RESEARCH COMMUNICATION

Evidence against involvement of the acid lysosomal sphingomyelinase in the tumour-necrosis-factor- and interleukin-1-induced sphingomyelin cycle and cell proliferation in human fibroblasts

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The hydrolysis of sphingomyelin (SPM) has been reported to mediate a number of responses to extracellular agents, including cytokines. The so-called SPM cycle may result from the activation of different types of sphingomyelinases (SPMases). We investigated the hypothetical contribution of acid lysosomal SPMase in the SPM signal-transduction pathway. We examined the ability of human skin fibroblasts with a genetic deficiency of acid lysosomal SPMase activity to respond to tumour necrosis factor α (TNF- α) or interleukin-1 β (IL-1 β). We report that both cytokines promoted SPM hydrolysis in fibroblasts derived from

patients with Niemann–Pick disease or I-cell disease, similar to that observed in normal cells. Treatment of normal fibroblasts with cationic amphiphilic drugs resulted in inhibition of acid SPMase activity, but had no effect on cytokine-induced SPM turnover. In addition, TNF- α and IL-1 β stimulated [3 H]thymidine incorporation in Niemann–Pick fibroblasts, as in normal cells. Thus our results argue against a role for acid endolysosomal SPMase in mediating the cytokine-induced SPM signalling cascade.

INTRODUCTION

The sphingomyelin (SPM) pathway is a recently identified signal-transduction pathway [1–4] which mediates the action of various extracellular agents, including dihydroxyvitamin D₃ [5], tumour necrosis factor α (TNF- α) [6–11], interferon- γ [6], interleukin-1 β (IL-1 β) [12,13] and nerve growth factor [14]. Stimulation of mammalian cells with these agents results in a SPM cycle, i.e. a rapid hydrolysis and then resynthesis of this sphingolipid. This cycle transiently generates ceramide, a putative intracellular second messenger which can ultimately lead to cell differentiation [5,6], inhibition [10] or stimulation [15] of cell growth, or apoptosis [16].

A major question raised by these studies relates to the mechanism responsible for the generation of ceramide. Indeed, the nature of the sphingomyelinase (SPMase) which is activated in this signal-transduction pathway is still a matter of debate. Both the plasma-membrane-bound neutral Mg-dependent SPMase [5,7,17] and the acidic SPMase [8,18] activities have been reported to be stimulated. A novel cytosolic neutral SPMase has also been implicated to mediate the SPM cycle in HL60 cells [19]. In order to evaluate the role of the acidic lysosomal SPMase in the SPM pathway, we investigated the effects of two cytokines in human skin fibroblasts with genetically or chemically deficient lysosomal SPMase activity. We show that TNF- α and IL-1 β can trigger both the SPM cycle and cell proliferation regardless of the activity of acidic SPMase.

MATERIALS AND METHODS

Materials

Human recombinant TNF- α was supplied from PeproTech-Tebu (Le Perray en Yvelines, France) and human recombinant

IL-1 β from Genzyme (Cambridge, MA, U.S.A.). [3 H]Choline chloride (79.2 Ci/mmol) and [3 H]thymidine (84.2 Ci/mmol) were obtained from DuPont–NEN (Les Ulis, France). Desipramine, perhexiline maleate and quinacrine were from Sigma. All solvents and other reagents obtained from Merck or SDS (Peypin, France) were of analytical grade.

RPMI 1640 medium, penicillin, streptomycin, L-glutamine, trypsin/EDTA and fetal-calf serum were from Gibco BRL (Cergy-Pontoise, France).

Fibroblast cultures

Human normal skin fibroblasts were obtained from our laboratory or from the Laboratoire de Culture de Peau (CHU Rangueil, Toulouse, France). Mutant fibroblasts were derived from patients affected with Niemann–Pick disease (NPD) Type A (lines Lan and GM 370) or Type B (line Par) or with I-cell disease (line F2892). The cell line Par was from our laboratory. The GM 370 cell line was purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.). Fibroblast cell lines Lan and F2892 were kindly provided by Professor G. Bach (Department of Human Genetics, Hadassah University Hospital, Jerusalem, Israel) and Dr. M. T. Zabet (Laboratoire de Biochimie, Hôpital Debrousse, Lyon, France) respectively. Cells were routinely grown in a humidified 5% CO₂ atmosphere at 37 °C in RPMI 1640 medium containing L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml) and heat-inactivated fetal-calf serum (10%).

