Overexpression of a neutral sphingomyelinase (nSMase1) in Jurkat T cells: Effect on ceramide production and apoptosis sensitivity.

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The abbreviations used are: Cer, ceramide; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate; GlcCer, glucosylceramide; nSMase, neutral sphingomyelinase; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin.

SUMMARY

We previously observed that ceramide (Cer) formed during the execution phase of apoptosis is derived from plasma membrane sphingomyelin (SM), most likely by a neutral sphingomyelinase activity. In this study, we characterize a putative human neutral sphingomyelinase (nSMase1), which was cloned on the basis of homology with other phosphohydrolases [Tomiuk et al. 1998]. We show that the \textit{in vitro} hydrolyzing activity of nSMase1 is specific for SM and requires a neutral pH. Substitution of predicted catalytic residues (Glu$^{49}$, Asn$^{180}$ and His$^{272}$) by Ala abolishes the catalytic activity. We transduced Jurkat T cells with either wildtype or catalytically inactive nSMase1 (containing all three point mutations) to investigate the role of nSMase1 in SM metabolism. Cells overexpressing wildtype nSMase1 showed dramatically elevated \textit{in vitro} nSMase activity. However, nSMase1 gene transduction (wildtype or mutant) did not alter steady state levels of SM, Cer or glucosylceramide. CD95-induced Cer production and apoptosis were identical in both vector- and nSMase1- transduced cells. Immunolocalization studies in transduced HeLa cells showed no association of nSMase1 with the plasma membrane, but rather with the endoplasmic reticulum. These data lead to the conclusion that an enzyme other than nSMase1 is involved in the conversion of SM to Cer during apoptosis.
CHAPTER 7

INTRODUCTION

In previous studies we have shown that the execution phase of apoptosis is accompanied by ceramide (Cer) production (Tepper, 1997; Tepper, 1998). Our most recent work revealed the mechanism underlying this late phase of Cer production (Tepper, 2000). Outer leaflet plasma membrane sphingomyelin (SM) flips to the inner leaflet during a process of phospholipid scrambling, concomitant with cell surface exposure of phosphatidylserine (PS). Subsequently, internalized SM is hydrolyzed by a sphingomyelinase (SMase). A neutral SMase is most likely responsible for the slow Cer production in Jurkat cells upon CD95 ligation or DNA-damaging regimens (etoposide or radiation), because we found no role for acid SMase (Boesen-de Cock, 1998) or de novo biosynthesis (Tepper, 2000). We suggest that the physiological role of late Cer production has to be sought in the removal of plasma membrane SM, rather than in the formation of Cer. Via tight interactions with cholesterol, SM is a critical determinant of stability and impermeability of the lipid bilayer. SM hydrolysis during the execution phase of apoptosis will reduce SM/cholesterol interactions and thereby facilitate morphological changes to occur, such as membrane blebbing and apoptotic body formation.

Despite several purification attempts, the identity of nSMase has remained elusive, and for a long time no molecular tools were available to evaluate the role of neutral SMase. However, Tomiuk et al. cloned a putative human Mg²⁺-dependent neutral SMase (nSMase₁) by a database search method using a generalized profile of Mg²⁺-dependent phosphohydrolases (Tomiuk, 1998). Overexpression of the cloned gene showed only minor effects on rapid (within 10 min) Cer production in TNFα-stimulated U937 cells. nSMase₁ overexpression in HEK293 cells had no effect on cell growth or morphology (Tomiuk, 1998). On the other hand, nSMase₁ was implicated in T cell receptor-induced Cer production and apoptosis on the basis of an nSMase antisense RNA approach (Tonnetti, 1999). To examine whether nSMase₁ is involved in Cer accumulation during CD95-induced apoptosis, we retrovirally transduced Jurkat cells with nSMase₁. Furthermore, we designed and overexpressed a catalytically inactive nSMase₁ mutant with the aim to study possible dominant-negative effects. The subcellular localization of nSMase₁ was determined using nSMase₁-transduced HeLa cells.

