The tumour necrosis factor-sensitive pool of sphingomyelin is resynthesized in a distinct compartment of the plasma membrane

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Sphingomyelin (SM) biosynthesis is believed to occur in the early Golgi apparatus, plasma membrane and recycling endosomes. In the present study, the localization of the SM synthesis that follows its hydrolysis upon activation of the SM signal-transduction pathway was investigated in human skin fibroblasts treated with tumour necrosis factor (TNF-α). After TNF-α-induced degradation, the intracellular SM levels returned to baseline levels within 30–60 min in cells treated at 37°C. Pre-treatment or co-incubation of cells with bacterial sphingomyelase or phospholipase C, decreasing the SM and phosphatidylcholine content in the external leaflet of the plasma membrane respectively, did not inhibit SM resynthesis. However, SM resynthesis was not observed when TNF-α-treated cells were continuously exposed to exogenous sphingomyelinase, suggesting that under these particular conditions the resynthesized SM becomes accessible to the enzyme. Furthermore, whereas inhibition of vesicular traffic/endocytosis at 4°C blocked exoplasmic SM resynthesis, it did not alter SM resynthesis in TNF-α-treated fibroblasts, negating the role of endosomes and the Golgi apparatus. This was further evidenced by the finding that after SM resynthesis, TNF-α was again able to promote SM turnover, even at 4°C. In addition, when the exoplasmic leaflet SM was hydrolysed by treating fibroblasts with bacterial sphingomyelase, resynthesis of SM occurred at 37°C much more slowly than after TNF-α treatment. These findings support strongly the conclusion that the SM, which is resynthesized after TNF-α-induced hydrolysis, resides in the cytosolic leaflet of the plasma membrane, and that the process involved in this resynthesis displays characteristics different from those of the previously described SM syntheses.

INTRODUCTION

Sphingomyelin (SM, ceramide phosphocholine) is a major structural constituent of mammalian cell membranes, plasma lipoproteins and the myelin sheath ([1,2], also reviewed in [3–5]). Although SM is distributed widely among the various intracellular membranes [1,2,6], it is predominantly found in the plasma membrane of mammalian cells [2,5,7]. Within this subcellular compartment, most SM resides in the outer, exoplasmic membrane leaflet [6,8–11]. However, we [12] and others [13,14] have provided evidence for the existence of a particular pool of SM, most probably located in the inner, cytoplasmic leaflet of the plasma membrane, which is involved in the so-called SM signalling pathway.

During the last years, a crucial function of SM in signal transduction has been documented [15–20]. Indeed, SM has emerged as a key participant in the signalling pathway of cytokines, such as tumour necrosis factor α (TNF-α), neurotrophins, antibodies and various stress agents (see [15–21] for reviews). In this pathway of cell regulation, SM is hydrolysed by a sphingomyelinase to ceramide, which in turn serves as a second messenger, mediating certain effects of the above extracellular agonists [15,16,21–23]. In many instances, the examination of the intracellular SM levels has shown a biphasic phenomenon, that is, a gradual decrease (often within minutes) in response to the treatment of cells by the agonist, which is followed by a return to basal levels. The term ‘SM cycle’ has been proposed to describe this phenomenon [16]. Since the first description in HL-60 leukaemia cells treated by the differentiating agent vitamin D3 [24], a number of studies have reported such an SM cycle using different stimuli and cell types [25–33].

The observation that intracellular SM levels can be restored rapidly in the absence of extracellular SM (most experiments being performed in serum-free media) suggests strongly that SM is resynthesized. However, the topology and mechanism of this resynthesis have not yet been investigated. Biosynthesis of SM in mammalian cells appears to be catalysed by phosphatidylcholine (PtdCho):ceramide phosphocholine transferase (also termed SM synthase), which directly transfers the phosphocholine headgroup from PtdCho to ceramide (reviewed in [3–5]). Whereas the subcellular location of SM synthase is still a matter of debate, the main site is the luminal side of cis/medial Golgi apparatus [34–38], from which SM is transported to the plasma membrane by vesicular flow [5,34,35]. Other reported sites responsible for the synthesis of cell-surface SM include the plasma membrane [37–41] and recycling endosomes [42].

In this report, the localization of the SM synthesis that immediately follows its hydrolysis induced by TNF-α was investigated in cultured human skin fibroblasts. Evidence is presented for the first time that, after cytokine-induced SM hydrolysis, SM is resynthesized in a plasma-membrane pool that is inaccessible to exogenous sphingomyelinase. We also show that this process differs from the resynthesis of SM molecules, present in the cell-surface exoplasmic leaflet, that have been degraded by exogenous sphingomyelinase.

