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Lactosylceramide is required in apoptosis induced by N-Smase

Sergio F. Martin · Niesha Williams · Subroto Chatterjee

Abstract Lactosylceramide (LacCer) is a member of the glycosphingolipid family which has been recently recognized as a signaling intermediate in the regulation of cell proliferation and cell adhesion. In this paper, we present our studies pointing to a potential role of LacCer in inducing apoptosis. In our studies we employed a human osteosarcoma cell line MG-63 (wild type, WT) and a neutral sphingomyelinase (N-SMase) deficient cell line CC derived from MG-63 (mutant) cells. We observed that WT cells were highly sensitive to tumor necrosis factor-α (TNF-α), ceramide and LacCer-induced apoptosis. In contrast, the mutant cells were insensitive to TNF-α-induced apoptosis as they did not generate ceramide and LacCer. However, the exogenous supply of ceramide and/or LacCer rendered the mutant cells apoptotic. Interestingly, preincubation of cells with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an inhibitor of glucosylceramide synthase and lactosylceramide synthase, abrogated ceramide-induced apoptosis but not LacCer-induced apoptosis in both WT cells and the mutant cells. Moreover, TNF-α and LacCer-induced apoptosis required the generation of reactive oxygen species (ROS) in WT cells. However, since mutant cells did not produce significant amounts of LacCer and ROS in response to TNF-α treatment they are insensitive to TNF-α-induced apoptosis. In summary, our studies suggest that TNF-α-induced N-SMase activation and production of ceramide is required to activate the apoptosis pathway in human osteosarcoma cells. But it is not sufficient to induce apoptosis. Rather, the conversion of ceramide to LacCer and ROS generation are critical for apoptosis.

Keywords ROS · Sphingomyelinase · Apoptosis · Lactosylceramide · TNF-α

Abbreviations

LacCer	LactosylCeramide
TNF-α	Tumor Necrosis Factor-α
N-SMase	Neutral Sphingomyelinase
D-PDMP	D-threo-1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol
ROS	Reactive Oxygen Species
MnSOD	Manganese Superoxide Dismutase
NAC	N-acetyl-L-cysteine
PBS	Phosphate-Buffered Saline
DTT	Dithiothreitol
ATP	Adenosine Tris-Phosphate
DAPI	4′,6-Diamidine-2′-phenylindole dihydrochloride
DCFH-DA	diachlorofluorescein diacetate
TNFR1	TNF-α receptor 1
SM	sphingomyelin; Wild type
WT	human osteosarcoma cell line MG-63
Mutant	N-SMase deficient osteosarcoma cell line CC

Introduction

Glycosphingolipids are characteristic components of plasma membranes, which have been previously considered mainly

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as structural lipids. However, glycosphingolipids are increasingly recognized to function as mediators of cell-cell interaction, as receptors for microbes and their toxins, and as modulators of the sensitivity of cancer cells to anticancer drugs [1–4]. Moreover, recently there has been a surge in reports suggesting that glycosphingolipids may be implicated as second messengers in mediating diverse molecular events, including cell proliferation [5–7], programmed cell death (apoptosis) [8], and adhesion by way of increasing the expression of cell adhesion molecules [9].

Lactosylceramide (LacCer) is a ubiquitously occurring glycosphingolipid which plays a pivotal role in the biosynthesis of complex glycosphingolipids [10]. Recently, an exciting development in the field of LacCer research shows that this molecule regulates critical phenotypic changes such as cell adhesion and proliferation in mammalian cells [11–15]. Further studies have revealed that, by virtue of activating NADPH oxidase and producing superoxide, LacCer regulates such diverse phenotypic changes.

Previously, reactive oxygen species (ROS) has been implicated to play an important role as a regulator in apoptosis [16,17]. Pro-oxidants and redox cycling agents [18–21] can induce apoptosis. Other apoptotic stimuli, such as treatment with TNF-α [22] lipopolysaccharide [23], growth factor withdrawal [24], and human immunodeficiency virus infection [25], can stimulate ROS production. Studies with TNF-α have shown that these agents activate ROS generation by mitochondria [22]. Our recent studies with human aortic smooth muscle cells have revealed that GD_3, a disaloganglioside, can induce apoptosis by way of producing large levels of ROS. The recent proposed model of p53-induced apoptosis also placed ROS as a central signaling event [26,27]. On the other hand, antioxidants and thiol reductants, such as *N*-acetyl-L-cysteine (NAC) [28], overexpression of thioredoxin [29], and Manganese Superoxide Dismutase (Mn-SOD) [30], can block or delay apoptosis. ROS can also provide protective mechanisms under some conditions, such as the activation of NFκB by TNF-α-induced ROS production [31,32].

Although ceramide has been implicated in apoptosis, studies from several laboratories including our own reveal that ceramide does not produce ROS [6,14]. Therefore, we hypothesized that ceramide may well be glycosylated to LacCer/GD_3 to produce ROS and subsequently induce apoptosis. In this article, we show that LacCer is implicated in the apoptotic signaling through ROS production. We have employed human osteosarcoma cell line MG-63 (wild type, WT) in which the TNF-α inducible apoptotic pathway via neutral sphingomyelinase is intact. Moreover, in our studies we have also employed N-SMase-deficient osteosarcoma cell line CC (mutant) in our studies that are insensitive to TNF-α and Fas ApoI/CD95-induced apoptosis (data unpublished). We observed that in WT cells TNF-α stimulated ceramide and LacCer production. Subsequently, LacCer stimulated $O_2^{\cdot -}$ generation and apoptosis. This phenotypic change was abrogated by preincubation of cells with D-PDMP; an inhibitor of glucosylceramide synthase and LacCer synthase. On the other hand, in the mutant cells TNF-α did not significantly produce ceramide and/or LacCer and did not generate $O_2^{\cdot -}$ and apoptosis. However, the exogenous supply of LacCer induced apoptosis in the mutant cells. Thus LacCer production is critical to apoptosis induced by TNF-α in human osteosarcoma cells.

