Biochemical identification of a neutral sphingomyelinase 1 (NSM1)-like enzyme as the major NSM activity in the DT40 B-cell line: absence of a role in the apoptotic response to endoplasmic reticulum stress

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DT40 cells have approx. 10-fold higher Mg^{2+} -dependent neutral sphingomyelinase (NSM) activity in comparison with other B-cell lines and contain very low acidic sphingomyelinase activity. Purification of this activity from DT40 cell membranes suggested the presence of one major NSM isoform. Although complete purification of this isoform could not be achieved, partially purified fractions were examined further with regard to the known characteristics of previously partially purified NSMs and the two cloned enzymes exhibiting *in vitro* NSM activity (NSM1 and NSM2). For a direct comparative study, highly purified brain preparations, purified NSM1 protein and *Bacillus cereus* enzyme were used. Analysis of the enzymic properties of the partially purified DT40 NSM, such as cation dependence, substrate specificity, redox regulation and stimulation by phosphatidylserine, together with the localization of this enzyme to the endoplasmic reticulum (ER), suggested that this NSM from DT40 cells corresponds to NSM1. Further studies aimed to

correlate presence of the high levels of this NSM1-like activity in DT40 cells with the ability of these cells to accumulate ceramide and undergo apoptosis. When DT40 cells were stimulated to apoptose by a variety of agents, including the ER stress, an increase in endogenous ceramide levels was observed. However, these responses were not enhanced compared with another B-cell line (Nalm-6), characterized by low sphingomyelinase activity. In addition, DT40 cells were not more susceptible to ceramide accumulation and apoptosis when exposed to the ER stress compared with other apoptotic agents. Inhibition of *de novo* synthesis of ceramide partially inhibited its accumulation, indicating that the ceramide production in DT40 cells could be complex and, under some conditions, could involve both sphingomyelin hydrolysis and ceramide synthesis.

Key words: apoptosis, ceramide.

INTRODUCTION

Changes in sphingolipid metabolism, particularly accumulation of ceramide, have been implicated in a number of cellular responses to stress, including cell-cycle arrest, senescence and apoptosis [1–3]. Accumulation of ceramide has been demonstrated in many cell types undergoing apoptosis, characterized by a number of changes, such as activation of caspases, DNA fragmentation, poly(ADP-ribose) polymerase cleavage and loss of membrane integrity. Although the relationship between the accumulation of ceramide and the induction of changes accepted as apoptotic hallmarks has not been proven directly, several lines of experimental evidence support this link. For example, exogenously added ceramides are generally able to mimic stress-induced apoptosis in a stereospecific manner, and inhibition of *de novo* synthesis of ceramide has been, in some cases, reported to inhibit the progression of apoptosis [1]. In addition to *de novo* synthesis of ceramide, a number of stress responses result in the conversion of sphingomyelin (SM) into ceramide by the action of sphingomyelinases [4]. Two main types of sphingomyelinase have been studied extensively, acidic sphingomyelinase and Mg^{2+} -dependent neutral sphingomyelinase (NSM), and many agonists (e.g. tumour necrosis factor and Fas) have been reported to activate both of these activities.

Studies of different B-cell lines suggested that, at least in some systems, ceramide in apoptotic cells could accumulate as a result of activation of a Mg^{2+} -dependent NSM. In WEHI 231 B-cells, an increase of ceramide in response to B-cell receptor activation is accompanied by an increase in NSM activity [5]. In addition, a derived cell line resistant to B-cell-receptor-triggered apoptosis has been described and it was suggested that the basis of this resistance could be the failure to activate NSM activity [5]. In another study, the response of lymphoid cells transformed by Epstein–Barr virus to the activation of the CD40 receptor included activation of NSM, ceramide generation and several morphological changes associated with apoptosis [6]. Furthermore, apoptosis was mimicked by cell-permeant ceramide or bacterial NSM and could also be observed in acid-sphingomyelinase-deficient cells. However, the molecular nature of the NSM that could be involved in apoptosis in B-cells remains unclear. More generally, the roles of NSMs that have been described either as highly purified preparations from brain [7,8] or cloned recently [9,10] remain to be established. For example, two of the mammalian enzymes (designated NSM1 and NSM2) have been identified based on sequence similarity with *Bacillus cereus* NSM [9,10], but their relationships with activities obtained by purification or their involvement in the generation of ceramide in cells need to be clarified further. The NSM1 enzyme has been

Abbreviations used: NSM, neutral sphingomyelinase; ER, endoplasmic reticulum; SM, sphingomyelin; lysoPC, lyso-3-phosphatidylcholine; DTT, dithiothreitol; TUNEL, terminal transferase deoxynucleotidyl uridine end labelling; AraC, cytosine α -arabinofuranoside.

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found to be associated with the endoplasmic reticulum (ER) [11–13]. Although NSM1 has been shown to be involved in apoptosis triggered by T-cell-receptor activation [14], overexpression of this enzyme showed minor effects on apoptosis triggered by tumour necrosis factor [9,15] or H_2O_2 [16], suggesting that NSM1 could be involved in apoptosis of lymphoid cells upon activation of lymphoid-cell-specific receptors [14].

Results from the laboratories of Y. A. Hannun and L. Obeid [17] have shown that a critical aspect of ceramide function is the subcellular compartmentalization of ceramide formation. Moreover, accumulating evidence suggests that organelles such as the ER are major points of integration of pro-apoptotic signalling, which can locally activate signal-transduction pathways leading to apoptosis [18]. Interestingly, there has been no report on the putative involvement of endogenous NSM1 enzymes in the apoptotic response to ER stress.

In this study, we analysed different B-cell lines for NSM enzymes and identified DT40 lymphoma B-cells as exceptionally rich in NSM activity. Purification and characterization of this activity, together with studies of cellular localization, revealed that it is highly similar to the enzyme described previously as NSM1. Further studies of ceramide accumulation in apoptotic B-cells did not provide supporting evidence for the involvement of this endogenous NSM1-like activity in DT40 apoptosis and suggested that some of the ceramide accumulation could result from *de novo* synthesis, as suggested by others [19]. More particularly, our results also suggested that this NSM1-like enzyme, which is located in the ER, was not involved in apoptosis triggered by ER stress.

