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Ceramide generated by acidic sphingomyelinase contributes to tumor necrosis factor-α-mediated apoptosis in human colon HT-29 cells through glycosphingolipids formation

Possible role of ganglioside GD3

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Abstract In the present study we assessed the contribution of acidic sphingomyelinase (ASMase), a ceramide generating enzyme, in tumor necrosis factor (TNF)-mediated apoptosis in human colon HT-29 cells. TNF induced apoptosis in HT-29 cells in a time- and dose-dependent fashion. Downregulation of the active endogenous ASMase form prevented TNF-stimulated ASMase activity and apoptosis. Furthermore, inhibition of glucosylceramide synthase, which blunted TNF-stimulated GD3 levels, abolished TNF-mediated cell death. Immunocytochemical staining revealed the co-localization of GD3 with mitochondria induced by TNF. The knockdown of targeted GD3 synthase by antisense expression vector protected HT-29 cells against TNF-induced cell death. Thus, ASMase plays a key role in TNF-induced cell death in human colon epithelial cells possibly through GD3 generation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Death ligand; Apoptosis; Ceramide; Glycosphingolipid; Mitochondrion

1. Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine that induces a wide variety of cellular responses including apoptosis. The mechanisms involved in TNF-induced apoptosis are complex and involve protein–protein interactions and generation of signaling intermediates [1–4].

The TNF binding to its plasma membrane receptor-1 has been reported to generate ceramide, a sphingolipid that has been implicated in apoptosis [5,6]. Ceramide levels can increase through several mechanisms, including the de novo synthesis from L-serine and palmitoyl-CoA catalyzed by the action of serine-palmitoyl transferase and the activation of ceramide synthase [7–9]. Indeed, the latter has been reported to mediate TNF-mediated cell death in MCF7 cells [10]. On the other hand, ceramide levels can increase by hydrolysis of membrane sphingomyelin by the action of sphingomyelinases (SMases). Several SMases have been identified, although not all of them have been fully characterized. The magnesium-dependent, membrane-bound, neutral SMase (NSMase) has been linked to apoptosis mediated by chemotherapeutic agents, serum starvation, TNF, and Fas [10–12]. Acidic SMase (ASMase), which displays an optimum pH around 4.8, has been further subclassified into the endosomal/lysosomal ASMase and a secretory Zn^{2+}-dependent SMase [5–7,13–15]. Although ASMase has been shown to mediate developmental apoptosis and ionizing radiation-mediated cell death [16,17], its role in death-ligand-induced apoptosis is controversial. Decreased ceramide production by ASMase due to overexpression of acid ceramidase has been shown to protect L929 cells from TNF-induced cell death [18]; however, inhibition of ASMase early but not late during TNF signaling protected ML-1a cells against TNF [19]. Finally, hepatocytes deficient in ASMase showed resistance to Fas-mediated apoptosis [20,21], but studies using lymphocytes derived from Niemann–Pick patients with minimal ASMase have yielded conflicting results in Fas-mediated cell death [22,23].

In addition to its involvement in apoptosis signaling, ceramide also functions as a precursor for the synthesis of glycosphingolipids (GSLs). GSLs participate in the regulation of various cellular functions, including cell adhesion and modulators of signal transduction [24,25]. In particular, GD3, a sialic acid-containing GSL, has been identified as a lipid death effector due to its ability to interact and recruit mitochondria to apoptotic pathways, contributing to the mitochondrial-dependent apoptosome activation triggered by death ligands [26–31]. Increased GD3 levels have been reported to mediate Fas-mediated cell death in lymphoid cells [31,32].

Despite the emerging function of GSLs, e.g. GD3, in apoptosis, their role in TNF-mediated cell death in human colon epithelial cells has not been examined. Thus, the purpose of our work was to assess the role of ASMase in TNF-mediated

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Abbreviations: ASMase, acidic sphingomyelinase; CEA, carcinoembryogenic antigen; GSLs, glycosphingolipids; GD3, ganglioside GD3; GM1, ganglioside GM1; GM3, ganglioside GM3; GluCer, glucosylceramide; HPTLC, high performance thin layer chromatography; LactCer, lactosylceramide; NB-DNJ, *N*-butyldeoxynojirimycin; NSMase, neutral sphingomyelinase; TNF, tumor necrosis factor-α

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apoptosis in HT-29 cells and to estimate the relative contribution of GSLs in this function. In this paper, we provide evidence that the ceramide pool contributing to TNF-induced apoptosis derives from ASMase and this role is accomplished through GSLs formation, possibly through the specific generation of ganglioside GD3.