Materials and Methods

Cell lines and culture conditions

A human osteosarcoma cell line MG-63 (WT) was obtained as a gift from Dr. F. Ramirez. These cells had the full complement of enzymes and signaling cascades requisite for TNF-α-induced apoptosis (unpublished data). These cells and antisense technology were employed to prepare an N-SMase-deficient human osteosarcoma cell lines: CA, CB and CC (mutant, data described above). The WT and mutant cells were cultured in Dulbecco's modified Eagle's minimum-essential medium (Grand Island Biological Company-Bethesda Research Laboratories (GIBCO-BRL), Bethesda, MD) supplemented with 10% fetal calf serum (Hyclone, Salt Lake City, UT), 100 units/ml penicillin, and 100 mg/ml streptomycin. Cell lines were cultured in a 37°C humidified environment containing 5% CO_2 in air. Cell viability was determined by trypan blue exclusion.

Ribozyme construction

The pU1/NSM vector was constructed using a modification of techniques previously described [8]. Briefly, the SV40 promoter, polyA, and polylinker were excised from pZeoSV (Invitrogen) prokaryotic/eukaryotic expression at the BamHI sites. A U1 snRNA expression cassette (gift of K Beemon) was ligated into the BamHI sites of the modified pZeoSV. Unique EcoRI and SpeI restriction sites were introduced flanking the Sm protein binding site by site-directed mutagenesis. Two complementary oligonucleotides were synthesized to include the 24 highly conserved nucleotides of hammerhead ribozymes, as well as the U1 snRNA stem-loops. These oligonucleotides were annealed at 40°C such that the remaining 5′ and 3′ overhangs were exactly complementary to the overhangs left by the EcoRI and SpeI digests of the vector. After ligation of the oligonucleotide duplex into the vector (now pNSMRz), the sequence of the insertion was confirmed. The corresponding sequence was

analyzed using a program that predicts secondary structure (http://ibc.wustl.edu/~zucker/rna/).

Transfection of the MG63 cells

MG63 cells were grown to 60% confluence and transfected with either linearized pNSMRz or an unmodified reporter gene construct (pZeoSVLacZ, Invitrogen) used as a control. The transfections were performed using Lipofectamine Plus (Life Technologies) according to manufacturer's instructions. Cells were grown in MEM media (Cellgro) with 10% fetal calf serum and 250 μg/ml of zeocin (Invitrogen). Cell death was evident after 48 h. To remove dead and dying cells, cultures were rinsed daily with PBS and overlaid with fresh zeomycin-containing medium. After 14 days, widely spaced clonal colonies of 10–100 cells were observed and harvested using 8×8 mm cloning cylinders. After treatment with trypsin, cells were transferred to single wells of 24-well tissue culture plates and were clonally expanded. Zeomycin selection (250 μg/ml) was maintained throughout all phases of experimentation. Ninety-six clones were selected for Northern analysis (data not shown), and those with reduced N-SMase mRNA were analyzed for N-SMase activity as described below. The clone with the lowest demonstrable N-SMase activity is described in all further work as the "ribozyme transfected clone".

Measurement of NADH/NADPH oxidase assay in intact cells

Lucigenin, an acridylium compound that emits light upon reduction and interaction with O_2, was used to measure O_2^- production. Briefly, cultured cells were harvested and washed twice in phosphate-buffered saline (PBS). Cell pellets were suspended in a balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 35 mM phosphoric acid, and 20 mM HEPES, pH 7.4). NADH/NADPH oxidase assay was measured as described by Li and Shah [33]. Photon emission was measured every 60 seconds for O_2^- produced at each time point calculated by comparison with a standard curve 10 minutes in a scintillation counter (Packard TOP counter, Wellesley, MA). The amount of generated superoxide was measured using xanthine/xanthine oxidase as described [6]. The viability of the suspended cells as determined by the trypan blue exclusion principle was >90%. Protein content was measured by the method of Lowry with bovine serum albumin serving as a standard.

Measurement of ceramide in cells

Ceramide was quantified by the diacylglycerol kinase assay as described previously [34]. Briefly, after incubation with different chemical compounds, cells were pelleted by centrifugation (300×g for 10 min), washed twice with ice-cold PBS, and extracted with 600 μl1N NaCl and 720 μl, 2% acetic acid in methanol. The organic-phase extract (containing mostly lipids) was dried under N_2 atmosphere. The sample was resuspended in a 100 μl reaction mixture containing 150 μg of cardiolipin (Avanti Polar Lipids, Alabaster, AL), 280 μM diethylenetriaminepentaacetic acid (Sigma, St. Louis, MO), 50 mM octyl-D-glucopyranoside (Calbiochem, San Diego, CA), 50 mM NaCl, 50 mM imidazole, 1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.7% glycerol, 70 μM ß-mercaptoethanol, 1 mM adenosine triphosphate (ATP), 10 μCi [^{32}P]ATP (3,000 Ci/mmol; Dupont New England Nuclear, Boston, MA), and 35 μg/ml Escherichia coli diacylglycerol kinase (Calbiochem, San Diego, CA) at pH 6.5. After 30 min at room temperature, the reaction was stopped by extraction of lipids with 3 mL of chloroform:methanol (1:2, v/v); 1 mL chloroform, and 1 mL 1N NaCl. The lower organic phase was washed twice with 1% perchloric acid. This lower organic phase was dried under N_2. Ceramide 1-phosphate was resolved by thin-layer chromatography on silica gel 60 plates (Whatman, Fairfield, NJ) using a solvent system consisting of chloroform-methanol-acetone-acetic acid:H_2O (50:20:15:5:2, v/v) and detected with iodine vapors. The level of ceramide was determined by comparison with a standard curve generated concomitantly using known amounts of ceramide (ceramide type III; Sigma).