MATERIALS AND METHODS

Materials

The [*methyl*- ^{14}C]SM, [*palmitoyl*- ^{14}C]lyso-3-phosphatidylcholine (lysoPC), [^{14}C]acetic acid and FPLC columns were supplied by Amersham Bioscience; the hydroxyapatite CHTII columns were from Bio-Rad. UDP-galactose {[4,5- $^3H(N)$]galactose} was from NEN. The Cell Death Detection ELISA and complete protease inhibitor tablets were from Roche. The Apoptosis Detection System was from Promega. Phosphatidylserine was supplied by Avanti. Anti-Grp94 antibodies were from Bioquote, and anti-Giz2 and anti-caveolin antibodies were from Santa Cruz Biotechnology. All other reagents, including poly-lysine-coated slides, were from Sigma.

Cell culture

DT40 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% chicken serum and 2 mM glutamine. All other B-cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine, except BAF3 cells, which were additionally supplemented with 2% interleukin-3 and 10 μ M β -mercaptoethanol.

Except for DT40 (chicken) and BAF3 (mouse) cells, all the B-cell lines used were human.

Partial purification of NSM activity from DT40 cells

DT40 cells (from 25 litres of culture) were washed with cold PBS and resuspended in 40 ml of PBS, containing 2 mM dithiothreitol (DTT), 2 mM EDTA and protease inhibitors (lysis buffer). Cells were then subjected to centrifugation for 1 h at 100 000 *g*. The pellet was rehomogenized in 40 ml of lysis buffer containing 1 M NaCl, stirred for 30 min and centrifuged again. The pellet, containing most of the NSM activity, was washed three more times using lysis buffer and the centrifugation repeated. The washed material was homogenized in 20 ml of 25 mM Tris/HCl, pH 7.5, 1% Triton X-100, 2 mM DTT, 2 mM EDTA and protease inhibitors, stirred for 2 h and again subjected to centrifugation. The supernatant was saved (extract 1) and the extraction repeated. The two extracts were pooled and used as starting material for the purification.

The active sample was loaded on to a Q-Hi Trap column (5 ml) equilibrated with 25 mM Tris/HCl, pH 7.5, 1 mM DTT and 0.05% Triton X-100 (buffer A). Proteins were eluted with a 50 ml linear gradient (0–0.5 M NaCl in buffer A). Active fractions were pooled, transferred to 10 mM potassium phosphate, pH 7, 1 mM DTT and 0.05% Triton X-100 (buffer B) and loaded on to a 5 ml hydroxyapatite CHTII column. DT40 NSM activity was eluted using a 70 ml linear gradient (10–200 mM) of potassium phosphate. Samples were pooled, buffer was exchanged for 25 mM Tris/HCl, pH 6.5, 1 mM DTT and 0.1% Triton X-100 (buffer C) and applied to a Mono Q 5/5 column, and proteins were eluted by a 20 ml linear gradient of 0–0.5 M NaCl in buffer C. Active fractions were pooled, concentrated to 200 μ l and loaded on to a Superose 12 column equilibrated in buffer A containing 100 mM NaCl. Fractions containing NSM activity were pooled, transferred to buffer A and applied to a 1 ml heparin–Hi Trap column. Proteins were eluted using a 15 ml linear gradient of 0–0.5 M NaCl in buffer A. Active fractions were pooled, exchanged to buffer A and subjected to chromatography on a Mini Q column. Proteins were eluted using a 3 ml linear gradient of 0–0.5 M NaCl in buffer A. Active fractions were adjusted to 5% glycerol and stored at $-20^\circ C$. Specific activities were measured after the different steps are shown in Table 1.

Partial purification of NSM from rat brain

A total of 25 rat brains were washed with cold PBS and homogenized in 100 ml of lysis buffer (25 mM Tris/HCl, pH 7.5, 2 mM DTT, 2 mM EDTA and protease inhibitors) using a blender and a pestle glass homogenizer. The crude extract was subjected to centrifugation for 1 h at 100 000 *g*. The resulting pellet was re-homogenized in 150 ml of lysis buffer containing

Table 1 Purification of the main NSM activity from DT40 cells

Step	Total activity (units)	Total protein (μ g)	Specific activity (units/ μ g)	Purification (fold)	Yield (%)
Triton extract	18 073	629 000	0.028	1	100
Q-Hi Trap	4480	7000	0.64	23	25
Hydroxyapatite CHTII/Mono Q/Superose 12	1486	510	2.91	104	8
Heparin–Hi Trap	216	70	3.08	110	1.1
Mini Q	47	9	5.22	187	0.2

1 M NaCl and stirred for 30 min at 4 °C, and again subjected to centrifugation. The pellet was washed four more times with 150 ml of lysis buffer as described above. The pellet was then homogenized in 60 ml of lysis buffer containing 1 % Triton X-100, stirred for 1 h at 4 °C and subjected to centrifugation. The supernatant was saved and the pellet was homogenized again in lysis buffer containing Triton X-100. A total of seven Triton X-100 extractions were performed and extracts 2–5 were pooled and used as the starting material to purify rat brain NSMs. All steps were performed at 4 °C using a FPLC or a SMART system. The pooled extract was loaded on to a Q-Hi Trap column (10 ml, two columns in tandem) equilibrated with buffer A. Proteins were eluted with a 150 ml linear gradient of 0–0.5 M NaCl in buffer A. Fractions containing NSM activity eluted at the end of the gradient (350 mM NaCl) were pooled and buffer transferred to buffer C. The sample was then applied to a Mono Q 5/5 column equilibrated with buffer C and proteins were eluted with a 20 ml linear gradient of 0–0.5 M NaCl in buffer C. Fractions containing NSM activity were transferred to buffer B. Proteins were loaded on to a 5 ml hydroxyapatite CHTII column equilibrated with buffer B. The bound proteins were eluted with a 100 ml linear gradient of 10–150 mM potassium phosphate. Active fractions were pooled, exchanged to buffer A and loaded on to a 5 ml heparin–Hi Trap column equilibrated with the same buffer. The activity was eluted by a 50 ml linear gradient of 0–0.5 M NaCl, the active sample concentrated to 200 μ l and loaded on to a Superose 12 column equilibrated with buffer A containing 100 mM NaCl. Fractions containing NSM activity were pooled, transferred to buffer A and subjected to chromatography on a Mini Q column and proteins eluted with a 3 ml linear gradient of 0–0.5 M NaCl. Active fractions were adjusted to 5 % glycerol and stored at –20 °C. This purification protocol resulted in an approx. 1100-fold purification, starting from the Triton X-100 extract, with the specific activity of the final preparation being approx. 100 units/ μ g and the yield being 0.03 %.