2. Materials and methods

2.1. Cell culture and experimental conditions

The human colon cancer cell line, HT-29, was purchased from the European Collection of Animal Cell Cultures (Salisbury, Wilts, UK). HT-29 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose in the presence of 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum. Cells (5×10^5 cells/ml) were seeded into six-well plates and confluent cell monolayers were then incubated for various periods of time with human recombinant TNF (14–280 ng/ml, Promega, Barcelona, Spain). In some cases, cells were pretreated with imipramine or *N*-butyldeoxynojirimycin (NB-DNJ) before exposure to TNF. Under the experimental conditions (TNF treatment in the absence or presence of imipramine or NB-DNJ) HT-29 cells did not undergo enterocytic-like differentiation as verified by the absence of stimulated release of carcinoembryogenic antigen (CEA) into the medium as described before [33].

2.2. Determination of cell death and apoptosis

Cell survival was quantitated by the MTT assay by adding MTT (20 μl of 5 mg/ml MTT in phosphate-buffered saline (PBS)) and monitoring the absorbance at 570 nm. This approach was validated by the release of LDH into the medium, expressed as percentage of total LDH (cells plus medium). Apoptotic features of cell death were determined by the staining of cells with the DNA-binding fluorochrome H33258 assessing chromatin condensation by fluorescence microscope analyses or nuclear DNA fragmentation by gel electrophoresis.

2.3. ASMase downregulation

Endogenous ASMase was depleted before treatment with TNF by incubation of HT-29 cells with imipramine (20 μM), which stimulates the proteolytic degradation of ASMase [34]. HT-29 cells were washed twice in PBS and resuspended in 1 ml of buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 1 mM phenylsulfonyl fluoride). After incubation for 15 min on ice, cells were homogenized using a Potter–Elvehjem homogenizer. Nuclei and cell debris were removed by low speed centrifugation ($800\times g$). Supernatants containing cytosolic and membrane proteins (50–60 μg) were resolved by SDS–PAGE and proteins were transferred to nitrocellulose membranes. Blots were incubated with goat anti-ASMase polyclonal IgG (1:2500), provided by Dr. Sandhoff (Universität Bonn), and goat anti-IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:5000) followed by ECL-based detection (Amersham, Madrid, Spain). ASMase activity in cellular extracts was determined by monitoring [*N*-methyl-^{14}C]sphingomyelin (56.6 mCi/mmol) hydrolysis as described before [35].

2.4. Sphingolipids analyses

Cellular lipids after treatment were extracted in chloroform:methanol (1:1, v/v). Ceramide levels were determined by the diacylglycerol kinase assay in thin layer chromatography (TLC) plates developed in chloroform:acetic (9:1, v/v) or high performance liquid chromatography (HPLC) after derivatization of the sphingoid base with *O*-phthaldehyde following deacylation of ceramide as characterized previously [35]. The aqueous phase containing glycolipids was loaded in a Sep-Pak C18 cartridge and applied to high performance thin layer chromatography (HPTLC) plates, resolved and developed as described previously [28]. The levels of GSLs were calculated by densitometric analyses of HPTLC plates, compared with a standard curve generated using known amounts of glucosylceramide (GluCer), lactosylceramide (LactCer), GM1, GM3 and GD3 (Matreya, Pleasant Gap, PA, USA, 99% purity by TLC). The variability between individual experiments was below 18%. Alternatively, GD3 was detected and quantitated by immunostaining in aluminum-baked silica gel 60 HPTLC plates followed by incubation with 0.5% poly-isobutylmetacrylate in hexane and exposure to mouse anti-GD3 R24 antibody (Matreya, Pleasant Gap, PA, USA) [31]. Plates were then treated with horseradish peroxidase-conjugated goat anti-mouse IgG, and GD3 was visualized by immunoperoxidase staining.