Histochemical detection of nuclear apoptotic bodies by DAPI

The morphologic changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome (DAPI or 4′,6-Diamidine-2′-phenylindole dihydrochloride). Cells were grown on sterilized glass coverslips. Following incubation with various compounds as indicated in the legend, cells were washed twice with PBS and incubated in 30% acetic acid in methanol for 3 min at room temperature. After the fixative was removed and cells were washed twice in PBS, cells were stained with 10 μl aliquot of 10 mg/mL DAPI reagent [35] and 500 cells per slide were scored to quantify apoptotic cells. The slides were viewed under a fluorescence microscope. Cells with three or more chromatin fragments were considered apoptotic.

Detection of ROS by flow cytometric analysis

Intracellular levels of H_2O_2 were analyzed by flow cytometry using diachlorofluorescein diacetate (DCFH-DA) as a specific fluorescent dye probe [36]. Cells were incubated

with DCFH-DA at a final concentration of 5 mM in M199 medium for 15 min at 37°C, washed twice and placed with M199 medium with or without reagents or serum-deprived medium for the indicated periods of time. Finally, cells were washed with HBSS, incubated with trypsin, and neutralized with trypsin neutralizing solution (Clonetics, Walkersville, MD). Cells were analyzed by FACScan (Becton Dickinson, USA) with excitation and emission wave lengths of 475 and 525 nm, respectively. For each analysis 5000 cells were recorded.

Determination of sphingolipid biosynthesis employing [^{14}C]palmitate incorporation

A confluent culture of cells was incubated for 24 h in medium containing lipoprotein-deficient serum (1 mg/mL) and [^{14}C]palmitate (2 μCi/mL). Next, the medium was removed and cells were washed twice in the medium to remove nonspecifically bound radioactivity. Fresh medium with and without TNF-α (10 ng/mL) was added and at 0, 2.5, 5, 15, and 60 min cells were extracted twice with 5 mL of hexane-isopropanol (3:2, v/v) for 5 min at room temperature. The total lipid extract was dried in N_2 atmosphere. One mL 1 N NaOH was added to the dishes, and the extracts used for the determination of protein employing bovine serum albumin as a standard. The total lipid extracts were solubilized in chloroform and subject to silicic acid column chromatographic separation as described [37]. Briefly, the neutral lipids, glycolipids, and phospholipids were eluted from the column with chloroform, acetone:methanol (9:1, v/v), and methanol, respectively. Next, the total neutral lipids, glycolipids, and phospholipids were separated into individual molecular species by HPTLC employing the following solvents: heptane-ethylether-acetic acid (85:15:1, v/v), chloroform-methanol-water (100:42:6, v/v) and chloroform-methanol-acetic acid (65:25:4, v/v). The HPTLC plates were calibrated with known lipid standards. Following development, the plates were air dried and exposed to iodine vapors. The gel area corresponding to standard lipids was scraped and radioactivity measured by scintillation spectrometry. The data was expressed as [^{14}C]palmitate incorporated into individual molecular species of lipid/mg protein.

Detection of mitochondrial cytochrome c release

Following incubation with agonists, cells were washed, harvested in ice-cold phosphate-buffered saline, and suspended in 100 μl of extraction buffer containing 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 0.25 mM sucrose. Cytosol was prepared, and the level of cytosolic cytochrome c was determined by Western blotting using a monoclonal anti-cytochrome c antibody (Santa Cruz Biotechnology, Santa Cruz, CA)

Caspase-3, p47-phox and p92-phox immunohistochemical staining

Proteins in cell lysates were size-fractionated by SDS-PAGE and electro blotted onto nitrocellulose membranes (Amersham Life Science Inc., Arlington Heights, IL). Prior to immunoblotting, non-specific binding of antibodies to membranes was blocked by overnight incubation in 5% skimmed milk, 1% casein, 0.05% Tween-20. Membranes were probed with 10C6 or 11B4 anti-caspase-3 mAbs (Santa Cruz Biotechnology, Santa Cruz, CA) (1 μg/mL), anti-p92-phox (Santa Cruz Biotechnology, Santa Cruz, CA) (5 μg/mL), and anti-p47-phox (Santa Cruz Biotechnology, Santa Cruz, CA) (5 μg/mL) followed by goat anti-rat IgG antibodies conjugated to HRP (Southern Biotechnology, Birmingham, AL) and detection by enhanced chemiluminescence (ECL; Amersham International, Little Chalfont, Buckinghamshire, England).

Incorporation of [^{3}H]LacCer in cells

The incorporation of [^{3}H]LacCer into osteoblastic cells was carried out exactly as described by Sonderfeld *et al*. [38]. Briefly, a confluent monolayer of osteoblastic cells was incubated in minimum essential medium containing 0.3% heat-inactivated fetal bovine serum and 200 nmol [^{3}H]LacCer (specific activity 75 Ci/mol). At the indicated time intervals, medium was removed and the monolayers were washed three times with ice cold PBS. Total radioactivity in the washed cell pellets was measured by scintillation spectrometry using Aquasol-II as the scinttilation fluid (New England Nucelar-Dupont, Boston, MA). Next the cells were harvested following trypsinization (0.25% trypsin for 15 min at 37°C and centrifuged (1,000 × g 5 min, 4°C). The cell pellets were washed thrice with PBS, centrifuged, and radioactivity associated with the cells was measured by scintillation spectrometry.