Preparation of recombinant glutathione S-transferase-NSM1

Recombinant glutathione S-transferase-NSM1 was prepared as described previously [11,20].

Measurement of sphingomyelinase and lysophospholipid phospholipase C activities

The activities of both acidic sphingomyelinase and NSM were measured using [14 C]SM as described previously [11,20]. NSM activity was measured in the presence of MgCl_2 and reducing agents. Following 1 h of incubation, the assay was quenched, the phases were separated and 200 μ l of the aqueous phase was analysed by scintillation counting. One unit of activity was defined as the amount of enzyme that generates 1000 d.p.m./20 μ l per h.

Measurement of lyso-PC-hydrolysing activity was performed as described previously [16,20] with [*palmitoyl*- 14 C]lysoPC as a substrate and in the presence of 0.05 % Triton X-100.

Sucrose density-gradient fractionation of DT40 cell extracts and biochemical determination of subcellular markers

Approx. 2.5×10^8 DT40 cells were used for each gradient. Cells were washed twice in 250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid and 1 mM EDTA, pH 7.5, and then resuspended in 2–3 ml of the same buffer containing protease inhibitors and left on ice for 10 min. The cells were broken using a stainless steel ball-bearing homogenizer and subjected to centrifugation at 2000 *g* at 4 °C for 10 min to generate the post-nuclear super-

natant. The post-nuclear supernatant was layered on to 10.2–40 % (w/w) linear sucrose gradients with a 1 ml 65 % sucrose cushion and fractionated as described by Lewis et al. [21].

Galactosyltransferase activity (a Golgi marker) was assayed according to Kim et al. [22]. Each fraction (30 μ l) was assayed in the presence of 2.5 μ Ci of UDP-[3 H]galactose for 30 min. Following washing, the cell pellet was resuspended in 0.5 % SDS and analysed by scintillation counting. Protein disulphide-isomerase (an ER marker) was analysed by monitoring the reduction of insulin [23] caused by 50 μ l of each fraction. Both 5' nucleotidase [24] and Na^+/K^+ -ATPase [25] activities were used to monitor the plasma-membrane distribution. Lactate dehydrogenase activity (a cytosolic marker) was analysed exactly as described by Storrie and Madden [26]. Although the ER (protein disulphide-isomerase) and Golgi (galactosyltransferase) markers overlap, their peaks occur in separate fractions, the ER marker preceding the Golgi marker.

Labelling and lipid analysis of DT40 and Nalm-6 cells

DT40 and Nalm-6 cells were grown in the presence of 0.5 μ Ci/ml [14 C]acetate for at least four growth cycles so that the lipids were labelled to equilibrium. Following the labelling period, the cells were spun down and resuspended at 10^7 /ml in media without the label. Labelled cells were seeded and stimulated as indicated for up to 48 h. Following stimulation, aliquots containing 2×10^6 cells were washed twice and resuspended in 50 μ l of PBS. Lipids were extracted according to standard methods; 750 μ l of chloroform/methanol (1:2, v/v) was added to the extract and the phases were separated by the addition of 250 μ l of chloroform and 250 μ l of water. The lipid phase was removed and one aliquot used to assess total radioactivity incorporated into the lipids. The remainder was dried under vacuum and resuspended in 50 μ l of chloroform. Neutral lipids and known standards were separated by TLC in the solvent system benzene/diethyl ether/ethanol/acetic acid (500:400:4:1, by vol.) [27] and visualized with iodine. Ceramide-containing spots were removed and placed in scintillation vials. Lipids were extracted by the addition of 250 μ l of methanol. Radioactivity incorporated into ceramide was expressed as a percentage of the total radioactivity in the lipid phase.

Detection of apoptosis

Following stimulation of apoptosis, an aliquot containing 10^5 cells was removed for cell-death detection by ELISA and processed according to the instructions accompanying the kit from Roche. For the TUNEL (terminal transferase deoxynucleotidyl uridine end labelling) assay, DT40 cells were grown at the indicated densities for up to 48 h. An aliquot containing 5×10^6 cells was allowed to attach to poly-lysine-coated slides for 10 min. Cells were then fixed for 25 min with 4 % paraformaldehyde and the protocol for the kit was followed. Fluorescein-stained cells were then examined by confocal microscopy.

Other methods

Preparation of stock reagents of compounds used to induce apoptosis was as follows. Cytosine d-arabinofuranoside (AraC) was dissolved in PBS (stock solution, 1 mM), thapsigargin (stock, 1 mM) and tunicamycin (stock, 1 μ g/ml) were dissolved in DMSO and fumonisins B1 (stock, 20 mM) was resuspended in methanol. All reagents were aliquoted and stored at –20 °C.

Samples for gel electrophoresis were combined with 4 $\times$ sample buffer and boiled for 5 min. Following separation by SDS/PAGE, proteins were transferred to PVDF membrane. Mem-

branes were probed with anti-Giz2 (1:1000), anti-caveolin (1:1000) or anti-Grp94 (1:2200) antibodies for 1 h. Following the addition of horseradish peroxidase-conjugated secondary antibodies, ECL was used for detection. Samples from purification steps were stained after SDS/PAGE with colloidal Coomassie Brilliant Blue stain (Sigma).