MATERIALS AND METHODS

Materials

Human recombinant TNF-α was supplied from PeproTech-Tebu (Le Perray en Yvelines, France). [methyl-3H]Choline chloride

Abbreviations used: SM, sphingomyelin; PtdCho, phosphatidylcholine; TNF-α, tumour necrosis factor α; C6-NBD, N-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)hexanoic acid; DMEM, Dulbecco’s modified Eagle’s medium.

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(81 Ci/mmol), was obtained from NEN (Le Blanc Mesnil, France). Sphingomyelinase (Bacillus cereus; sphingomyelin phosphodiesterase, E.C. 3.1.4.12) and C6-NBD (N-6 -(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)-ceramide were from Sigma; phospholipase C (Bacillus cereus, grade I, devoid of sphingomyelinase activity) was from Boehringer Mannheim (Meylan, France). All solvents and other reagents, obtained from Merck or SDS (Peypin, France), were of analytical grade. Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, Glutamax and trypsin-EDTA were from Gibco BRL (Cergy-Pontoise, France); fetal calf serum was from Gibco (Gagny, France). Bacterial sphingomyelinase was added again in the medium after 60 min of incubation, the radioactive medium was removed and cells were chased for 2 h in fresh, serum-free medium. Then cells were incubated for 30 min in the absence or presence of bacterial sphingomyelinase (100 m-units/ml) or phospholipase C (1 unit/ml). Thereafter, cells were treated, or not, with TNFα (3 nM) at 37 °C or 4 °C in the absence or presence of bacterial sphingomyelinase or phospholipase C as indicated. In some experiments, bacterial sphingomyelinase was added again in the medium during the TNFα treatment. At the indicated incubation times, cells were collected and washed with ice-cold PBS, harvested by scraping with a rubber policeman and sedimented at 4 °C by low-speed centrifugation. Cell pellets were immediately frozen at −20 °C.

Metabolic labelling of cellular choline-phospholipids
Confluent fibroblasts were incubated in DMEM containing 1 % fetal calf serum and [methyl-3H]choline (2 µCi/ml). After 72 h incubation, the radioactive medium was removed and cells were chased for 2 h in fresh, serum-free medium. Then cells were incubated for 30 min in the absence or presence of bacterial sphingomyelinase (100 m-units/ml) or phospholipase C (1 unit/ml). Thereafter, cells were treated, or not, with TNFα (3 nM) at 37 °C or 4 °C in the absence or presence of bacterial sphingomyelinase or phospholipase C as indicated. In some experiments, bacterial sphingomyelinase was added again in the medium during the TNFα treatment. At the indicated incubation times, cells were washed with ice-cold PBS, harvested by scraping with a rubber policeman and sedimented at 4 °C by low-speed centrifugation. Cell pellets were immediately frozen at −20 °C.

RESULTS
After TNFα treatment, SM resynthesis occurs in the absence of vesicular traffic/endocytosis
In previous experiments on different cell types, including U937, HL-60, human skin fibroblasts and smooth-muscle cells [12,29,32,33,45], we observed that the TNFα-induced SM hydrolysis was followed by a rapid increase in intracellular SM levels. This phenomenon is exemplified in Figure 1(A); SM level returned to baseline value within 30–60 min of the peak hydrolysis. This increase was seen by monitoring the SM levels not only after metabolic labelling to equilibrium with radioactive choline but also by mass quantification by lipid phosphorus assay [12].

Since we demonstrated that TNFα can trigger SM degradation even when the SM present in the plasma membrane outer leaflet has been hydrolysed by treatment with exogenous sphingomyelinase [12], we investigated whether SM resynthesis can occur under these conditions. Fibroblasts were incubated with bacterial sphingomyelinase, then washed and treated with TNFα. As shown in Figure 1(A), after hydrolysis of cell-surface SM, the levels still returned to the value observed before cytokine treatment, and within the same time frame as in the absence of a pretreatment by exogenous sphingomyelinase. Of note is that the SM levels in cells not treated by TNFα remained stable during this time interval, indicating no substantial resynthesis of the SM degraded by bacterial sphingomyelinase. This suggested that the SM hydrolysed by exogenous sphingomyelinase and that degraded upon TNFα activation have different metabolic fates.