Results

N-SMase activity is essential for TNF-α induced apoptosis

In the mutant cell lines, TNF-α did not alter apoptosis (Fig. 1A) and ceramide generation (Fig. 1C). In sharp contrast, in WT cells TNF-α markedly increased apoptosis (13-fold), and a 3.5-fold increase in ceramide levels; and this was highly statistically significant ($p < 0.01$) (Fig. 1D).

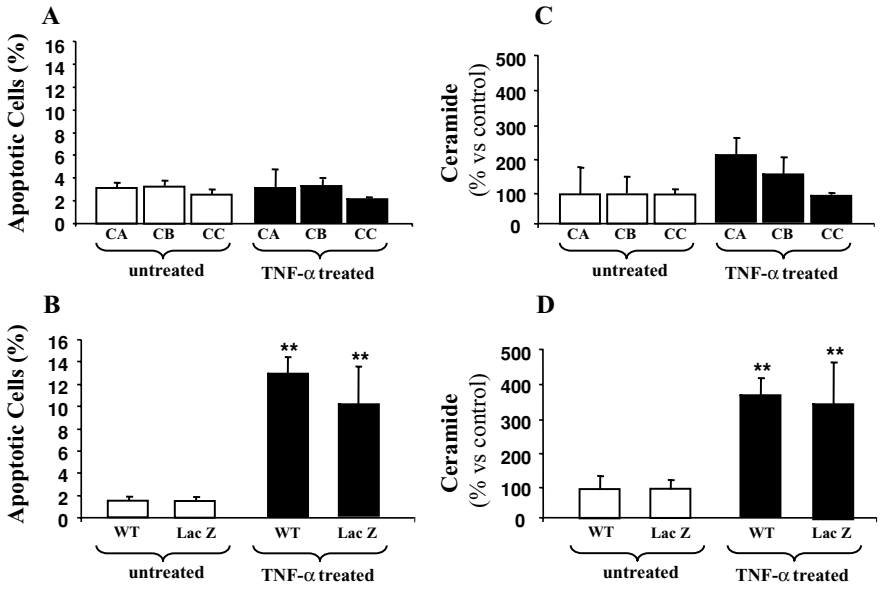

Fig. 1 Mutant cells are resistant to TNF-α-induced ceramide production and apoptosis, but not the human osteosarcoma WT cell line. Mutant (A,C) and WT (B,D) osteosarcoma cells were cultured in serum-free media in the absence (open bars) or in the presence of 25 ng/mL TNF-α (closed bars) for 24 h. Ceramide production and apoptosis were determined by diacylglycerol kinase assay and DAPI respectively (see Material and Methods). At least three independent experiments (three determinations each) were carried out. (**) Significant differences, $p < 0.01$

TNF-α does not stimulate LacCer biosynthesis in mutant cells

The basal incorporation of [^{14}C]palmitate into ceramide in WT cells and the mutant cell line was identical (Fig. 2A). However, in WT cells upon the addition of TNF-α, there was a time-dependent increase in the radioactivity incorporated into ceramide which increased to 2.5-fold in 10 min and returning to baseline value in 30–60 min (Fig. 2A). In contrast, TNF-α did not significantly alter the radioactivity incorporated into ceramide in a mutant cell line.

As expected, in WT cells, TNF-α exerted a time-dependent decrease in the radioactivity incorporated into sphingomyelin. A 3-fold decrease was observed in 10 min.

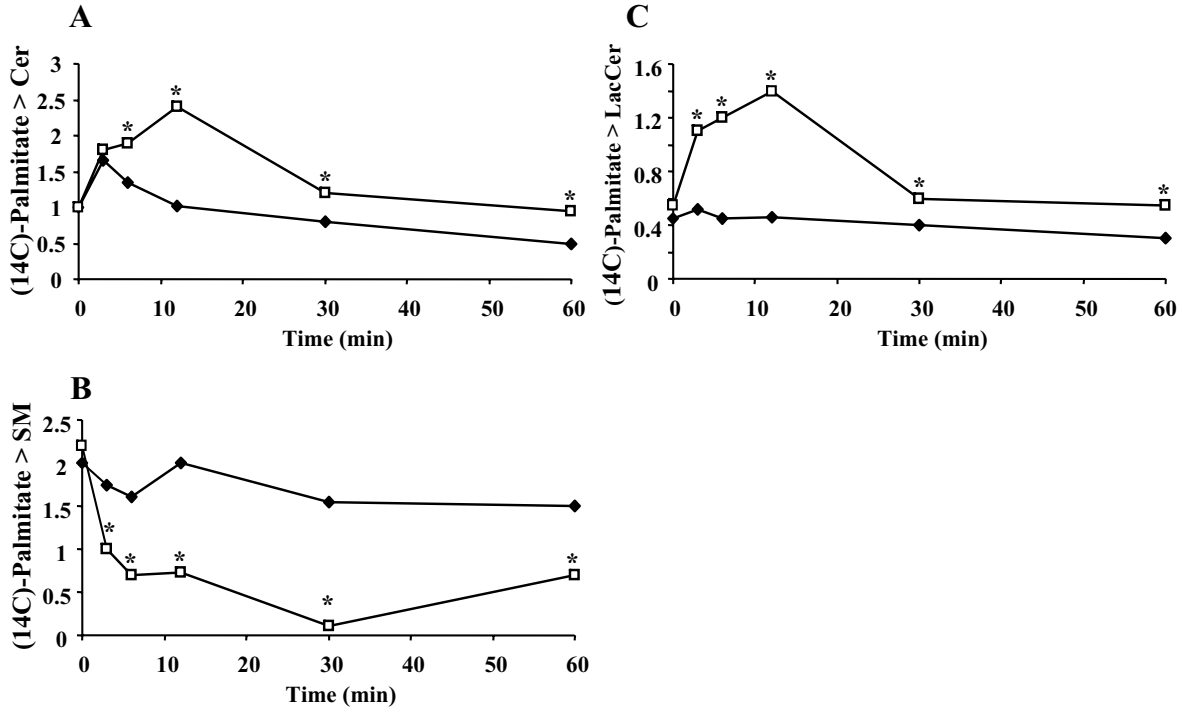

Fig. 2 The de novo biosynthesis of ceramide, sphingomyelin, and LacCer is impaired in mutant cells. Confluent culture of cells were incubated for 24 h in medium containing lipoprotein-deficient serum (1 mg/mL) and [^{14}C]palmitate (2 μCi/mL). After total lipid extraction, ceramide (A), sphingomyelin (B) and LacCer (C) in mutant (◆) and WT (□) ostesarcoma cells were we quantified (see Material and Methods). Three determinations each were carried out. SD was less than 10% in each point. (*) Significant differences, $p < 0.05$