RESULTS

Sphingomyelinase activity in various B-cell lines

Several B-cell lines undergo cell death by apoptosis that, in many cases, has been accompanied by accumulation of ceramide [5,6,28,29]. It has been suggested that a pathway responsible for this ceramide production could involve activation of sphingomyelinases; in particular a neutral Mg^{2+} -dependent enzyme [5,6,28]. To analyse sphingomyelinase activities present in B-cells, a panel of nine different cell lines has been tested for hydrolysis of SM *in vitro*, under conditions optimized for measurements of either Mg^{2+} -dependent NSM or acidic sphingomyelinase activity (Figure 1). For the majority of B-cell lines (including B85, BAF3, BC3, KF, KM3 and LCL3) the cell extracts had higher levels of acidic than neutral sphingomyelinase activity. However, DT40 cells demonstrated exceptionally high levels of the Mg^{2+} -dependent NSM activity (about 10-fold higher than the other cell lines tested) and quite low levels of SM hydrolysis under acidic conditions (Figure 1). This cell line has been studied previously for apoptotic responses that could be induced readily by a variety of conditions, including UV and γ irradiation, surface cross-linking of Ig molecules, chemotherapeutic drugs, and agents that induce osmotic, oxidative or nutritional stress [30–34]. Therefore, we extended our studies of DT40 cells, firstly to characterize the Mg^{2+} -dependent NSM in

Figure 1 Comparison of (A) neutral and (B) acidic sphingomyelinase activities in various B-cell lines

(A) Extracts were generated from individual cell lines and 20 μ g of protein from each extract was assayed at pH 7.5 in the presence of 10 mM Mg^{2+} and 10 mM DTT. (B) Assay conditions were as in (A) except that the pH was 5.1 and no DTT and Mg^{2+} were present in the buffer.

Figure 2 Partial purification of DT40 NSM activity

Colloidal Coomassie Brilliant Blue staining of 20 μ l of Triton X-100 extract from DT40 cell membranes (lane 2) and of 20 μ l of DT40 NSM1 purified partially on a Mini Q column (lane 3). Molecular mass markers are shown in lane 1.

this cell line and secondly to test its potential link with ceramide accumulation in B-cells undergoing apoptosis.

Purification and characterization of NSM in DT40 cells: comparison with NSM1 and a NSM from brain

Preliminary analysis of NSM activity of DT40 cells showed that it was present exclusively in cellular membranes (specific activity, 0.06 unit/ μ g). Thus to purify NSM from DT40 cells, a particulate fraction containing cell membranes was subjected to extraction in the presence of 1% Triton X-100. The solubilized protein was enriched in NSM activity; this extraction step therefore contributed to purification that was estimated to be about 5-fold. Subsequent purification steps, using chromatography on Q-Hi Trap, hydroxyapatite CHTII, Mono Q, Superose 12, heparin-Hi Trap and Mini Q columns, are summarized in Table 1. These purification steps resulted in a further approx. 200-fold purification with an overall purification from the DT40 membranes of up to 1000-fold (Table 1). Further purification steps led to important inactivation of the NSM activity (results not shown). Analysis of the proteins present in the final preparation by SDS/PAGE revealed that a partial purification was achieved with several protein bands present (Figure 2). Nevertheless, in all purification steps a single peak of activity was observed and the final preparation was considered to correspond to a single isoform of NSM. Also, this preparation was sufficiently pure for the characterization of enzymic properties.

For the characterization of NSM from DT40 cells, other previously described NSM activities were included for comparison when indicated. These were: purified recombinant protein encoded by the NSM1 clone (described in [11,20]), an activity partially purified from rat brain particulate fraction (similar to activities described previously [7,8]) and commercially available NSM from *B. cereus*. For the partial preparation of a rat brain NSM, a new purification procedure was developed (see the Materials and methods section) that resulted in about 1000-fold purification from the Triton X-100 extracts and overall purification of up to 5000-fold. This preparation did not contain any other, potentially interfering, enzyme activities and the protein was not recognized by the antibody to NSM1, which was separated at earlier purification steps as described previously for mouse brain preparations [11].

Figure 3 Characterization of NSM from DT40 cells

(A) Top panel: partially purified NSM from DT40 cells was incubated in the presence of 10 mM of the indicated cation under standard reaction conditions, including 10 mM DTT. Bottom panel: the DT40 enzyme was preincubated with 10 mM DTT, 10 mM reduced glutathione (GSH), 10 mM oxidized glutathione (GSSG) or 500 μ M H₂O₂ for 15 min at room temperature prior to incubation with [¹⁴C]SM for 1 h in the presence of 10 mM Mg²⁺. (B) Partially purified rat brain NSM (■), DT40 enzyme (▲) and NSM1 preparation (●) were incubated with increasing concentrations of phosphatidylserine in the presence of 10 mM Mg²⁺ and 10 mM DTT.

Characterization of the partially purified NSM from DT40 cells is shown in Figure 3 and Tables 2 and 3. SM hydrolysis was stimulated by Mg²⁺ and Mn²⁺, whereas Ni²⁺, Ca²⁺ and Zn²⁺ were inhibitory (Figure 3A, top panel). The presence of reducing agents (DTT and reduced glutathione) was required for maximal activity and a reversible inhibition was observed in the presence of oxidizing compounds (oxidized glutathione and H₂O₂; Figure 3A and Table 2). The NSM1 protein, analysed in the same experiment, gave essentially the same results as in our previous publications [11,20], demonstrating similar cation dependence and redox regulation for NSM1 and the DT40 enzyme. In addition, gel filtration on a Superose 12 column (results not shown) indicated that DT40 NSM was eluted at approx. 90–

Table 2 Redox regulation of DT40 NSM1-like enzyme

Partially purified DT40 NSM activity was preincubated for 15 min with or without oxidizing agents prior to a 1 h incubation with [¹⁴C]SM in the presence or absence of 10 mM DTT.

