholesterolemia. Sphingomyelinase activity, measured in vitro by conventional methods, was not altered in fibroblasts from familial hypercholesterolemia. The sphingomyelin uptake was notably lower in familial hypercholesterolemia than in controls. The decrease in exogenous sphingomyelin degradation was also found in lipoprotein-deficient medium, suggesting that it is not related to the low density lipoprotein receptor impairment which exists in familial hypercholesterolemia. These results are discussed in relation to sphingomyelin and cholesterol metabolism, and possible abnormalities of the cell membrane in familial hypercholesterolemia are suggested.

Sphingomyelin degradation *Familial hypercholesterolemia* *Cultured fibroblast*

1. INTRODUCTION

Several papers have pointed out a close relationship between sphingomyelin and cholesterol metabolisms. Authors in [1] described an inhibition of LDL internalization in fibroblasts treated with positively charged liposomes containing SM. We previously reported [2] alterations of cholesterol metabolism in fibroblasts from patients with Niemann-Pick disease, a genetic disorder characterized by SM accumulation, and in vitro [3] and in situ [4] inhibition of SM degradation by sterols. All these results suggest that cholesterol and SM, closely related in membranes, also have closely related metabolisms, and probably some common regulatory mechanisms. This hypothesis is especially supported by the results in [5], demonstrating an inhibition of SM biosynthesis by LDL in normal fibroblasts. This suggests a role of LDL in SM metabolism, and it may be supposed that the lack of LDL receptors which exists in familial hypercholesterolemia (FH) could result in alterations of SM metabolism. We thus investigated SM catabolism in FH fibroblasts. We report here a marked decrease of the in situ degradation of SM in FH fibroblasts as compared to normal fibroblasts. This phenomenon was also found in LDL-deficient medium, suggesting that it is not related to the LDL receptor deficiency, but rather to an as yet unknown abnormality of FH cells.

Abbreviations: LDL, low density lipoprotein; SM, sphingomyelin; FH, familial hypercholesterolemia; WM, whole medium; LDM, lipoprotein-deficient medium

2. MATERIALS AND METHODS

2.1. *Cell culture*

Control fibroblasts were obtained from skin biopsy, and FH (heterozygotes and homozygotes) from the Mutant Genetic Cell Repository, Camden, USA. Cells were cultivated in 60-mm

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petri dishes containing Ham F10 medium supplemented with 10% fetal calf serum (Gibco), at 37°C, in 5% CO_2 humidified atmosphere. Experiments were performed on confluent cells.

2.2. *Degradation of exogenous sphingomyelin*

The 'in situ' method in [7] was used. [*choline-methyl*-^{14}C]Sphingomyelin (NEN, 44 mCi/mmol) was incubated overnight (17 h) with cells either in Ham F10 medium containing 10% fetal calf serum (WM), or in medium with 10% lipoprotein-deficient serum (LDM). The lipid was introduced in ethanolic solution (final ethanol concentration 0.2%). The specific activity was 0.1 μCi/ml. Final concentration was about 2.5 nmol/ml for labeled SM, and about 12–15 nmol/ml for unlabeled SM. After incubation, the cells were washed 4 times with phosphate-buffered solution, and then harvested with a rubber policeman. Lipid analyses were performed by thin-layer chromatography on silica gel plates after direct application of an aliquot of the cell suspension as in [8]. Plates were developed in chloroform/methanol/water (65:25:4, v/v), and the radioactive spots located by autoradiography or with a radiochromatogram reader (Chromelec 101, Numelec-France). Spots were then cut out, and the radioactivity measured by liquid scintillation in an Intertechnique-spectrometer. Protein determination was performed on aliquots of the cell suspension as in [9]. The uptake of SM is expressed in pmol/mg cellular protein. The in situ degradation was evaluated by the percentage of undegraded SM recovered in the cells (i.e., the ratio of the radioactivity recovered in the SM spot over the total radioactivity recovered in the cells). Under these experimental conditions, as we previously described [7], the degradation of exogenous SM is mainly achieved by lysosomal sphingomyelinase, and the phosphorylcholine thus produced is rapidly reincorporated into phosphatidylcholine (PC) in normal fibroblasts. The lipidic radioactivity was recovered in SM and PC only. The radioactivity recovered at the starting point (free bases: choline and/or phosphorylcholine) was always less than 5% of the total radioactivity found on plates.