To investigate whether the SM resynthesis which follows TNFα treatment is sensitive to conditions known to block vesicular transport of sphingolipids, and SM in particular [12,34,46], fibroblasts were incubated at low temperature (a condition that does not inhibit TNFα-induced SM turnover [29]). As illustrated in Figure 1(B), resynthesis of SM occurred at 4 °C as efficiently as at 37 °C. This was also observed in cells pretreated with exogenous sphingomyelinase to degrade the plasma-membrane outer-leaflet SM. These results agree well with previous observations from this laboratory, which showed TNFα-induced SM degradation and SM resynthesis in cells incubated at 14 °C or in the presence of hypertonic medium, i.e. under conditions that interrupt endocytosis [29]. Collectively, these data support the conclusion that the resynthesis of SM happening after cytokine-stimulated SM breakdown requires neither vesicular traffic nor endocytosis.

After exogenous sphingomyelinase and TNFα treatment, resynthesized SM is accessible to exogenous sphingomyelinase
In order to examine whether the resynthesized SM is present in the outer leaflet of the plasma membrane, cells were treated as above but were exposed continuously to bacterial sphingomyelinase. Under these particular conditions, no SM resynthesis could be observed in cells incubated with TNFα (Figure 2). (In cells treated only by sphingomyelinase, but not by TNFα, re-addition of sphingomyelinase during the first 2 h was without further effect on the SM levels, supporting the conclusion that exogenous sphingomyelinase did not get access to the inner SM compartments). The data of Figure 2 suggest that, under conditions where the whole cell-surface SM has been hydrolysed by bacterial sphingomyelinase, the SM which is synthesized after...
TNFα treatment (see Figure 1) is accessible to the exogenous enzyme.

To further explore this possibility, after exogenous sphingomyelinase treatment, cells were incubated with TNFα in medium devoid of bacterial sphingomyelinase to allow SM resynthesis (see Figure 1), and then were exposed again to sphingomyelinase. As illustrated in Figures 3(A) and 3(B), the SM that was resynthesized either at 37 °C or at 4 °C became accessible to exogenous sphingomyelinase. Thus, after hydrolysis of the cell-surface pool of SM, addition of TNFα is followed by the appearance of a newly synthesized SM on the outer leaflet of the plasma membrane.

The data obtained at 4 °C suggest that SM resynthesis did not occur in the Golgi apparatus. Indeed, if SM had been synthesized in this organelle, its transport to the cell surface would have been blocked at 4 °C [12,34,46] and, consequently, the SM would not have been degraded by bacterial sphingomyelinase.

After TNFα treatment only, resynthesized SM is not accessible to exogenous sphingomyelinase

To investigate whether the localization to the cell surface of the resynthesized SM in fibroblasts pretreated with exogenous sphingomyelinase was related to this pretreatment, experiments were repeated in which cells were not preincubated with the bacterial phospholipase. Figure 4 demonstrates that TNFα-induced SM hydrolysis was followed by SM resynthesis and that this SM was not accessible to exogenous sphingomyelinase. Thus, from the findings of Figures 1 and 4, it is tempting to propose that the SM synthesized after TNFα stimulation of fibroblasts most probably resides in the inner leaflet of the plasma membrane. However, under those particular, non-natural conditions of complete degradation of the outer leaflet of the plasma membrane by exogenous sphingomyelinase (Figures 2 and 3), the SM that is synthesized rapidly after TNFα treatment goes to the external leaflet, perhaps, first of all, for regenerating the cell-surface compartment.

After TNFα treatment, SM resynthesis occurs at 4 °C and regenerates the SM signalling pool

Previous work has demonstrated that the SM signalling pool probably resides in the plasma-membrane inner leaflet [12–14]. To further study the localization of the SM resynthesis in fibroblasts treated with TNFα, cells were incubated at 4 °C and treated again with the cytokine. Should the SM pool of the
cytoplasmic leaflet of the plasma membrane be regenerated, shortly afterwards, TNFα should be able to again promote SM turnover. Figure 5 indeed demonstrates that after SM resynthesis (i.e. after 60 min incubation), repeated additions of TNFα resulted in the stimulation of SM hydrolysis and resynthesis. Most importantly, this was found to occur at 4 °C (Figure 5), that is, under well-established conditions where the vesicular flow from the Golgi to the plasma membrane is completely blocked. This observation suggested strongly that, after TNFα treatment, SM resynthesis did not take place in the Golgi apparatus. Further support for this notion came from the finding that, when the Golgi compartment was labelled at low temperature with C6-NBD-ceramide [5,34], TNFα did not activate significantly the conversion of this fluorescent analogue into SM. Indeed, the C6-NBD-SM levels in TNFα-treated cells (60, 90,
Sphingomyelin signalling pool is resynthesized in plasma membrane

Figure 6  SM resynthesis in fibroblasts treated with B. cereus sphingomyelinase (SMase) alone

Normal skin fibroblasts were labelled for 72 h with [3H]choline as described in Materials and methods. After 2 h chase in serum-free medium, cells were incubated at 37 °C for 30 min in the presence of 100 m-units/ml of B. cereus SMase. Then, at time 0, the medium was removed and cells were incubated at 37 °C or at 4 °C without TNFα or SMase. At the indicated time points, incubations were stopped and SM levels determined as described in Materials and methods. The SM levels are expressed as a percentage of the value observed before treatment with SMase (at time −30). The values correspond to the mean ± S.E. of 3–5 independent experiments for the short time course (A), and to a representative experiment for the extended time course (B).