Metabolic labelling of cellular phospholipids

Confluent fibroblasts from one 25-cm² flask were trypsin-treated, plated in three 25-cm² flasks and grown for 24 h in RPMI medium supplemented with 10% fetal-calf serum. Then the

Abbreviations used: SPM, sphingomyelin; SPMase, sphingomyelinase; TNF- α , tumour necrosis factor α ; IL-1 β , interleukin-1 β ; NPD, Niemann–Pick disease.

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culture medium was replaced by fresh medium containing 1% fetal-calf serum and [methyl- ^3H]choline (1 $\mu\text{Ci}/\text{ml}$). After 48 h incubation, the radioactive medium was removed and cells were chased for 2 h in 1% fetal-calf-serum-enriched medium. Then $\text{TNF-}\alpha$ or $\text{IL-1}\beta$ was added to the cell cultures at the indicated concentrations. After various times of incubation at 37°C, the cells were harvested with a rubber policeman and sedimented by slow-speed centrifugation. Cell pellets were immediately frozen at -20°C.

Lipid extraction and analyses

Cell pellets were suspended in 0.6 ml of distilled water and cells were disrupted by brief sonication (10 s) using a Soniprep MSE probe sonicator. A portion was taken for protein determination [20]. The remaining was extracted with 2.5 ml of chloroform/methanol (2:1 v/v), vortex-mixed and centrifuged at 1000 g for 15 min [21]. The lower phase was washed once with a synthetic upper phase consisting of chloroform/methanol/water (3:48:47, by vol.) to remove free radioactive choline, and evaporated under nitrogen. Then the lipid extract was subjected to mild alkaline hydrolysis [22] as follows: the residue was dissolved in 0.25 ml of chloroform and 0.25 ml of methanolic 0.5 M NaOH and incubated overnight at 37°C. After adding 0.85 ml of chloroform, 0.25 ml of methanolic 0.5 M HCl, 0.43 ml of water and 0.5 ml of chloroform/methanol (2:1, v/v), mixing and centrifuging, the upper phase (containing the [^3H]choline label released from phosphatidylcholine) was counted for radioactivity by liquid scintillation (Packard Tri-Carb 4530 spectrometer) using 5 ml of Aquasafe 300 (Zinsser BAI). The lower phase was washed twice with a synthetic upper phase and the washings were counted for radioactivity. Then the organic phase (containing the SPM) was evaporated under nitrogen, dissolved in a smaller volume of chloroform/methanol (2:1, v/v) and counted for radioactivity. [Preliminary experiments using pure natural or [^3H]choline-labelled standard phospholipids indicated that this technique yielded maximal (99.5%) hydrolysis of phosphatidylcholine, without (less than 2%) accompanying SPM hydrolysis. Under our conditions of mild alkaline hydrolysis, even 2 h of incubation was sufficient to hydrolyse completely the phosphatidylcholine present in the fibroblast extracts.] Results for SPM are expressed as d.p.m./mg of cell protein.

[^3H]Thymidine Incorporation assay

This assay was performed under the optimal conditions previously described for fibroblasts by Vilček et al. [23]. Cells were seeded in 6-well (35 mm) plates (about 60000 cells/well) in RPMI medium supplemented with 5% fetal-calf serum. After 18 h incubation, TNF (5 nM) was added, and the cultures were incubated for 48 or 72 h. Then [^3H]thymidine was added to the cells (0.2 $\mu\text{Ci}/\text{ml}$). After overnight incubation, the radioactive medium was removed, cells were rinsed with saline, harvested with a rubber policeman and sedimented. Cell pellets were washed with 2 $\times$ 1 ml of ice-cold 5% trichloroacetic acid, and the precipitated material was dissolved in 0.25 ml of 0.25 M NaOH, which was transferred to scintillation vials for counting the radioactivity.

Other procedures

The mass of SPM was quantified by measuring its phosphate content [24]. DNA was assayed fluorimetrically by using 4,6-diamidino-2-phenylindole [25]. Statistical analysis was performed by the Mann and Whitney test.