2.5. Confocal microscopy

Cellular distribution of GD3 was accomplished by immunocytochemical staining using the mouse anti-GD3 R24 antibody (1:500) as described before [31]. The co-localization of GD3 with respect to mitochondria was assessed using human anti-mitochondrial serum (1:2000) obtained from the serum of a patient with primary biliary cirrhosis, shown to recognize the E2 epitope of the mammalian mitochondrial pyruvate dehydrogenase complex [36]. Briefly, cells were fixed for 10 min in 3.7% paraformaldehyde in 0.1 M phosphate buffer prior to permeabilization with 0.1% saponin in 0.5% bovine serum albumin/PBS buffer for 15 min. The anti-mitochondrial antibody was detected using a biotin-SP-conjugated mouse anti-human IgG followed by a streptavidin–FITC conjugate (BioSource International, Leeds, UK), whereas the anti-mouse IgG-Cy3-conjugated antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used to detect the mouse anti-GD3 R24 antibody. After final washes in PBS, cells were mounted in Mowiol. Confocal images were collected using a Leica TCS-NT laser scanning confocal microscope equipped with an argon–krypton laser and a 63× Leitz Plan-Apo objective (NA 1.4). More than 200 cells per condition at different fields were examined for GD3 and mitochondrial co-localization.

2.6. Stable transfection of HT-29 cells

Stably transfected HT-29 cells were generated using the mammalian expression vector pCR3.1 (Invitrogen) containing the antisense GD3 synthase cDNA, a gift of Drs. Yu and Zeng (Medical College of Georgia) described previously [37], in the presence of Lipofectamine2000 (Invitrogen). Isolation of single clones was accomplished by selection adding geneticin (Invitrogen) to the culture medium at 500 μg/ml. After 3 weeks, 15–20 geneticin-resistant clones were pooled and expanded. Plasmid integration was tested by PCR using total DNA isolated with DNAzol reagent (Invitrogen) from transfected cells. cDNA inserts were visualized by PCR using specific primers for the pCR3.1 vector (5′-GGGAAGAGCCTGTGGTATGA-3′) and GD3 synthase cDNA (5′-CACCTTCCAGGGTCAAGGAA-3′, GenBank™ accession number U53883). PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. GD3 synthase activity was determined using CMP-[^{3}H]-*N*-acetylneuraminic acid (New England Nuclear, Boston, MA, USA, 19.7 mCi/mmol) as described before in detail [37]. Glycolipid products were separated by Sephadex G50 gel filtration and their radioactivities measured by liquid scintillation counting.

2.7. Statistical analyses

Results are expressed as the mean ± S.D. and are averages of three to five values per experiment. Statistical analyses of mean values for multiple comparisons were made by one-way analysis of variance.

3. Results and discussion

3.1. Downregulation of ASMase protects HT-29 cells against TNF-mediated apoptosis

In order to assess the specific role of ASMase, a ceramide generating enzyme of relevance in TNF signaling [5,6], in TNF-induced cell death in HT-29 cells, we first characterized their susceptibility to TNF exposure. As shown, TNF induced a dose-dependent loss of viability that increased over time (Fig. 1A). At the lower dose of TNF tested (14 ng/ml) apoptosis became significant after more than 20 h of incubation (Fig. 1A). To confirm the incidence of apoptosis, we monitored chromatin morphology by fluorescence microscopy. As seen, TNF disrupted chromating integrity, an indication of apoptotic cell death (Fig. 1B). The magnitude of ceramide generation by TNF was dependent on the dose used (264 ± 21% and 442 ± 48% at 14 ng/ml and 280 ng/ml, respec-