Figure 7  Effect of B. cereus phospholipase C on the SM resynthesis that occurs in fibroblasts after TNFα and B. cereus sphingomyelinase pretreatment

Normal skin fibroblasts were labelled for 72 h with [3H]choline as described in Materials and methods. After 2 h chase in serum-free medium, cells were incubated at 37 °C for 30 min with 100 m-units/ml of B. cereus sphingomyelinase in the absence (+ TNF−−PLC) or presence (+ TNF−+PLC) of 1 unit/ml of B. cereus phospholipase C. Then, at time 0, the medium was removed, and cells were incubated at 37 °C (A) or at 4 °C (B) with 3 nM TNFα in the absence of sphingomyelinase but with or without B. cereus phospholipase C. At the indicated times, incubations were stopped and SM levels determined as described in Materials and methods. The SM levels are expressed as a percentage of the value observed at time 0 [after phospholipase treatment(s) and in the absence of TNFα]. No difference was observed for the SM levels obtained after sphingomyelinase treatment alone or in the presence of phospholipase C. On the other hand, PtdCho levels approximated 606±306 (n=8) dpm/mg before and after phospholipase C treatment, respectively. The values correspond to the mean ± S.E. of 3 independent experiments.

120 or 180 min after addition of TNFα) represented 106±4% (n=8) of the values measured in the absence of the cytokine.

After exogenous sphingomyelinase treatment, SM resynthesis does not occur at 4 °C and the resynthesized SM is present at the cell surface

In the next experiments, we compared the fate of SM hydrolysed upon TNFα stimulation with that of SM degraded by exogenous sphingomyelinase. Cells were exposed to bacterial sphingomyelinase, washed and allowed to resynthesize SM. As shown in Figure 6(A), no SM resynthesis could be detected during the first 3 h following exogenous sphingomyelinase treatment (in sharp contrast with the rapid SM resynthesis observed in Figure 1 after stimulation with TNFα). A prolonged incubation indicated a progressive restoration of SM levels, which was almost complete 24 h after exposure to bacterial sphingomyelinase (Figure 6B). However, in contrast with the SM resynthesis that followed TNFα treatment (Figure 1B), this resynthesis did not take place at 4 °C (Figures 6A and 6B). In addition, the SM resynthesized at 24 h post-treatment was found to be partially accessible to exogenous sphingomyelinase (results not shown).

The cell-surface PtdCho is not involved in SM resynthesis after TNFα treatment

Since SM synthesis is believed to implicate PtdCho as a donor of phosphocholine [3–5], we examined whether the PtdCho present in the plasma-membrane external leaflet was involved in the resynthesis of SM that follows TNFα treatment. For this purpose,
fibroblasts were exposed to bacterial phospholipase C to hydrolyse the cell-surface PtdCho. As noted in a previous study [12], phospholipase C treatment did not interfere with the ability of TNFα to trigger the breakdown of cellular SM. Incubation of the fibroblasts with phospholipase C proved to be without effect on the SM resynthesis that occurred at 37 °C or at 4 °C after TNFα treatment (Figure 7). This indicates that the PtdCho present in the exoplasmic leaflet of the plasma membrane is not involved in the TNFα-stimulated SM resynthesis.

DISCUSSION

A growing body of evidence suggests that the SM-ceramide pathway plays a pivotal role in the regulation of numerous functions of eukaryotic cells (see [15–23] for reviews). As, in the vast majority of instances, SM appears to be the source of the sphingolipid mediators, it is conceivable that tightly regulated mechanisms help in maintaining the intracellular levels of the signalling pool of SM. Accordingly, in the early studies of the SM-ceramide pathway, it was noted that agonist-induced SM hydrolysis occurred during a short period of time and that it was followed by a return to baseline SM levels. This phenomenon, described tentatively as SM turnover, reversible SM hydrolysis or the SM cycle, and confirmed by many authors on various experimental systems [24–33], indicates that the living cell is able to resynthesize the SM.