2.3. *Sphingomyelinase measurements*

Sphingomyelinase activity was measured on aliquots of cell suspension as in [10]. The activity is

Table 1

Sphingomyelin uptake and degradation by cultured fibroblasts from controls and patients with familial hypercholesterolemia, in whole medium or in lipoprotein-deficient medium

Strain	No. of experiments	SM uptake (pmol/mg)	Undegraded SM
In whole medium			
Controls	6	886 (598-1180)	0.22 (0.14-0.30)
GM 283 (hete.)	2	586 (481-671)	0.33 (0.30-0.36)
GM 376 (hete.)	2	532 (485-579)	0.36 (0.31-0.41)
GM 2000 (homo.)	4	233 (129-354)	0.80 (0.73-0.85)
GM 701 (homo.)	2	308 (254-362)	0.83 (0.78-0.88)
GM 488 (homo.)	2	189 (147-231)	0.87 (0.84-0.90)
In lipoprotein-deficient medium			
Controls	8	1298 (768-1657)	0.27 (0.16-0.35)
GM 2000 (homo.)	4	321 (264-369)	0.81 (0.76-0.84)
GM 701 (homo.)	4	410 (360-492)	0.87 (0.82-0.90)

Undegraded SM is the ratio of the radioactivity recovered in SM over the total radioactivity recovered in the cells. [*choline-methyl*-^{14}C]Sphingomyelin (44 mCi/mmol), was incubated overnight with cells in Ham F10 medium supplemented either with 10% fetal calf serum, or 10% lipoprotein-deficient serum. SM was introduced in ethanolic solution (ethanol, 0.2%; SM 0.1 μCi/ml). Range in brackets

expressed in nmol/h per mg cellular protein (triplicates).

3. RESULTS

It can be seen in table 1 that in the presence of whole medium (WM), the percentage of undegraded SM was 0.15–0.30 in controls, increased about 1.5-fold in heterozygotes, and increased 4-fold in homozygotes. The SM uptake by cells was reduced 30–40% in heterozygotes, and 60–75% in homozygotes, compared to controls.

From these results, it could be supposed that SM uptake by normal cells in WM is mainly achieved by way of lipoproteins, especially LDL, and that the LDL-receptor deficiency in FH resulted in a marked decrease in SM uptake. In this interpretation, the decrease of exogenous SM degradation in FH fibroblasts could be explained if receptor-mediated endocytosis, which results in a more direct access of LDL-SM to lysosomes, is depressed in these cells. A possible deficiency in sphingomyelinase activity is ruled out by the results presented in table 2, which shows a large overlap between controls and FH cells.

To check this possibility, we repeated the experiments in LDM. It can be clearly seen in table 1 that:

(i) The SM uptake by controls was not reduced but rather increased in LDM as compared to WM. This suggests that there are other ways for SM delivery to cells than by lipoproteins.

Table 2

Sphingomyelinase activity, measured in vitro on cell homogenates from controls and patients with familial hypercholesterolemia

Strain	Sphingomyelinase activity (nmol/h per mg protein)	Range
Controls	89.9	72.3-103.7
Heterozygotes		
GM 283	81.4	73.2-97.8
GM 376	84.5	71.0-94.6
Homozygotes		
GM 2000	79.8	68.6-91.9
GM 701	83.4	74.0-95.2

Measurements were performed in 0.2 M acetate buffer (pH 4.5) containing 0.1% Triton X-100

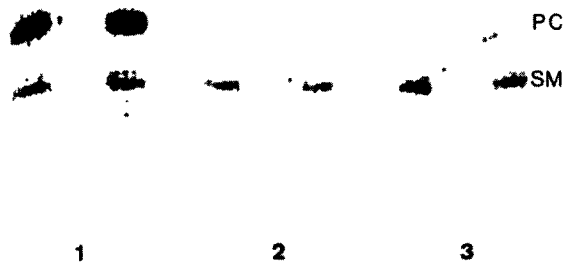

Fig.1. Degradation of exogenous sphingomyelin by cultured fibroblasts from control (1), GM 2000 (2), and GM 701 (3), in lipoprotein-deficient medium. Each sample in duplicate.