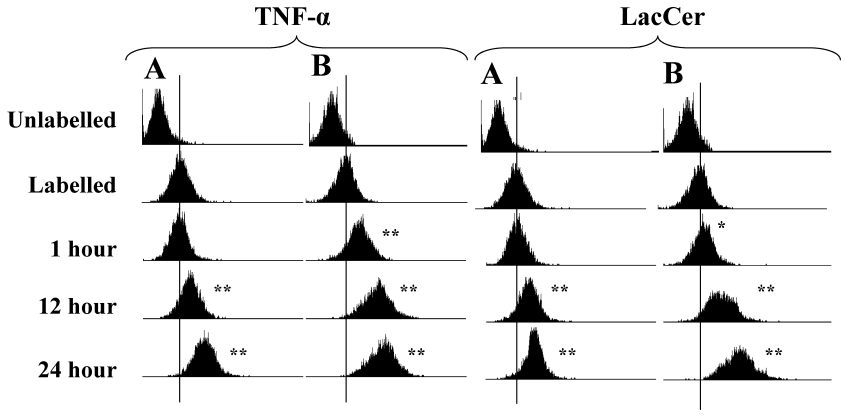

Fig. 3 TNF-α and LacCer induce ROS in WT and mutant cells in long time incubation. WT (A) and mutant (B) osteosarcoma cells were incubated with vehicle (unlabelled) or DCFH-DA (label) at a final concentration of 5 mM in M199 medium for 15 min at 37°C, washed twice and placed with M199 medium with or without TNF-α (25 ng/mL) or LacCer (20 ng/mL) for the indicated periods of time. Finally, cells were analyzed in FACScan as described in Material and Methods. At least three independent experiments (three determinations each) were carried out. SD was less than 10% in each point. Significant differences, (*) $p < 0.05$ and (**) $p < 0.01$

In contrast, in the mutant cell line TNF-α did not significantly alter the radioactivity incorporated into sphingomyelin (Fig. 2B).

The basal level of [^{14}C]palmitate incorporated into lactosylceramide was quite similar in the WT cells and in the mutant cells. In WT cells, TNF- α markedly increased (3-fold) the incorporation of [^{14}C]palmitate into LacCer in 10 min and then returned to baseline values (Fig. 2C). In contrast, in the mutant cells TNF-α did not alter the incorporation of [^{14}C]palmitate into LacCer throughout the incubation period up to 60 min (Fig. 2C).

Long term incubation of cells with LacCer or TNF-α increases ROS production

WT cells (Fig. 3B) but not mutant cells (Fig. 3A) began producing ROS within 1 h of incubation with TNF-α or LacCer. Howeover, at 12 h both WT cells and mutant cells generated ROS in response to incubation with TNF-α or LacCer. Clearly, WT cells (Fig. 3B) produced more ROS in the presence of TNF-α and/or LacCer in 12–24 h compared to the mutant cells (Fig. 3A).

The uptake of LacCer is not impaired in the mutant cells

Time kinetic analysis of [^{3}H]LacCer uptake revealed that both WT cells and the mutant cell line took up LacCer equally well over the time period investigated (Fig. 4) as well as up to 24 h (data not shown). Moreover, trypsin insensitive incorporation of [^{3}H]LacCer was also similar in WT and mutant cells (data not shown).

Lactosylceramide induces the apoptotic cascade

As shown in Fig. 5. LacCer induced the release of cytocrome-c, activated caspase-3, and increased apoptosis (5-fold) in WT cells. In the mutant cells, LacCer also activated the apoptotic cascade 2–3 fold as compared to untreated cells.

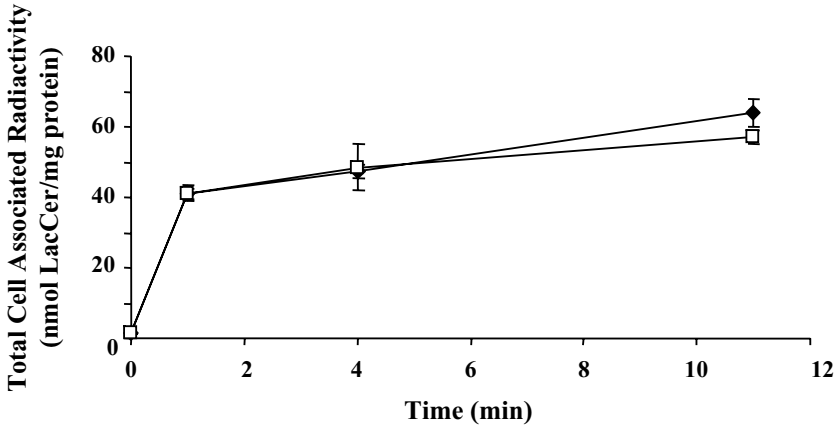

Fig. 4 Uptake of [^{3}H]LacCer is not impaired in WT and mutant cells. WT (□) and mutant (◆) osteosarcoma cells were incubated at 37°C with [^{14}C]palmitate (2 μCi/mL). At the indicated time periods, total cell-associated radioactivity was measured by scintillation spectrometry as was described in Material and Methods. Data represent mean value ±SD