Preincubation	Activity (% of control)	
	Reaction buffer without DTT	Reaction buffer with DTT (10 mM)
No oxidizing agent	100	206
GSSG (10 mM)	68	193
H ₂ O ₂ (500 μ M)	32	113

Table 3 SM/lysoPC hydrolysis ratios

Partially purified preparations of NSM from DT40 cells and brain, GST-tagged NSM1 or *B. cereus* sphingomyelinase (0.0025 units/ml) were incubated for 1 h with either [¹⁴C]SM or [¹⁴C]lysoPC. Following analysis by TLC, the SM/lysoPC hydrolysis ratio was determined.

NSM	SM/lysoPC hydrolysis ratio
<i>B. cereus</i>	24.2:1
Brain	27.0:1
NSM1	5.4:1
DT40	6.9:1

Figure 4 Localization of NSM in DT40 cells

(A) Extracts from DT40 cells were fractionated on a linear sucrose density gradient and the resulting fractions (20 μ l) assayed for NSM activity in the presence of 10 mM Mg²⁺ and 10 mM DTT. Organellar distribution was determined using either biochemical assays or Western blotting (see the Materials and methods section). The fractions containing specific markers, in the part of the gradient also containing sphingomyelinase activity, are underlined. (B) Aliquots (50 μ l) of each fraction were subjected to Western blotting using anti-Grp94 (ER marker), anti-Glc2 or anti-caveolin (plasma-membrane markers) antibodies.

Figure 5 Ceramide increases when DT40 cells are stimulated to apoptose

(A) DT40 cells were labelled to equilibrium with [¹⁴C]acetate and then seeded at either 10⁵ cells/ml (●) or 10⁷ cells/ml (■). At the indicated time points, aliquots were analysed for apoptosis (ELISA kit; top panel) and for changes in ceramide levels (bottom panel). (B) Using the TUNEL assay for detection of apoptosis, fluorescein-stained DT40 cells were visualized by confocal microscopy. The cells were seeded at either 10⁵ cells/ml (left-hand panel) or 10⁷ cells/ml (right-hand panel) for 48 h. (C) [¹⁴C]Acetate-labelled DT40 cells (5 × 10⁵ cells/ml) were incubated in the presence of 2.5 μM AraC, 5 μM thapsigargin or 1 μg/ml tunicamycin or at a density of 10⁷ cells/ml. After 48 h, the samples were processed as in (A) to analyse changes in levels of apoptosis (top panel) and ceramide (bottom panel). (D) DT40 cells were treated as in (C) and the extract generated from 10⁵ cells. Protein samples (18 μg) were subjected to Western blotting using anti-Grp94 antibodies.

100 kDa, as obtained previously for the purified mouse NSM1 enzyme [11]. In our experiments, the partially purified enzyme from rat brain also had similar properties, in particular the cation dependence (results not shown). However, NSM1 and the rat brain preparation were clearly different when analysed in the presence of increasing amounts of phosphatidylserine, which stimulated the brain preparation but not NSM1 (Figure 3B). The recently described NSM2 was reported to be stimulated under similar conditions [10]. The NSM from DT40 cells, as with NSM1, was not stimulated by phosphatidylserine (Figure 3B). It has been reported previously that NSM1 can hydrolyse not only SM but also some lysophospholipids, such as lysoPC [16,20,35]. NSM from *B. cereus* used both substrates *in vitro*; however, it had a much greater preference for SM [12]. When these enzymes were compared directly with DT40 sphingomyelinase, the DT40 enzyme had a SM/lysoPC ratio of about 7:1, similar to the ratio obtained for NSM1 (approx. 5:1; Table 3). In contrast, the rat brain sphingomyelinase had a ratio of 27:1, similar to the *B. cereus* enzyme (24:1; Table 3). Therefore the characterization of

DT40 NSM demonstrated that the enzyme is Mg²⁺-dependent and shares many enzymic properties with the previously described NSM1 enzyme.

Subcellular localization of DT40 NSM activities

To characterize the DT40 enzyme further, experiments of cell fractionation have been carried out. The presence of NSMs has been reported in different subcellular fractions, including the plasma membrane and intracellular compartments (reviewed in [3]). Several studies established localization of NSM1 in the ER [11,13,20]. Using a sucrose density gradient, DT40 cell extract was fractionated and the fractions containing the ER, Golgi and plasma-membrane markers were identified using standard enzyme assays (see the Materials and methods section) and Western blotting (Figure 4). Analysis of the fractions for Mg²⁺-dependent NSM activity *in vitro* demonstrated that the major peak of activity was present in fractions containing the ER and overlapping Golgi markers while only a small portion of SM-

hydrolysing activity was detected in the plasma-membrane fractions. The peak of NSM activity corresponds to the peak of ER markers and the presence of NSM activity in Golgi fractions could be due to cross-contamination, as observed for human NSM1 by Tomiuk et al. [13]. These data suggest that the main activity found in DT40 cells, capable of hydrolysing SM *in vitro*, resides in the ER/Golgi fraction. Together with biochemical evidence, these cell-fractionation studies further support the identification of the main DT40 NSM activity as an NSM1-like enzyme.

Production of ceramide in apoptotic DT40 and Nalm-6 cells

A number of different conditions have been reported to trigger apoptosis in DT40 cells [30–34]. To test the possible involvement of sphingomyelinase in apoptosis, in addition to detection of apoptosis (using ELISA and TUNEL assays), the levels of ceramide in cells were also measured (Figure 5). A range of conditions, including high density of DT40 cells and the addition of the anti-cancer drug AraC or compounds known to result in the ER stress responses, thapsigargin and tunicamycin, were tested over different periods of time. As illustrated for the high cell density (Figure 5A), an increase in both apoptosis and ceramide production was prominent between 24 and 48 h. However, although an increase in apoptosis (caused under different conditions) was always accompanied by the accumulation of ceramide, it did not strictly correlate with the fold increase in ceramide (Figure 5C). For example, thapsigargin and tunicamycin both induced the ER stress, monitored by an increase in Grp94 (Figure 5D), but the higher increase in ceramide by thapsigargin resulted in somewhat lower levels of apoptosis. Since the main DT40 enzyme, capable of hydrolysing SM *in vitro*, resides in the ER, the ER stress could act to cause its stimulation or translocation. The data showing that the ER stress was not much more potent in inducing ceramide or apoptosis than the other conditions not affecting the ER (cell density or AraC; Figures 5C and 5D) did not provide evidence for a link between high levels of NSM1-like activity in the ER, accumulation of ceramide and apoptosis.