RESULTS

Cytokine-induced SPM turnover in normal and acid lysosomal SPMase-deficient fibroblasts

The responsiveness to $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ was studied on human fibroblasts derived from normal individuals and from patients with a genetic deficiency of the lysosomal SPMase activity. Cellular SPM was quantified after labelling to equilibrium of the choline-containing lipids by a well-established procedure using radioactive choline [5–10,12,13,17,26] and measurement by a method using mild alkaline hydrolysis [22] (see also the Materials and methods section). The latter procedure proved to be more reliable than the t.l.c. separation method, which can lead to large errors in SPM determination [26], and resembles the bacterial SPMase assay recently described by Jayadev et al. [26]. In

Figure 1 SPM turnover in normal and acid lysosomal SPMase-deficient fibroblasts treated with $\text{TNF-}\alpha$ and $\text{IL-1}\beta$

Human skin fibroblasts derived from normal subjects and from patients affected with NPD or I-cell disease (ICD) were labelled with [^3H]choline and then incubated in the absence or presence of $\text{TNF-}\alpha$ (2 nM in **a** and **c**, varying concentration in **b**) or 6 pM $\text{IL-1}\beta$ (**d**). At the indicated time points, incubations were stopped and SPM levels (d.p.m./mg of cell protein) determined as described in the Materials and methods section. Total cell-associated radioactivity averaged 1540900 ± 270280 and 1611960 ± 352270 d.p.m./mg of protein in normal and mutant cells respectively. The SPM levels are expressed as percentage of the value observed at zero time. (**a**) Time course of $\text{TNF-}\alpha$ -stimulated SPM hydrolysis in normal fibroblasts. The values are means $\pm$ S.E.M. of four independent experiments (except for the cell line Normal 2, where a representative experiment is shown). (**b**) TNF dose-dependence of SPM hydrolysis in normal fibroblasts. (**c**) and (**d**) Time course of SPM hydrolysis in acid SPMase-deficient fibroblasts stimulated by $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, respectively. The broken lines correspond to the SPM levels in cells not treated with the cytokines. NPD 1 cell line is GM 370; NPD 2 cell line is Lan.

Figure 2 TNF-induced SPM turnover in normal fibroblasts treated with cationic amphiphilic drugs

Normal skin fibroblasts were labelled with [^3H]choline as described in the Materials and methods section. After 48 h labelling, the medium was removed and cells were chased in medium containing 1% fetal-calf serum. At 30 min before addition of TNF- α , the cationic amphiphilic drug was added in aqueous or dimethyl sulphoxide solution to the culture medium at the following final concentrations: desipramine and quinacrine, 10 μM ; perhexiline, 5 μM . A control experiment was simultaneously carried out in the absence of amphiphilic drugs. SPM levels were determined as described in the Materials and methods section. The inset shows the activity of acid SPMase *in vitro* determined [36] in the treated cells. The data are means $\pm$ S.E.M. of 2–4 separate experiments. Total cell-associated radioactivity averaged 2266530 $\pm$ 159490, 1519100 $\pm$ 42360 and 2097320 $\pm$ 149620 d.p.m./mg of protein in cells treated with desipramine, perhexiline and quinacrine respectively (control cells without inhibitor, 2432090 $\pm$ 92110).

Table 1 Stimulation of [^3H]thymidine incorporation in normal and lysosomal-SPMase-deficient fibroblasts

Normal and mutant skin fibroblasts were plated in 6-well dishes and grown in medium containing 5% fetal-calf serum. At 1 day after seeding, TNF- α (5 nM) or IL-1 β (6 pM) was added and the cultures were further incubated for 48 h. Then [^3H]thymidine was added for the next 18 h of incubation. Cells were processed as described in the Materials and methods section. Results are expressed relative to control values observed in the absence of cytokines. Each value is the mean $\pm$ S.E.M. of triplicate determinations from a representative experiment (ND, not determined). Similar results were obtained in two additional experiments.