EXPERIMENTAL PROCEDURES

Materials - The DOTAP transfection reagent was obtained from Boehringer Mannheim. Zeocin was from Kyla. [N-methyl-³¹⁵]sphingomyelin (55.0 mCi/mmol), L-3-phosphatidyl[N-methyl-³¹⁵]choline (58.0 mCi/mmol), and L-[³¹⁵]serine (54.0 mCi/mmol) and the enhanced chemiluminescence system (ECL) were purchased from Amersham Pharmacia Biotech. Horseradish peroxidase-conjugated rabbit anti-mouse Ig was obtained from Dako (Glostrup, Denmark). Polyclonal (rabbit) anti-calreticulin antibody (catalogue nr. PA3-900) was purchased from Affinity BioReagents, Golden, CO, USA.
Cell culture and transfection - COS7 and HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 8% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM L-glutamine. The Jurkat clone J16, which has been selected for sensitivity to CD95 stimulation and T cell antigen receptor expression, was cultured as described (Tepper, 1997). COS7 cells were transfected with 1 μg of plasmid DNA according to the DEAE-dextran method (Sambrook, 1989). Cells were used for experiments 48 h after transfection.

Cloning of the human nSMase1 cDNA - Human nSMase cDNA was assembled using EST clones ID2158126 and ID351494 identified in the LifeSeq™ database of IncyteGenomics. The complete cDNA was cloned in the pcDNA3 expression vector (Invitrogen). In comparison to the sequence published by Tomiuk et al. (1998) (AJ222801) our sequence shows two polymorphic alterations: A T - C transition at position 367 on nucleotide level (AJ222801) leads to a L - P exchange at position 3 on aminoacid level and a T - G transition at position 1154 causes a S - R exchange at position 265. These polymorphisms are known from different GenBank entries. For example one can find the T - C transition in the entries AI739480, AI911456, AA282975, AA283028, AI005003 and AA749303 while the T - G transition can be identified in the entries AL136222 and AL673685.

Construction of expression vectors - A Myc-tag, a new stop codon, and a XhoI site were added to the 3'-end of the nSMase cDNA in pcDNA3 by PCR. The correct sequence was verified by deoxy nucleotide sequencing. nSMaseMyc and nSMaseAAAMyc cDNA were cloned into the BamHI and XhoI sites of the retroviral vector LZRS-MS-IREZeo/pBR (F. Michiels, our institute), a derivative of LZRS-pBMN-lacZ which was provided by Dr. G. Nolan (Stanford University School of Medicine).

Site-directed mutagenesis - The single point mutants Glu49Ala, Asn180Ala and His272Ala were constructed using the Quickchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with pcDNA3-nSMaseMyc as template. A construct containing all three mutations was obtained by the same procedure. The correct sequences of all constructs were verified by automated nucleotide sequencing.

Retroviral gene transduction - Plasmid LZRS-nSMaseMyc-IREZeo, LZRS-nSMaseAAAMyc-IREZeo, or empty vector were transfected into the ΦNX-Ampho producer cell line by calcium phosphate precipitation (Kinsella, 1996). Virus-containing supernatant was harvested 48 h after transfection and stored at -80°C until further use. Jurkat cells were transduced at a density of 0.5 x 10⁶/ml of virus-containing supernatant in the presence of 10 μg/ml DOTAP. HeLa cells were transduced at approximately 20% confluency with 1 ml virus supernatant per 3 cm dish. Supernatants were removed after overnight incubation and cells were cultured in fresh medium. Selection of transduced cells was initiated after 24 h by the addition of 200 μg/ml Zeocin.

Western analysis - COS7 cells were washed once in ice-cold PBS and lysed in reducing SDS sample buffer. Lysates were heated for 10 min at 95°C and separated on 12% SDS-polyacrylamide gels (equivalents of 0.8-1 x 10⁶ cells per lane). For immunoprecipitation, 40 x 10⁶ Jurkat cells were lysed for 30 min at 4°C in 500 μl of 10 mM triethanolamine-HCl, pH 7.8, 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, containing 1% Nonidet P-40, 0.02 mg/ml trypsin inhibitor and 0.02 mg/ml leupeptin. Lysates were centrifuged, and supernatants were incubated with 10 μl 9E10 (anti-Myc) antibody and 10 μl protein A-Sepharose CL4B beads which had been
preincubated with rabbit anti-mouse serum. Protein isolates were washed four times in lysis buffer and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes. Immunoblotting was performed with 9E10 hybridoma supernatant (anti-Myc tag) at a dilution of 1:250. Horseradish peroxidase-conjugated rabbit anti-mouse (1:7500) and ECL were used to detect the primary antibody.