A comparison was also made between DT40 cells and another B-cell line, Nalm-6, containing very low levels of both neutral and acidic sphingomyelinase activities (Figure 1). When Nalm-6 cells were analysed under the same conditions as DT40 cells, AraC, thapsigargin and tunicamycin, but not the high cell density, resulted in an increase of ceramide accumulation and apoptosis (Figure 6). Again, an increase in apoptosis was accompanied by an increase in ceramide and was most prominent in response to thapsigargin (Figure 6). These experiments show that the presence of high levels of NSM1-like activity in DT40 cells is not linked to a greater accumulation of ceramide or susceptibility to apoptosis, compared with Nalm-6 cells containing very low levels of sphingomyelinase activity.

Considering the results described above, we also investigated the possibility that accumulation of ceramide in apoptotic cells might result from *de novo* ceramide synthesis rather than the hydrolysis of SM. To test this, DT40 and Nalm-6 cells were treated with fumonisin B1, used widely as an inhibitor of ceramide synthesis. Some decrease, but not complete inhibition, was observed in both cell lines (results not shown). The most potent inhibition by fumonisin B1, $75 \pm 6\%$ inhibition in DT40 and $67 \pm 5\%$ inhibition in Nalm-6 cells, was detected when ceramide accumulation was triggered by thapsigargin. Although synthesis of ceramide may not completely account for its accumulation, it could be an important source of ceramide, at least under some conditions that induce apoptosis.

Figure 6 Effect of apoptotic stimuli on the levels of apoptosis and ceramide in Nalm-6 cells

Nalm-6 cells were labelled with [¹⁴C]acetate to equilibrium and then seeded at 5×10^5 cells/ml and treated with the indicated agent for 48 h. Following the incubation, cell aliquots were analysed for apoptosis and ceramide changes as described in Figure 5.

DISCUSSION

This study was aimed at exploring the molecular properties of NSM that could be involved in ceramide generation in cells undergoing apoptosis. Since the processes of apoptosis and ceramide accumulation have been well documented in different lymphoid cells [1–3], a number of B-cell lines were analysed and the DT40 cell line was identified as being particularly rich in NSM activity (Figure 1). Previous studies of this cell line have demonstrated that apoptosis can be triggered by a variety of conditions, including ligation of the B-cell receptor, oxidative stress, exposure to γ -radiation or cell-permeant (C2) ceramide [30–34]. Although some further insights into the involvement of specific protein components (e.g. tyrosine kinase Btk or ataxia telangiectasia protein) in apoptosis have been obtained using DT40 cells deficient in these components [31–34], the generation of ceramide has not been studied in this cell line. It is shown here (Figure 5) that in response to diverse inducers of apoptosis, such as an increase in cell density (presumably acting through a tumour necrosis factor-family receptor), the ER stress (triggered by thapsigargin or tunicamycin) and treatment with the anti-cancer drug AraC, DT40 cells accumulate ceramide. As found in some other lymphoid cell lines [5,28,36], accumulation of ceramide was observed at later time points (Figure 5). Several studies have suggested that this ceramide formed after prolonged exposure to stress, and not the early changes in ceramide observed in some systems, could have a role in apoptosis [1–3]. Although B-cells in avian species are produced in a specific organ, the Fabricius bursa (in contrast with mammalian B-cells, which are

produced in the bone marrow), we are not aware of experimental evidence regarding specific properties/functions of avian B-cells that could be obviously linked to the high level of NSM activity in the chicken DT40 cells.

Purification and characterization of the main NSM from DT40 cells (Figures 3 and 4, Tables 1–3) revealed several characteristics common with NSM1 enzyme described previously [9,11,13,16,20,35]. Furthermore, several characteristics not only show similarity to NSM1 but point out differences when compared with other previously described activities, including highly purified preparations from brain [7,8] or the cloned protein designated NSM2 [10]. As shown by a direct comparison with the NSM1 protein and a preparation of NSM from brain, the DT40 activity and NSM1 were insensitive to increasing concentrations of phosphatidylserine, whereas the brain preparation was stimulated several-fold (Figure 3). Stimulation by phosphatidylserine has also been reported to be a property of NSM2 [10], and in this respect the DT40 enzyme clearly differs from this enzyme. Regarding substrate specificity, it has been described previously how several NSMs can to some extent hydrolyse lysophospholipids (e.g. lysoPC) in addition to SM [20]. However, consistent with previous studies [16,20,35], NSM1 showed less preference for SM when compared with the NSM from rat brain or *B. cereus* (Table 3). In addition, when DT40 enzyme was analysed in the same experiment, it demonstrated a similar ratio of lysoPC/SM hydrolysis as NSM1, distinct from the brain preparation or *B. cereus* enzyme (Table 3). Another characteristic, the presence of most of the SM-hydrolysing activity in DT40 cells in fractions containing the ER and Golgi markers (Figure 4), is consistent with properties reported previously for NSM1 [11,13,20].