Cell line	Relative [^3H]thymidine incorporation	
	TNF- α	IL-1 β
Cytokine ...		
Normal 3	1.90 $\pm$ 0.13	ND
Normal 5	2.84 $\pm$ 0.12	2.77 $\pm$ 0.40
Normal 6	ND	3.03 $\pm$ 0.93
NPD 1	2.27 $\pm$ 0.28	ND
NPD 3	1.75 $\pm$ 0.29	6.44 $\pm$ 0.54
I-cell disease	1.52 $\pm$ 0.13	1.73 $\pm$ 0.12

addition, to account for variability between samples (see [26]), SPM levels were normalized by dividing the SPM d.p.m. by mg of total cell protein, but not by total [^3H]choline-labelled phospholipids [6,16,26], because TNF and IL-1 induced turnover of both SPM and phosphatidylcholine (results not shown; see the Discussion section).

TNF- α stimulated a time-dependent SPM hydrolysis in normal human fibroblasts (Figure 1a). As previously observed [26], the

time course of the cytokine effect varied slightly, depending on the cell batch utilized. In general, maximal hydrolysis (about 30%; $P < 0.01$ relative to controls) was detected at 30–60 min (Figures 1a and 1c); SPM levels then returned toward baseline by 90–120 min. In normal fibroblasts, TNF- α caused a dose-dependent breakdown of SPM (Figure 1b), with initial effect at 0.2 nM and peak effect observed at 1 nM. In addition, the cellular SPM mass, as assessed by the lipid phosphorus content, decreased upon incubation with 2 nM TNF (from about 54 to 42 nmol/mg of cell protein). IL-1 β also induced a time-dependent hydrolysis of SPM with a peak response seen at about 60 min (Figure 1d).

As illustrated in Figure 1(c), TNF- α could stimulate SPM hydrolysis in fibroblasts with a genetic deficiency in the acid lysosomal SPMase activity, i.e. Niemann–Pick disease Type A fibroblasts, as in normal cells. This finding was confirmed on a cell line derived from a patient with I-cell disease (Figure 1c), in which the activity of acid SPMase averaged 4% of controls (results not shown). The ability of lysosomal SPMase-deficient cells to respond to cytokines was further demonstrated with IL-1 β . Similar to TNF- α , IL-1 β was also found to cause a SPM cycle in both NPD and I-cell-disease fibroblasts (Figure 1d).

These data strongly suggest that the acid lysosomal SPMase is not implicated in the cytokine-induced SPM hydrolysis. Additional evidence for this conclusion was provided by examining the response to cytokines in cells treated with lysosomotropic compounds. Preincubation of normal fibroblasts for 30 min with desipramine, perhexiline or quinacrine resulted in a marked decrease (up to 95%) of the acid SPMase activity *in vitro* (see Figure 2, inset). However, under these conditions, the TNF-induced SPM cycle remained unaffected (Figure 2).

Mitogenic effect of cytokines on lysosomal SPMase-deficient fibroblasts

Previous studies have reported that TNF- α enhances incorporation of [^3H]thymidine into fibroblasts [23]. Although the mitogenic effect of TNF has not been shown to be mediated by the SPM cycle, ceramide, the immediate product of SPM degradation, was found to stimulate proliferation of fibroblasts [15]. Therefore, indirect evidence suggests that the SPM cycle may occur as an early biochemical event preceding proliferation of TNF-treated fibroblasts, as already shown for cell differentiation or apoptosis [3,4]. Thus, if the acid lysosomal SPMase does not participate in the TNF-induced SPM cycle, one would expect that TNF should be able to stimulate the growth of lysosomal SPMase-deficient fibroblasts. Table 1 shows that TNF- α and IL-1 β indeed enhanced [^3H]thymidine incorporation in NPD and, to a lesser extent, I-cell-disease cells. This mitogenic activity was further established by the observation of increased DNA and protein contents in treated cells (results not shown).

DISCUSSION

Although there is accumulating evidence for the existence in different cell types of a new signal-transduction pathway involving hydrolysis of cellular SPM [1–4], the SPMase which is activated in response to various stimuli remains undefined. Until very recently, four mammalian SPMases had been characterized [27–30]: the acid lysosomal enzyme; the neutral, Mg^{2+} - or Mn^{2+} -dependent SPMase, which has been reported to be externally oriented on the plasma membrane [31]; the nuclear dithiothreitol-dependent enzyme [32]; and the serum Zn^{2+} -dependent SPMase [33].