**Quantitation of SMase activity** - Neutral and acid SMase activities were determined as described by Jayadev et al. (Jayadev, 1997). Transfected COS7 cells were washed once with ice-cold PBS, scraped into 200 µl ice-cold lysis buffer (pH 7.4 or pH 5.0) and lysed via three cycles of freeze/thawing. Nuclei and unlysed cells were removed by centrifugation (1000 x g; 10 min; 4°C), and 50 µg of protein (determined with a Bradford protein assay) was assayed using [14C-choline]phosphorylcholine. The hydrolyzing activity towards phosphatidylcholine was measured using [14C-choline]phosphatidylcholine. Reactions were quenched using CHCl3/MeOH (2:1, v/v) and phase separation was induced with H2O. Aliquots of the aqueous phase containing the enzymatically released [14C]phosphocholine were counted in a liquid scintillation counter. To assay neutral SMase activity of transduced Jurkat cells, 2 x 10^6 cells were lysed in 100 µl of neutral lysis buffer and 40 µg of protein was used per assay.

**Sphingolipid quantification** - Intracellular Cer, SM and GlcCer levels were determined following metabolic labeling with L-[3-14C]serine as described previously (Tepper, 1997; Boesen-de Cock, 1998). Briefly, cells were incubated during 36-48 h with L-[3-14C]serine (0.3 µCi/ml). Following stimulation with 200 ng/ml anti-CD95 mAb (clone 7C11), total lipids were extracted and separated by TLC (Tepper, 1997). Radioactive lipids were quantified using Phospholmaging and sphingolipid levels were expressed relative to the radioactivity incorporated in phosphatidylserine (PS) and phosphatidylethanolamine (PE).

**Apoptosis assay** - Jurkat cells were seeded at 1 x 10^6 /ml and exposed to anti-CD95 mAb 7C11 at the indicated concentrations for different time periods. Cells were lysed in 0.1% sodium citrate, 0.1% Triton X-100, and 50 µg/ml propidium iodide (PI) (Nicoletti, 1991). Fluorescence intensity of PI-stained DNA was determined in 5000 cells on a FACscan (Beckton Dickinson Immunocytometry Systems, San Jose, CA). Fragmented, apoptotic nuclei are recognized by their subdiploid DNA content.

**Immunofluorescence** - HeLa cells seeded on glass cover slips were fixed in ice-cold methanol for 30 minutes. The cells were permeabilized for 15 min with PBS/0.1% Triton X-100 containing 1% BSA. Visualisation of nSMaseMyc and calreticulin was carried out with the primary anti-Myc (9E10; 1:10) and anti-calreticulin antibodies (1:50). Following washing (PBS/0.05% Triton X-100), the secondary antibodies goat-anti-mouse-FITC (1:200; Zymed) and goat-anti-rabbit-Texas Red (1:200; Molecular Probes Inc.) were added. All antibodies were incubated for one hour in PBS/0.05% Triton X-100/1% BSA at room temperature. After extensive washing, cells were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Fluorescence was evaluated with Confocal Scanning Laser Microscopy (CLSM), using laserlines 488 nm and 568 nm and appropriate filters.
RESULTS and DISCUSSION

Overexpression of nSMase1 in COS7 cells
When pcDNA3.nSMaseMyc was transiently transfected into COS7 cells, the Myc-tagged cDNA product could readily be detected in whole cell lysates (Fig. 1A). Its size of approximately 45 kDa is in good agreement with the reported molecular weight of nSMase1 (Tomiuk, 1998). Enzymatic characterization of nSMase1 was performed by transient expression in COS7 cells. The in vitro nSMase activity in total cell lysates of nSMase-expressing COS7 cells was increased by approximately 50-fold when compared to vector-transfected control cells (Fig. 1B). nSMase1 activity required a neutral pH and specifically hydrolyzed SM, because only a minimal increase in hydrolyzing activity towards phosphatidylcholine (PC) was observed under these conditions (Fig. 1B).