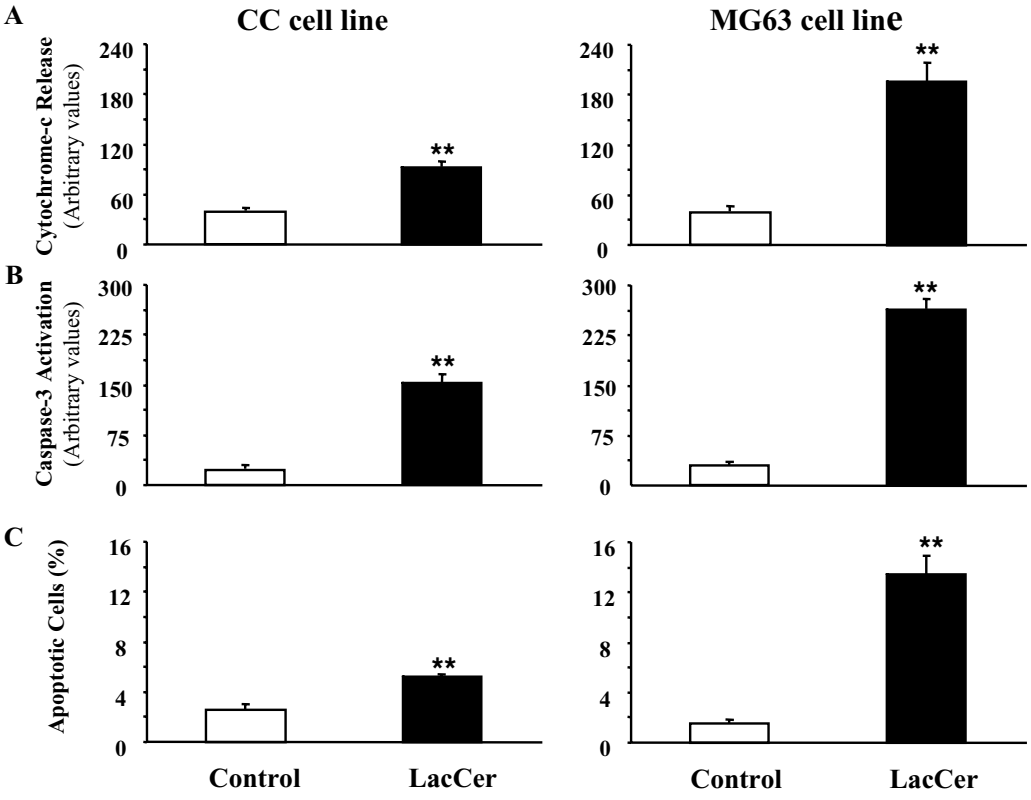

Fig. 5 LacCer induces cytochrome c release, caspase-3 activation and apoptosis in WT and mutant cells. A. Cytochrome c release was determined by western immunoblot assay. After incubation for 6 h at 37°C with serum free minimum essential medium and 20 ng/mL LacCer, these cells were processed as described in Material and Methods. B. Caspase-3 activation also was measured by western blot as described in Material and Methods. C. Apoptosis was measured by DAPI. Cells were grown on glass cover slips and incubated for 6 h with LacCer (20 ng/mL). Cells were fixed, stained with DAPI and photographed (X 50). At least three independent experiments (three determinations each) were carried out. Data represent mean value ±SD. Significant differences, (**) $p < 0.01$

Preincubation of cells with D-PDMP abrogates TNF-α induced apoptosis and this is bypassed by LacCer, but not by ceramide

As show in Fig. 6A, the mutant cell line was insensitive to TNF-α induced apoptosis. However, an exogenous supply of Ceramide or LacCer did induce apoptosis in these cells. The WT cells were prone to apoptosis when treated with TNF-α, ceramide or LacCer (Fig. 6B).

Quite remarkably, when the mutant cells were preincubated with D-PDMP and then treated with ceramide, apoptosis was compromised (Fig. 6A). This may suggest that

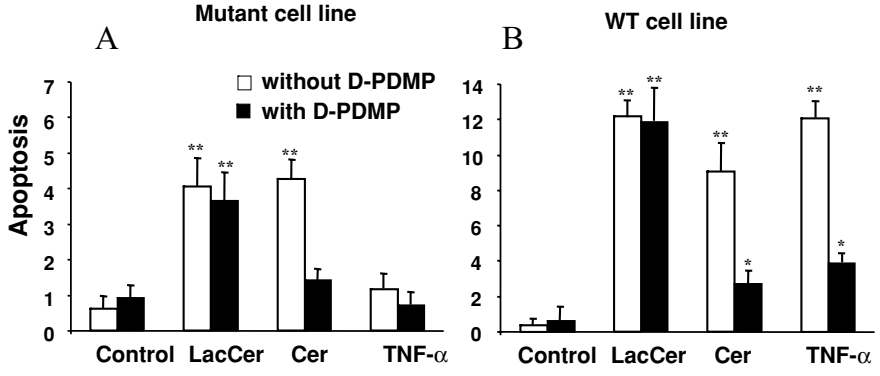

Fig. 6 LacCer is critical for inducing apoptosis in WT and mutant cells. Cells were grown on glass cover slips and incubated with vehicle, LacCer (20 ng/mL for 6 h), ceramide (20 ng/mL) and TNF-α (25 ng/mL for 24 hr) in the absence or presence of D-PDMP (15 μg/mL). Cells were fixed, stained with DAPI and photographed (X 50). At least three independent experiments (three determinations each) were carried out. Data represent mean value ±SD. Significant differences, (*) $p > 0.05$, (**) $p > 0.01$