The present studies, all performed on intact living cells, provide further insight into the metabolism and topology of the TNFα-sensitive SM pool. They establish for the first time (as far as we are aware) that in human skin fibroblasts, the SM synthesis that occurs after TNFα-stimulated hydrolysis of SM to ceramide most probably takes place in the inner leaflet of the plasma membrane. Indeed, our data demonstrate that the newly (re-)synthesized SM pool, and most probably the process involved in this SM synthesis: (i) is not accessible to exogenous bacterial sphingomyelinase; and (ii) is not located in the Golgi apparatus nor in the endosomes. The latter conclusion is supported by the finding that SM resynthesis is still active when vesicular flow/ endocytosis are blocked, for instance at 4 °C (this study and [29]). Indeed, a unique property of the enzymic system that catalyses this resynthesis is its ability to be fully active at 4 °C (this study and [29]). Moreover, we show that, while the Golgi SM synthase is not stimulated by TNFα, a new SM cycle can be triggered by the cytokine, even at low temperature, i.e. when vesicular transport to the cell surface is blocked. These features definitely distinguish the SM synthase that operates after cytokine-induced SM hydrolysis from the SM synthases present in the Golgi or recycling endosomes, which have been shown to be depressed strongly at temperatures close to 4 °C [34,41,46,47], or when endocytosis is inhibited in mitotic or energy-depleted cells [42]. The observation that the resynthesized SM pool is inaccessible to exogenous sphingomyelinase strongly supports the conclusion that this SM pool does not reside in the external monolayer of the cell surface, although the hypothesis of an as yet-undescribed sphingomyelinase-resistant SM domain present in the outer leaflet cannot be ruled out definitely.

Whether the resynthesis is mediated by the SM synthase located in the plasma membrane, described previously [37,41,48], still remains to be established. However, in contrast to our observations, the small proportion (about 10%) of total SM synthase activity detected by van Helvoort and coworkers at the plasma membrane was reported to be in the outer leaflet [41]. On the other hand, we provide evidence that the resynthesis of SM, which follows TNFα-induced SM degradation, differs from the pathway that resynthesizes the SM in plasma-membrane exo-

plasmic leaflet hydrolysed by exogenous bacterial sphingomyelinase. Whereas the former is active at 4 °C and restores SM initial levels within 30 min, the latter is poorly active at low temperature and requires several hours at 37 °C to resynthesize similar amounts of SM. In addition, it is unlikely that SM resynthesis following TNFα treatment occurs after translocation of the ceramide to the outer leaflet through the cell-surface PtdCho-SM interconverting enzyme [41]. If the ceramide produced in the inner leaflet could flip-flop (which seems quite unlikely [14]), it would be expected to follow the same resynthetic pathway as the ceramide generated on the outer leaflet by bacterial sphingomyelinase, which was not the case (see Figure 6). Moreover, degradation by exogenous phospholipase C of exoplasmic-leaflet PtdCho, the presumed donor of phosphocholine, did not prevent SM resynthesis.

The present findings on the topology of SM resynthesis in fibroblasts would be consistent with our previous observations regarding the localization of the SM signalling pool to the plasma-membrane cytosolic side [12], the activation of SM turnover in the absence of endocytosis [29] and the stimulation of a neutral, possibly membrane-bound or translocated at the membrane (Andrieu-Abadie, N. and Levade, T., unpublished work), rather than acidic, lysosomal, sphingomyelinase [45,49,50]. The presence in the plasma-membrane inner leaflet of both a distinct SM pool and an SM synthase is intriguing in relation to the SM signalling pathway. Of course, this synthase could be responsible for the generation of the SM signalling pool. However, our previous findings suggested strongly that the formation of this SM pool is dependent on vesicular traffic from the Golgi to the cell surface and is inhibited by agents that disrupt this transport route [12]. The plasma-membrane SM synthase may serve another purpose. As suggested by van Helvoort and colleagues [38,41], this enzyme, which also functions as a PtdCho synthase, may regulate the relative concentration of the lipid second messengers diacylglycerol and ceramide during signal-transduction processes. Alternatively, the SM synthase may have an important function not by removing ceramide from the plasma membrane, but also by regenerating the SM signalling pool. Finally, a provocative hypothesis would consider the TNFα-stimulated SM synthase and neutral sphingomyelinase as the same entity being able to function in opposite directions [41].

In summary, this study provides evidence for a unique SM synthesis process that is activated by TNFα (and probably other agents), and which is intimately linked to the so-called SM signal-transduction pathway. Whether this process is ubiquitous, having the same location in the plasma-membrane internal leaflet of other cells, awaits further investigation. Additional work should also aim to characterize the biochemical mechanism(s) involved in this synthesis, as well as the mode of regulation.

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