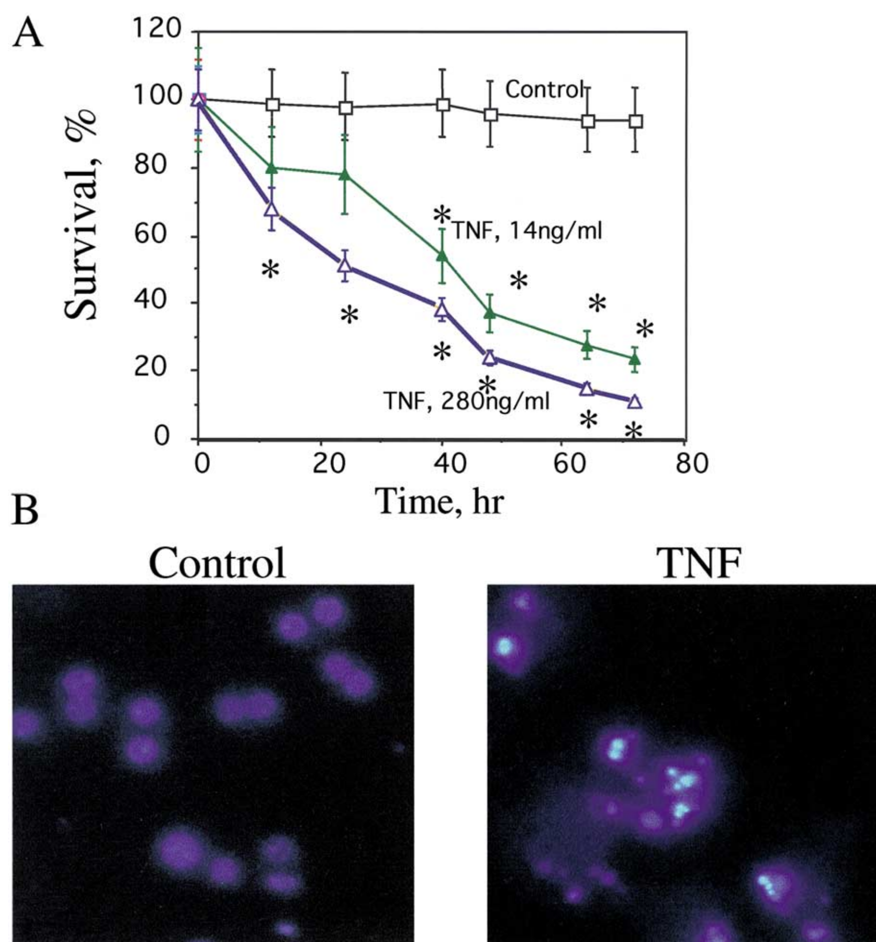

Fig. 1. Susceptibility of HT-29 cells to TNF. HT-29 cells were treated with TNF at the dose indicated for different time periods. Cells were then examined for viability by the MTT assay. Results are the mean ± S.D. of five independent experiments. $P < 0.05$ vs. control (A). Parallel samples of HT-29 cells after TNF (280 ng/ml) exposure were stained with DNA-binding fluorochrome H33258 to assess chromatin integrity by fluorescence microscopy, examining at least 5–10 random fields per condition. Representative images of control and TNF-exposed HT-29 cells are shown (B).

tively). This increase occurred between 1 and 2 h post-incubation, preceding the onset of cell death by TNF. Furthermore, TNF activated both NSMase and ASMase within 20 min after TNF exposure (not shown).

The next step was to evaluate the role of ASMase in TNF-mediated apoptosis. To this end endogenous ASMase was downregulated prior to TNF exposure. Imipramine, a tricyclic anti-depressant, has been described to induce the proteolytic degradation of the active ASMase form [34]. Although TNF increased the level of the active ASMase, HT-29 cells pretreated with imipramine exhibited lowered levels of ASMase, as revealed by Western blot (Fig. 2A). This effect of imipramine which was accompanied by decreased basal activity suppressed the stimulation of ASMase induced by TNF (Fig. 2B). Imipramine treatment, however, did not affect the TNF-stimulated activity of NSMase (Fig. 2C). Despite that imipramine preincubation prevented the stimulation of ASMase by TNF, this approach did not abolish the increase of ceramide induced by TNF (Fig. 2D). Finally, the role of imipramine preincubation was examined on the survival of HT-29 following TNF treatment. As shown, imipramine pretreatment abolished TNF-induced loss of cell viability (Fig. 2E). Under the present conditions, TNF, however, did not induce differentiation of HT-29 as verified by the lack of CEA release, findings that are in line with previous observations [33]. Thus, taken together, these data indicate that

Table 1
GSLs generation in HT-29 cells and HT-29 clones stably transfected with antisense GD3 synthase

	TNF	NB-DNJ+TNF	As-GD3	As-GD3+TNF
LactCer	187 ± 24*	37 ± 12**	83 ± 14	151 ± 14#
GM3	258 ± 31*	42 ± 17**	148 ± 21*	231 ± 18#
GM1	162 ± 21*	67 ± 14**	95 ± 12	147 ± 17#
GD3	267 ± 37*	81 ± 13**	32 ± 8*	57 ± 12*

HT-29 cells and clones expressing antisense GD3 synthase (As-GD3) were incubated with TNF (280 ng/ml) for 12 h and the pattern of GSLs was quantitated by densitometric analyses of HPTLC plates. Results are expressed as percentage of values seen in control HT-29 cells in the absence of TNF exposure (mean ± S.D., four independent determinations) (0.7 ± 0.04; 0.3 ± 0.02; 1.8 ± 0.2; 2.2 ± 0.3 nmol/mg protein, for LactCer, GM3, GM1 and GD3, respectively). *$P < 0.05$ vs. control; **$P < 0.05$ vs. TNF alone; #$P < 0.05$ vs. As-GD3.