(ii) The differences in exogenous SM degradation between controls and FH cells observed in WM were also found in LDM. This is also clearly illustrated in fig.1.

Thus, our first hypothesis which cannot explain such results must be ruled out. The decrease in exogenous SM degradation in HF does not appear to be directly related to the LDL receptor deficiency of FH cells, but rather to an as yet unknown alteration of SM metabolism in these cells.

4. DISCUSSION

Under our experimental conditions the degradation of exogenous SM appears to be mainly achieved by way of the lysosomal sphingomyelinase. Authors in [11] and [12], using slightly different experimental conditions, obtained similar results, but they also demonstrated the involvement of a membrane phosphocholine transferase, which catabolizes about 10–15% of the exogenous SM [12]. For a long incubation time (3–4 days) this results in significant recovery of the SM choline moiety into PC, even in lysosomal sphingomyelinase-deficient cells (Niemann-Pick disease, type A). In our experimental system (17 h incubation time, and lower amounts of SM with higher specific radioactivity), we were unable to detect a significant contribution of non-lysosomal processes in

the exogenous SM degradation [7].

It can also be seen in fig.1 that the radioactive material at the starting point of the chromatograms (free bases: choline and/or phosphorylcholine) was almost negligible, less than 5% of the total radioactivity recovered on plates, in FH as well as in controls. Moreover, [^{14}C]choline incorporation into PC was not significantly reduced in FH cells, which excludes a major impairment of PC synthesis (not shown). These results strongly suggest that the phenomenon described here is truly related to a decrease of SM degradation in FH cells. As sphingomyelinase activity measured in vitro was not significantly reduced, it can be supposed that it is the access of exogenous SM to the lysosomal compartment which is impaired in FH cells.

Several hypotheses could be considered to explain the impairment of the exogenous SM degradation observed in FH cells. We suggested [2–4] that the strong physical interactions which exist between SM and cholesterol [13] could result in reciprocal alterations of their metabolisms in some pathological situations: in Niemann-Pick disease, the metabolic basis is well identified (in types A and B) as a deficiency in lysosomal sphingomyelinase activity, but cholesterol accumulation is often as important as SM accumulation. In atherosclerotic lesions [14] and in aging [15], the increase in the intracellular content of cholesterol is often accompanied by a selective increase in SM without any sphingomyelinase deficiency when measured in vitro by conventional methods. The experiments described in [1] also showed that overloading cells with SM resulted in lowered LDL internalization and increased cholesterol synthesis. From these results, a first hypothesis is that alterations of cholesterol metabolism which take place in FH cells [16,17] could result in alterations of SM degradation. No increase in intracellular cholesterol content has been described in FH cells, but a marked increase in cholesterol synthesis is well documented [6,16,17]. It is possible that the endogenous and exogenous pools of cholesterol are differently localized in the cell, and interact differently with SM.

Another hypothesis is that as yet unknown abnormalities in the membrane structure or in lysosomal function may exist in FH cells, which could impair exogenous SM access to sphingomyelinase. Authors in [18] have described alterations of glycolipid levels and metabolism in FH fibroblasts. They found a 5-fold increase in SM content in homozygotes compared to normal, and concluded that alterations of the cell surface could be involved, in addition to the LDL receptor deficiency, in the accelerated atherosclerosis in FH. Our observations confirm impairment of SM metabolism in FH. It could be supposed that membrane alteration in FH results in lowered SM turnover, and thus, during the time of the experiment, in an apparent decrease in exogenous SM degradation by the cells. Experiments are now being performed to study the turnover of the main phospholipids (especially SM) in FH compared to controls.

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