**FIG. 1. nSMase1 protein expression and enzyme activity in COS7 cells.** A, Total cell lysates of COS7 cells transiently transfected with pcDNA3 vector (lane 1) or pcDNA3-nSMaseMyc (lane 2) were separated by SDS-PAGE and analyzed by Western blotting using an anti-Myc antibody. The only specific signal is a band at approximately 45 kDa. B, Cell homogenates of COS7 cells transiently transfected with vector (open symbols) or nSMaseMyc (filled symbols) were assayed at pH 7.4 for the ability to release water-soluble phosphocholine radioactivity from \[^{14}C\]-choline-labeled SM (circles) or PC (triangles). Acid SMase activity in the homogenates was monitored using \[^{14}C\]-cholineSM at pH 5.0 (squares).

Site-directed mutagenesis and catalytic activity of mutant enzymes
Human and mouse nSMase candidate genes were found to belong to a large superfamily of Mg\(^{2+}\)-dependent phosphodiesterases with neutral pH optimum, including exonuclease III and deoxyribonuclease I from *E. coli* (Tomiuk, 1998). The functionally important amino acids for the hydrolysis of the phosphodiester bond can be identified from the known crystal structures of these two nucleases (Mol, 1995). In human nSMase1, Glu49 is predicted to complex Mg\(^{2+}\), Asn180 would be involved in substrate binding and His272 is predicted to serve an essential role as catalytic base during the hydrolysis of the phosphodiester bond (Tomiuk, 1998). These amino acids are totally conserved between neutral SMase from mouse or man and the bacterial SMases and...
ORF Yer019w from yeast. We substituted each of the residues with alanine by site-directed mutagenesis. In addition, a triple point mutant (further referred to as AAA) containing all three mutations was generated. Mutant enzymes were expressed in COS7 cells and the catalytic activity toward SM was determined in vitro. As shown in Fig. 2, all three single point mutants completely lost their catalytic activity whereas mutant proteins were expressed to levels comparable to the wildtype enzyme. These data indicate that, whereas the substrates of SMase and DNase are quite different, the catalytic mechanisms responsible for cleavage of the phosphodiester bond in DNA or the lipid SM are very similar.

**FIG. 2. Point mutations in the nSMase1 active site abrogate catalytic activity.** Three single point mutations were introduced into the nSMase1-cDNA (WT) by site-directed mutagenesis. A triple point mutant containing all three mutations (AAA) was also generated. Constructs were transiently expressed in COS7 cells (upper panel) and the in vitro nSMase activity of cell homogenates was determined (lower panel). Data represent the mean values of two different experiments, each with duplicate samples ± SD.

**Generation of Jurkat T cells stably overexpressing nSMase1**

A polyclonal population of Jurkat cells stably overexpressing wildtype nSMase1 was generated by retroviral gene transduction. In addition, we transduced cells with the catalytically inactive triple mutant construct described above (nSMaseAAA), with the aim to create a system overexpressing an enzyme with possible dominant-negative nSMase function. Both gene products could be detected in anti-Myc immunoprecipitates from the transduced Jurkat cells (Fig. 3). *In vitro* activity measurements showed that nSMase activity in Jurkat cells overexpressing the wildtype enzyme was dramatically (~70-fold) increased compared to vector control cells (Fig. 3). Expression of the catalytically inactive nSMase mutant (nSMaseAAA) did not affect basal nSMase activity measured *in vitro*.

**FIG. 3. Retroviral transduction of nSMase1 in Jurkat cells increases nSMase activity.** Homogenates of empty vector-, nSMase- or nSMaseAAA-transduced Jurkat cells (clone J16) were assayed for SMase activity at pH 7.4 using \[^{3}^\text{H}-\text{choline}\]SM (upper panel). Expression of the Myc-tagged cDNA constructs was verified by Western blot analysis of anti-Myc immunoprecipitates (lower panel).
OVEREXPRESSION OF nSMase1

Steady state sphingolipid metabolism in transduced Jurkat cells
To examine how nSMase overexpression affects the basal turnover of SM, transduced Jurkat cells were labeled to equilibrium with [14C]serine which incorporates in the sphingoid backbone of all sphingolipids. After separation of total lipid extracts by TLC, the levels of Cer, SM and glucosylceramide were determined and normalized to the incorporation of [14C]serine into PS plus PE. Despite the large increase in in vitro nSMase activity, stable overexpression of wildtype nSMase did not result in altered basal levels of either Cer, SM or GlcCer (Fig. 4).