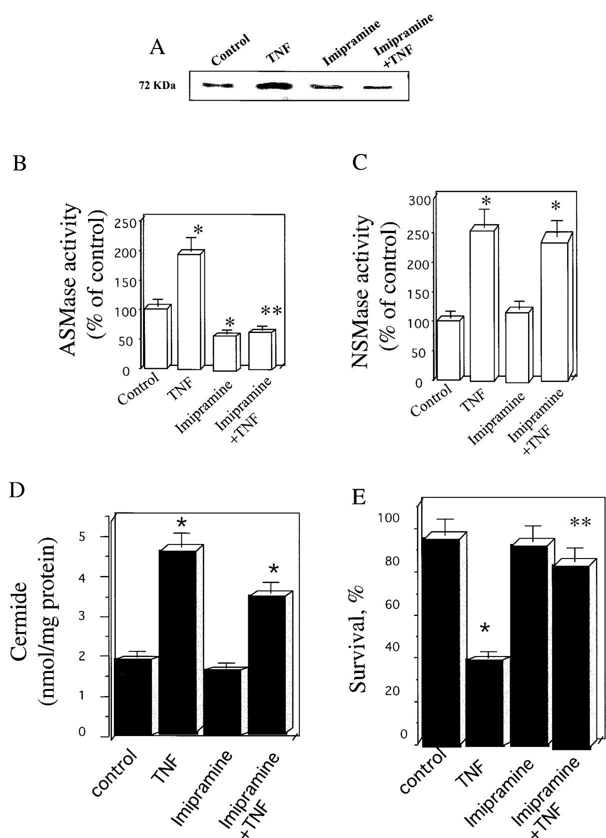

Fig. 2. Involvement of ASMase in TNF-mediated cell death. HT-29 cells were treated with TNF (280 ng/ml) for 12 h in the absence or presence of imipramine (20 μM). Cell extracts were isolated and resolved by SDS–PAGE followed by incubation with antiserum anti-ASMase (A). Parallel cell extract aliquots were used for ASMase (B) or NSMase (C) activity determined from [*N*-methyl-^{14}C]sphingomyelin (56.6 mCi/mmol) hydrolysis. Cells were then treated with chloroform:methanol and lipid extracts used for ceramide determination by HPLC (D). Survival of HT-29 cells after TNF (280 ng/ml) for 20 h with or without imipramine treatment was determined by MTT assay (E). Results are the mean ± S.D. of five independent experiments. *$P<0.05$ vs. control; **$P<0.05$ vs. TNF.

ASMase contributes to TNF-mediated apoptosis in HT-29 cells and that this effect is not due to the downregulation of ceramide levels. Since TNF generates distinct ceramide pools through activation of NSMase as well as ASMase, these data argue for a specific role of ceramide generated by ASMase in the TNF-mediated apoptosis.

3.2. *Inhibition of GluCer synthase prevents the susceptibility of HT-29 cells to TNF*

The preceding data indicate that ASMase is required for efficient TNF-α-mediated apoptosis in HT-29 cells. Since ASMase generates ceramide which, in turn, fuels GSLs formation, we next estimated the contribution of GSLs in this event. To address this issue, the generation of GSLs by TNF-α was determined with or without inhibition of GluCer synthase, a Golgi resident enzyme that forms GluCer, the precursor of the family of GSLs. GSLs formation, including the globoseries GSLs GluCer and LactCer, as well as gangliosides GM1, GM3 and GD3, was analyzed by HPTLC (Fig. 3A). TNF stimulated the levels of LactCer, GM1, GM3 and GD3 above control values (Table 1). NB-DNJ, a specific inhibitor