The substrate specificity, subcellular localization and failure to detect an increase in ceramide in transfected cells, raised doubt as to whether or not the NSM1 enzyme could act as a sphingomyelinase in cellular responses resulting in ceramide production [9,11,13,16,20,35]. In two previous studies [14,15] the involvement of NSM1 in apoptosis has been addressed more specifically, using T-cell lines (T-cell hybridoma 3DO and Jurkat cells) for the analysis. In the 3DO cell line undergoing apoptosis induced by T-cell receptor engagement both an increase in NSM activity and ceramide production has been observed [14]. Treatment of 3DO cells with a NSM1 antisense probe reduced ceramide production [14]. However, specificity of this probe or direct evaluation of the antisense effect on NSM1 expression have not been described. Involvement of NSM1 in apoptosis has also been tested in another T-cell line (Jurkat cells) [15], where the late responses to death-receptor stimulation (CD95) included ceramide accumulation, most probably derived from SM hydrolysis by a neutral Mg^{2+} -dependent activity [36]. Jurkat cells retrovirally transduced to overexpress either wild-type or catalytically inactive NSM1 (potentially, a dominant-negative form) did not show any changes in SM or ceramide levels, in either the absence or presence of CD95/Fas stimulation. Similarly, no changes in apoptosis sensitivity were observed [15]. From our observations of high SM-hydrolysing activity in DT40 cells that resides mainly in the ER (Figures 1 and 4), it could be expected that if the enzyme is involved in stress responses, greater amounts of ceramide would be produced in response to the ER stress or in this cell line compared with those with low sphingomyelinase activity. However, DT40 cells were not more susceptible to ceramide accumulation and apoptosis when exposed to the ER stress compared with other apoptotic agents and the responses were generally similar to those observed in another B-cell line characterized by low sphingomyelinase activity (Figures 5 and 6). A lack of quantitative correlation between NSM activity, ceramide

levels and apoptosis has been observed previously [1], which most likely reflects the complexity of the apoptotic process as well as the multiple roles of ceramide and sites of ceramide generation. While it remains possible that the involvement of NSM1 in apoptosis could be cell-type- or stimulus-dependent, taking into account the properties of this enzyme [9,11,13,16,20,35] and observations described in this (Figures 5 and 6) and previous [15] studies, the NSM1 enzyme is an unlikely candidate for a mediator of apoptosis. Interestingly, recent data showed that the targeting of *B. cereus* NSM to different cellular compartments such as the plasma membrane, Golgi apparatus, ER, mitochondria and nucleus resulted in an increase in total ceramide levels [17]. Moreover, specific targeting of this NSM to ER showed the highest increase in ceramide and the highest *B. cereus* NSM activity compared with other cellular compartments, suggesting that ER can be an important source of intracellular ceramide and an intracellular compartment that favours NSM activity [17]. However, the study by Birbes et al. [17] showed clearly that ceramide induces cell death specifically when generated in mitochondria by the bacterial NSM. These observations suggest that the generation of topologically restricted pools of ceramide by hydrolysis of SM by NSMs or *de novo* synthesis are associated with specific physiological responses.

Whatever the role of NSM1 might be, the finding that DT40 cells are a rich source of this ER enzyme identifies this cell line (known to be exceptionally amenable to genetic manipulations [30]) as a good model system to elucidate its function.

In addition to the generation of ceramide through hydrolysis of SM by NSMs, direct experimental evidence has emphasized the role of *de novo* ceramide synthesis in cell death [19]. Experiments described here, using the inhibitor fumonisin B1, support the importance of ceramide synthesis in B-cells, at least under some conditions used to induce apoptosis. Other observations, including a recent detailed study of ceramide synthesis in apoptotic B-cells [29], are consistent with this possibility.

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REFERENCES

- Hannun, Y. A. and Luberto, C. (2000) Ceramide in the eukaryotic stress responses. *Trends Cell Biol.* **10**, 73–80.
- Kronke, M. (1999) Involvement of sphingomyelinases in TNF signaling pathways. *Chem. Phys. Lipids* **102**, 157–166.
- Levade, T. and Jaffrezou, J.-P. (1999) Signalling sphingomyelinases: which, where, how and why? *Biochim. Biophys. Acta* **1438**, 1–17.
- Hannun, Y. A., Luberto, C. and Argraves, K. M. (2001) Enzymes in sphingolipid metabolism: from modular to integrative signaling. *Biochemistry* **40**, 4893–4903.
- Wiesner, D. A., Kilus, J. P., Gottschalk, A. R., Quantinas, J. and Dawson, G. (1997) Anti-immunoglobulin-induced apoptosis in WEHI 231 cells involves the slow formation of ceramide from sphingomyelin and is blocked by bcl-xL. *J. Biol. Chem.* **272**, 9868–9876.
- Segui, B., Andrieu-Abadie, N., Adam-Klages, S., Meilhac, O., Kreder, D., Garcia, V., Bruno, A. P., Jaffrezou, J.-P., Salvayre, R., Kronke, M. and Levade, T. (1999) CD40 signals apoptosis through FAN-regulated activation of the sphingomyelin-ceramide pathway. *J. Biol. Chem.* **274**, 37251–37258.
- Liu, B., Hassler, D. F., Smith, G. K., Weaver, K. and Hannun, Y. A. (1998) Purification and characterization of a membrane bound neutral pH optimum magnesium-dependent and phosphatidylserine-stimulated sphingomyelinase from rat brain. *J. Biol. Chem.* **273**, 34472–34479.
- Bernardo, K., Krut, O., Wiegmann, K., Kreder, D., Micheli, M., Schafer, R., Sickman, A., Schmidt, W. E., Schroder, J. M., Meyer, H. E., Sandhoff, K. and Kronke, M. (2000) Purification and characterisation of a magnesium-dependent neutral sphingomyelinase from bovine brain. *J. Biol. Chem.* **275**, 7641–7647.
- Tomiuk, S., Hofmann, K., Nix, M., Zumbansen, M. and Stoffel, W. (1998) Cloned mammalian neutral sphingomyelinase: functions in sphingolipid signaling? *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3638–3643.