The stimulation of the neutral Mg^{2+} -dependent SPMase ac-

tivity *in vitro* has been reported for HL60 cells and fibroblasts following incubation with vitamin D₃ or TNF- α [5,7,17]. However, also after exposure to TNF- α , acidic SPMase activity was found to be increased in U937 cells [8]; the acidic SPMase has also been shown to be activated by diacylglycerol [34]. Although Hannun and co-workers [4,19] have described a novel, cytosolic, neutral, Mg²⁺-independent SPMase as the mediator of vitamin D₃ or TNF- α action, Schütze et al. [8,18] recently suggested that TNF- α triggers the activation of both endo-lysosomal acidic and membrane-bound neutral SPMases.

As an approach to the identification of the enzymic pathway involved in signal transduction, we studied the ability of human cells with genetic deficiencies of the lysosomal SPMase activity to respond to cytokines. We show that both TNF- α and IL-1 β induce a SPM cycle in intact human cultured skin fibroblasts. This cycle closely resembles that already observed with TNF on living HL60 [6,9], U937 [8,16] and Jurkat [10] cells. Cytokine-induced SPM hydrolysis in cultured skin fibroblasts has already been reported [12,17]. However, our data differ from those obtained by Chatterjee [17] by the fact that TNF- α (and IL-1 β) stimulated a considerable (20–30 %) phosphatidylcholine breakdown parallel to the SPM cycle (results not shown). This agrees with the TNF-mediated hydrolysis of choline glycerophospholipids already described by Schütze et al. [8] and Yanaga and Watson [9]. This also explains why, in contrast with previous work [6,16,26], we did not monitor the amounts of SPM by the ratio of SPM to total choline-labelled phospholipids. Indeed, the SPM cycle would have been missed by using such a mode of expression.

The major finding in this study is that cells with a genetic deficiency of acid lysosomal SPMase activity could exhibit a SPM cycle in response to TNF- α and IL-1 β , exactly as their normal counterparts. This was first demonstrated by using fibroblasts from homozygous patients with NPD Type A (or B), i.e. cells having only 1–2 % residual acid SPMase activity *in vitro* [35,36], a SPM accumulation [37,38] and an almost complete inability to metabolize exogenously supplied SPM [39,40]. The fact that the cytokine-induced SPM cycle was unaffected by the deficient activity of acid SPMase was further attested by using cells from a patient with I-cell disease. Despite their nearly complete absence of lysosomal hydrolases in general [41,42] and of acid SPMase in particular [42,43], these fibroblasts exhibited a SPM cycle as normal cells. As the endosomal/prelysosomal compartment of I-cell-disease fibroblasts is devoid of mature lysosomal hydrolases [41,43], our data make very unlikely the hypothesis [8,18] for the involvement of an acidic endosomal SPMase in TNF signalling. Consistent with these data, treatment of normal fibroblasts with cationic amphiphilic drugs [44,45] did not alter the cytokine-stimulated SPM hydrolysis. However, as previously noted for fibroblasts [46,47] or other cells (J. P. Jaffrézou, G. Laurent and T. Levade, unpublished work), the activity of acid SPMase *in vitro* was considerably decreased by preincubation with these drugs.

In order to rule out the possibility that the lysosomal SPMase contributes somehow to the signal transduction cascade, we looked for a late effect of the cytokines. As previously reported for normal fibroblasts [23,48,49], a few days' incubation of NPD and I-cell-disease fibroblasts with TNF- α or IL-1 β resulted in stimulated cell proliferation, thus suggesting the absence of contribution of acid SPMase in the mitogenic effect of TNF and IL-1 β . Furthermore, as ceramide has been shown as a mediator of cell growth [15], the mitogenic activity of TNF or IL-1 on fibroblasts might be mediated by the SPM cycle.

In sum, several lines of evidence support the conclusion that the acid lysosomal SPMase is not involved in the SPM signal-

transduction pathway. Moreover, it seems unlikely that in cultured skin fibroblasts the SPM cycle is mediated by the neutral Mg²⁺-dependent SPMase, since its activity is experimentally almost undetectable in these cells [37,50]. In addition, in our hands, the activity of the neutral cytosolic SPMase described by Hannun's group [19] also remained undetectable in skin fibroblasts. Clearly, further investigations are required to identify and localize the enzyme system responsible for the SPM signalling pathway.

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