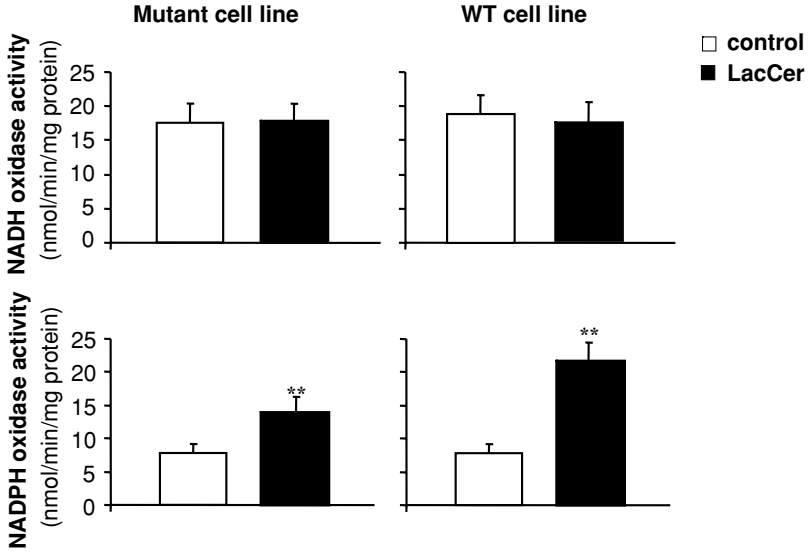

Fig. 7 NADPH oxidase is critical in LacCer induced apoptosis, but not NADH oxidase in mutant and WT cells. Mutant and WT osteosarcoma cells were harvested and suspended in balanced salt solution. After incubation for 0 min (□) or 15 min (■) at 37°C with serum-free minimum essential medium and 20 ng/mL LacCer, these intact cell suspensions were incubated in the presence of 50 mM NADH (for NADH oxidase activity) or 50 mM NADPH (for NADPH oxidase) in a 96-well black microtiter plate (Packard) for the measurement of superoxide generation by the lucigenin chemiluminescence method (see Material and Methods). Data represent mean value ±SD. Significant differences, (**) $p < 0.01$

the conversion/glycosylation of ceramide to LacCer is required to induce apoptosis. In contrast, treatment of cells with LacCer irrespective of exposure to D-PDMP induced apoptosis.

The WT cells were highly sensitive to TNF-α-induced apoptosis, and this was also abrogated by preincubation of cells with D-PDMP and bypassed by LacCer but not by ceramide (Fig. 6B).

NADPH oxidase was increased by LacCer, but NADH oxidase was not in both cell lines

NADH and NADPH oxidase were similar in both cell lines (Fig. 7). These redox activities presented an increment when cell lines were incubated with 20 μM LacCer in growth medium: While NADH was not increased in both cell lines, NADPH oxidase presented an increment about 2–3 fold (Fig. 7).

When we studied various components of NAD (P) H oxidase activity, such as p92-phox and p47-phox, we can observe a similar increment induced by LacCer (Fig. 8).

Discussion

The major findings in this paper are: i) human osteosarcoma cells (WT) are highly sensitive to TNF-α and LacCer-induced apoptosis. In contrast, a mutant cell line deficient in N-SMase activity, was resistant to TNF-α-induced apoptosis and this was bypassed by ceramide and LacCer; ii) metabolic labeling studies revealed that TNF-α transiently induced ceramide and LacCer production in the WT cells, but did not generate any newly synthesized LacCer in the mutant cell line. Also in the mutant cells, TNF-α failed to hydrolyze [^{14}C]palmitate-labeled sphingomyelin to ceramide; iii) TNF-α induced biosynthesis of LacCer, but not ceramide,

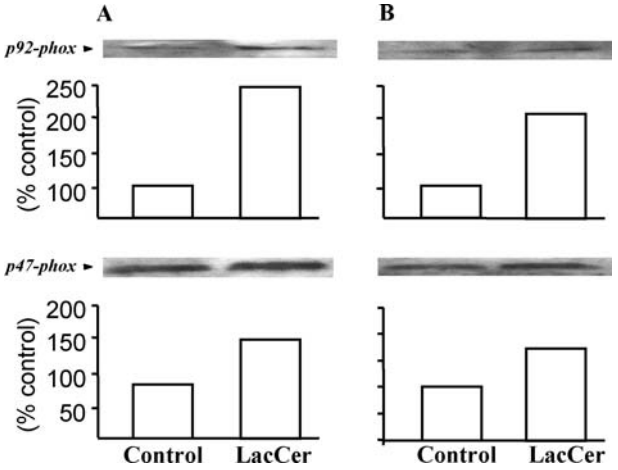

Fig. 8 p92-phox and p47-phox expressions are increased by LacCer in mutant and WT cells. After incubation for 15 min at 37°C with serum free minimum essential medium and 20 ng/mL LacCer, WT (A) and mutant (B) osteosarcoma cells were homogenated in lysis buffer and centrifuged 1 h at 50.000 × g at 4°C. The pellets (membrane fractions) were used for detection of p97-phox and p47-phox expression by western blot as described in Material and Methods

generated ROS and apoptosis in WT cells but not in mutant cells. Finally, since preincubation of cells with D-PDMP abrogated ceramide-induced apoptosis but not LacCer-induced apoptosis and since ceramide did not generate ROS but LacCer did, led us to conclude that the conversion of ceramide to LacCer is critical to induce apoptosis in these cells.