- 10 Hofmann, K., Tomiuk, S., Wolff, G. and Stoffel, W. (2000) Cloning and characterization of the mammalian brain-specific, Mg-dependent neutral sphingomyelinase? *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5895–5900
- 11 Fensome, A. C., Rodrigues-Lima, F., Josephs, M., Paterson, H. F. and Katan, M. (2000) A neutral magnesium-dependent sphingomyelinase isoform associated with intracellular membranes and reversibly inhibited by reactive oxygen species. *J. Biol. Chem.* **275**, 1128–1136
- 12 Neuberger, Y., Shogomori, H., Levy, Z., Fainzilber, M. and Futerman, A. H. (2000) A lyso-platelet activating factor phospholipase C, originally suggested to be a neutral-sphingomyelinase, is located in the endoplasmic reticulum. *FEBS Lett.* **469**, 44–46
- 13 Tomiuk, S., Zumbansen, M. and Stoffel, W. (2000) Characterization and subcellular localisation of murine and human magnesium-dependent neutral sphingomyelinase. *J. Biol. Chem.* **275**, 5710–5717
- 14 Tonnetti, L., Veri, M.-C., Bonvini, E. and D'Adamio, L. (1999) A role for neutral sphingomyelinase-mediated ceramide production in T cell receptor-induced apoptosis and mitogen-activated protein kinase-mediated signal transduction. *J. Exp. Med.* **189**, 1581–1589
- 15 Tepper, A. D., Ruurs, P., Borst, J. and Blitterswijk, W. J. (2001) Effect of overexpression of a neutral sphingomyelinase on CD95-induced ceramide production and apoptosis. *Biochem. Biophys. Res. Commun.* **280**, 634–639
- 16 Sawai, H., Domae, N., Nagan, N. and Hannun, Y. A. (1999) Function of the cloned putative neutral sphingomyelinase as lyso-platelet activating factor-phospholipase C. *J. Biol. Chem.* **274**, 38131–38139
- 17 Birbes, H., El Bawab, S., Hannun, Y. A. and Obeid, L. M. (2001) Selective hydrolysis of a mitochondrial pool of sphingomyelin induces apoptosis. *FASEB J.* **15**, 2669–2679
- 18 Ferri, K. F. and Kroemer, G. (2001) Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* **3**, 255–263
- 19 Dbaiho, G. S., El-Assaad, W., Krikorian, A., Liu, B., Diab, K., Idriss, N. Z., El-Sabban, M., Driscoll, T. A., Perry, D. K. and Hannun, Y. A. (2001) Ceramide generation by two distinct pathways in tumor necrosis factor alpha-induced cell death. *FEBS Lett.* **503**, 7–12
- 20 Rodrigues-Lima, F., Fensome, A., Josephs, M., Evans, J., Veldman, R. J. and Katan, M. (2000) Structural requirements for catalysis and membrane targeting of mammalian enzymes with neutral sphingomyelinase and lysophospholipid phospholipase C activities. *J. Biol. Chem.* **275**, 28316–28325
- 21 Lewis, V. A., Hynes, G. M., Zveng, D., Saibil, H. and Willison, K. (1992) T-complex polypeptide-1 is a subunit of a heteromeric particle in the eukaryotic cytosol. *Nature (London)* **358**, 249–252
- 22 Kim, Y. S., Perdomo, J. and Nordberg, J. (1971) Glycoprotein biosynthesis in small intestine mucosa. A study of glycosyl-transferase in microsomal subfractions. *J. Biol. Chem.* **246**, 5466–5476
- 23 Richarme, G. (1998) Protein-disulfide isomerase activity of elongation factor EF-Tu. *Biochem. Biophys. Res. Commun.* **252**, 156–161
- 24 Aronson, N. N. and Touster, O. (1974) Isolation of rat liver plasma membrane fragments in isotonic sucrose. *Methods Enzymol.* **31**, 90–102
- 25 Jorgensen, P. L. (1974) Isolation of Na/K-ATPase. *Methods Enzymol.* **32**, 277–290
- 26 Storrie, B. and Madden, E. A. (1990) Isolation of subcellular organelles. *Methods Enzymol.* **182**, 203–225
- 27 Allan, D. (2000) Lipid metabolic changes caused by short-chain ceramides and the connection with apoptosis. *Biochem. J.* **345**, 603–610
- 28 Dawson, G., Kilkus, J. P. and Schieven, G. L. (2000) Selective phosphotyrosine phosphatase inhibition and increased ceramide formation is associated with B-cell death by apoptosis. *FEBS Lett.* **478**, 233–236
- 29 Kroesen, B.-J., Pettus, B., Luberto, C., Busman, M., Sietsma, H., de Leij, L. and Hannun, Y. A. (2001) Induction of apoptosis through B-cell receptor cross-linking occurs via de novo generated C16-ceramide and involves mitochondria. *J. Biol. Chem.* **276**, 13606–13614
- 30 Lahti, J. M. (1999) Use of gene knockouts in cultured cells to study apoptosis. *Methods* **17**, 305–12
- 31 Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J. and Kurosaki, T. (1996) Btk as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. *Science* **273**, 1096–1100
- 32 Takao, N., Li, Y. and Yamamoto, K. (2000) Protective roles for ATM in cellular response to oxidative stress. *FEBS Lett.* **472**, 133–136
- 33 Maruo, A., Oishi, I., Sada, K., Nomi, M., Kurosaki, T., Minami, Y. and Yamamura, H. (1999) Protein tyrosine kinase Lyn mediates apoptosis induced by topoisomerase II inhibitors in DT40 cells. *Int. Immunol.* **11**, 1371–1380
- 34 Takata, M., Homma, Y. and Kurosaki, T. (1995) Requirement of phospholipase C-gamma2 activation in surface immunoglobulin M-induced B-cell apoptosis. *J. Exp. Med.* **182**, 907–914
- 35 Mizutani, Y., Tamiya-Koizumi, K., Irie, F., Hirabayashi, Y., Miwa, M. and Yoshida, S. (2000) Cloning and expression of rat neutral sphingomyelinase: enzymological characterization and identification of essential histidine residues. *Biochim. Biophys. Acta* **1485**, 236–246
- 36 Tepper, A. D., Ruurs, P., Weidmer, T., Sims, P. J., Borst, J. and Blitterswijk, W. J. (2000) Sphingomyelin hydrolysis to ceramide during the execution phase of apoptosis results from phospholipid scrambling and alters cell-surface morphology. *J. Cell Biol.* **150**, 155–164

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