FIG. 4. Effect of nSMase1-transduction on basal sphingolipid levels. J16 cells transduced with empty vector (black bars), nSMase (gray bars) or nSMaseAAA (open bars) were labeled to equilibrium with [14C]serine, and total lipids were separated by TLC and quantified using Phosphorimaging. Cer, SM and glucosylceramide (GlcCer) levels were normalized for total radioactivity in phosphatidylserine and phosphatidylethanolamine. Values represent the means ± SD of two independent determinations from two separate experiments.

Effect of nSMase overexpression on CD95-induced Cer formation
Next, we investigated whether overexpression of nSMase1 might enhance Cer production. CD95 ligation induced a slow and sustained accumulation of Cer in vector control cells (Fig. 5). However, neither the kinetics nor the absolute amount of Cer production were significantly affected by the overexpression of either a catalytically active (nSMase) or inactive (nSMaseAAA) nSMase1. These results indicate that nSMase1 plays no role in CD95-induced Cer formation.

FIG. 5. CD95-induced Cer production is not affected upon nSMase1 overexpression. J16 cells transduced with empty vector (circles), nSMase (squares) or nSMaseAAA (triangles) were labeled to equilibrium with [14C]serine and subsequently incubated in the absence (open symbols) or presence (filled symbols) of agonistic CD95 mAb (200 ng/ml). After the time periods indicated, total lipids were extracted and separated by TLC. Labeled Cer is expressed as described in Fig. 4. The data (means ± SD) represent two independent experiments performed in duplicate.
Apoptosis sensitivity of nSMase1-transduced Jurkat cells

We next determined the effect of nSMase1 overexpression on apoptosis sensitivity to CD95 ligation. As shown in Fig. 6, the rate and extent of apoptosis measured by nuclear fragmentation were very similar in vector-, nSMase- or nSMaseAAA- transduced Jurkat cells. In addition, these cell lines underwent identical morphological changes during apoptosis (data not shown).

![Graph](image)

**FIG. 6.** nSMase1 does not alter apoptosis sensitivity to CD95 ligation. J16 cells transduced with empty vector (circles), nSMase (squares) or nSMaseAAA (triangles) were incubated in the absence (open symbols) or presence (filled symbols) with agonistic CD95 mAb (7C11; 100 ng/ml). After the time periods indicated, nuclear fragmentation was determined by FACS analysis.

Subcellular localization of nSMase1

Because nSMase1 did not affect hydrolysis of plasma membrane SM, we investigated its subcellular localization. To this end, HeLa cells were transduced with the Myc-tagged nSMase1 cDNA (HeLa-Zeo-nSMase) or with empty vector (HeLa-Zeo). Confocal immunofluorescence microscopy of HeLa cells stably expressing nSMase1 showed a predominant staining of intracellular membranes (Fig. 7, panel C). This staining was specific for the transduced cDNA product as it was not observed in vector-transduced control cells (panel A).

The staining pattern of Myc-tagged nSMase1 seemed to correspond to endoplasmic reticulum (ER) staining. Indeed, double staining with anti-Myc antibody together with anti-calreticulin (known to stain the lumen of the ER) showed colocalization of nSMase with most of the calreticulin (Fig. 7). In addition, some of the nSMase protein localized to the Golgi complex. No staining of nSMase1 was observed at the plasma membrane. Although plasma membrane association of nSMase1 can not be fully excluded on the basis of confocal microscopy, these results indicate the predominant localization of nSMase1 at the ER membrane.

Taken together, we conclude that nSMase1 does not appear to be involved in basal SM metabolism nor in the generation of Cer during CD95-induced Jurkat cells. We could not validate whether the overexpressed inactive mutant acts as a dominant negative enzyme *in vivo*. The subcellular localization of nSMase1 raises intriguing questions regarding the biological role of nSMase1 in the ER, where its substrate SM level is thought to be relatively minor.
FIG. 7. Immunolocalization of nSMase1 in transduced HeLa cells detected by confocal laser scanning microscopy. HeLa cells stably transduced with empty vector (A and B) or Myc-tagged nSMase1 (C through H) were fixed and incubated with antibodies against the Myc-tag (9E10) together with an anti-calreticulin antibody. Primary antibodies were visualized with FITC- and Texas Red-conjugated secondary antibodies, respectively. A, C, E and G, anti-Myc; B, D, F and H, anti-calreticulin.
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Note added: During the course of this study, a paper appeared by Tomiuk et al. (2000, J Biol Chem 275:5710) with essentially similar results.

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