TNF-α induces apoptosis by engaging a cell surface receptor, TNF-α receptor 1 (TNFR1). Trimerization of TNFR1 by TNF-α induces the association of the receptor death domains. Cell surface expression of TNF-α receptors is necessary but not sufficient to induce a biological response, and post-receptor mechanisms are important in controlling the susceptibility to the cytotoxic action of TNF-α. The elucidation of the TNF-α signal transduction pathway is particularly challenging because of the extremely wide variety of TNF-α responses. Ceramide was reported to be an important lipid messenger in various pathways of TNF-α action [39,40]; but its direct implication in apoptosis has not been demonstrated. Several independent reports in the literature reveal that ceramide is not directly involved in inducing apoptosis in mammalian cells. First, cells overexpressing GD_3 synthase and exogenous supply of GD_3, were shown to render cells markedly sensitive to TNF-α-induced apoptosis [41]. Second, employing human aortic smooth muscle cells in our study and rat liver in another study, it was shown that GD_3 recruits ROS to induce apoptosis [42,26]. Third, fibroblasts from patients with ceramidase deficiency "Farbers disease" that results in the accumulation of large amounts of ceramide are equally as sensitive to TNF-α-induced apoptosis as normal fibroblasts (unpublished data). Fourth, treatment of osteoclasts with D-PDMP that raised the cellular level of ceramide inhibited proliferation but did not induce apoptosis [13]. This is in agreement with the findings in our work with osteosarcoma cells.

In mammalian cells, the bulk of ceramide is generated from the hydrolysis of sphingomyelin (SM) by the action N-SMase. Although ceramide has been proposed to be an intracellular mediator for many of the early cellular responses elicited by TNF-α, including apoptosis [43–46], the intracellular events connecting ceramide to these cellular responses remain unclear.

In our studies we assessed apoptosis employing multiple markers of apoptosis: cytochrome c release, caspase-3 activation, and the quantitation of apoptosis by DAPI. After a 24 h treatment we observed a significant increment of cytochrome c release, caspase-3 activation, and apoptosis in both cell lines. However, quantitatively WT cells were better responders to TNF-α/LacCer-induced apoptosis as compared to the mutant cells. Apparently, this was not due to a differential uptake of LacCer as our studies employing [^{3}H]LacCer revealed that both short term and long term kinetics of [^{3}H]LacCer uptake were similar in WT cells and the mutant cells. Moreover, the basal NAD(P)H oxidase activity p92-phox and p47-phox were also similar. In addition, LacCer induced NADPH oxidase and their enzymatic components in both cell lines.

Ceramide has multiple fates. One of them involves its glycosylation to glucosyl- and lactosylceramide. We observed that treatment with TNF-α of WT cells metabolically labeled with [^{14}C]palmitate resulted in a rapid rise in the production of ceramide and a concomitant decrease in radioactivity associated with sphingomyelin. This was also accompanied by a large increase in LacCer biosynthesis. In contrast, mutant cells failed to generate new LacCer in response to TNF-α treatment. However, the basal incorporation of [^{14}C]palmitate into ceramide and LacCer in WT cells and mutant cells was similar. This suggests that the biosynthetic pathway between ceramide and LacCer was intact and functional in these cells.

Recently, the functional role of LacCer in generating reactive oxygen species in particular superoxide via the activation of NAD(P)H oxidase has been reported [6]. In the present study we employed DCFH/flow cytometry to measure ROS production in WT cells and mutant cells treated with TNF-α and LacCer. We observed ROS production in response to TNF-α and/or LacCer treatment in both cell lines. However, this ROS production was quantitatively less and was produced later in mutant cells as compared to WT cells.

The most exciting result in our studies was the observation that D-PDMP abrogated ceramide-induced apoptosis in both the WT cells and the mutant cells. Moreover, this was bypassed by LacCer but not ceramide. D-PDMP was previously proposed to be a specific inhibitor for glucosylceramide synthase that catalyzes the transfer of glucose residues from UDP-glucose to ceramide to form glucosylceramide [47]. However, subsequently, we showed that in addition, D-PDMP also inhibits the activity of LacCer synthases directly [48]. Previously we have shown that D-PDMP can abrogate TNF-α [14] and fluid shear stress [12] induced ICAM-1, expression and cell adhesion in human endothelial cells. Moreover, D-PDMP was also shown to abrogate oxidized LDL, PDGF, and EGF-induced proliferation in human aortic smooth muscle cells [35] and recently in monocyte colony stimulating factor-induced proliferation in osteoclasts [13]. Collectively, our studies [7,12,14,42] suggest that LacCer-induced ROS generation is critical to inducing the phenotypic changes above in a variety of mammalian cells (Fig. 9). The present studies in WT cell line and mutant cell line are in accord with our proposed tenet above. In addition, our findings here extend the critical role of LacCer in inducing apoptosis and point to the suggestion that the conversion of ceramide to LacCer and ROS generation is essential for apoptosis to occur and that LacCer but not ceramide is the active compound that activates

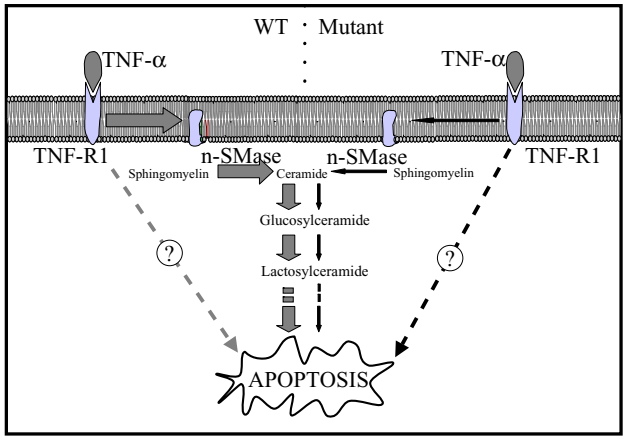

Fig. 9 Hypothetical model depicting the role of LacCer in inducing apoptosis in mutant and WT cells

an "oxygen sensitive signal transduction "cascade that leads to apoptosis.

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