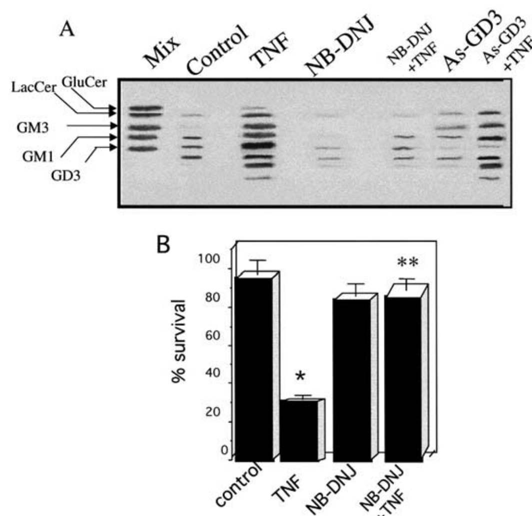

Fig. 3. Pattern of GSLs generation in response to TNF. A: Control HT-29 cells or HT-29 clones transfected with antisense GD3 synthase expression vectors (As-GD3) were treated with TNF (280 ng/ml) for 12 h in the absence or presence of NB-DNJ (30 μM). Cellular extracts were treated with chloroform:methanol for total lipid isolation and GSLs were resolved and identified by comparison with authentic standards as shown. B: HT-29 cells were treated with TNF (280 ng/ml) for 20 h in the presence or absence of NB-DNJ and cell survival determined by MTT assay. Results are the mean ± S.D. of five independent experiments. *$P<0.05$ vs. control; **$P<0.05$ vs. TNF.

of GluCer synthase, decreased the basal levels of GSLs (Fig. 3A), in agreement with previous in vivo findings [38]. Of greater relevance was the fact that NB-DNJ pretreatment prevented the TNF-stimulated generation of these GSLs (Table 1). The ability of NB-DNJ to downregulate GSLs, and to block the TNF enhancement of various GSLs is consistent with its mode of action inhibiting the first step in the stepwise addition of monosaccharides on to ceramide [38]. Indeed, in line with these observations, Bassi et al. have recently shown

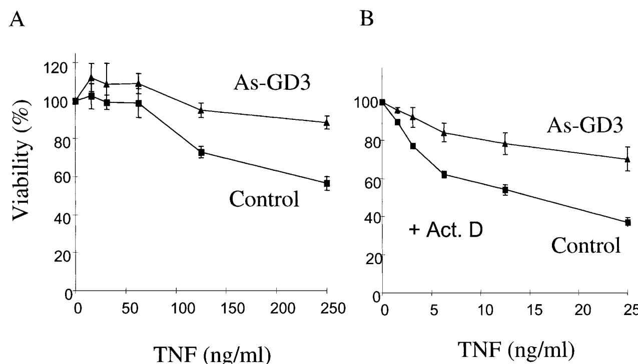

Fig. 4. Susceptibility of HT-29 cells expressing antisense GD3 synthase to TNF. HT-29 cells were transfected with mammalian expression vector pCR3.1 containing the antisense GD3 synthase cDNA (As-GD3) or empty vector alone (control). Selection of positive clones was determined by geneticin-resistant cells and insert verification was determined by PCR as described in Section 2. Control and As-GD3 cells were then treated with TNF in the absence (A) or presence of actinomycin D (2 ng/ml) (B) for 20 h and cell survival was determined by MTT assay. Please note the difference in scale of TNF in panels A and B. Results are the mean ± S.D. of five independent experiments. *$P<0.05$ vs. control.

that preincubation of C6 glioma cells with NB-DNJ diminishes the levels of ganglioside GM3 [39]. In order to address the question of whether GSLs are instrumental for efficient TNF-mediated apoptosis, we assessed the effect of NB-DNJ preincubation on the susceptibility of HT-29 cells to TNF-induced cytotoxicity. As shown, NB-DNJ protected HT-29 cells against TNF exposure (Fig. 3B). Since TNF enhanced the levels of various GSLs this outcome obtained upon blocking GluCer synthase clearly indicates the involvement of GSLs in TNF-mediated apoptosis. However, this approach does not allow us to incriminate a specific GSLs species as a key intermediate contributing to TNF-induced apoptosis in HT-29 cells.

3.3. Depletion of GD3 synthase prevents TNF-mediated cell death

Because of the limitation in the interpretation of the preceding findings and due to the putative role of GD3 as an emerging apoptotic lipid effector, we examined the specific role of GD3 in TNF-mediated apoptosis in HT-29 cells. To accomplish this aim we focused on GD3 synthase, the enzyme responsible for the synthesis of GD3 from its precursor GM3. GD3 synthase was downregulated by transfection with antisense expression vectors before exposure to TNF. As expected there was a significant downregulation of GD3 synthase activity by 45 ± 7% ($P < 0.05$), an approach that resulted in the depletion of GD3 levels in HT-29 clones expressing the antisense GD3 synthase (Table 1). This effect on GD3 was accompanied by enhanced GM3 levels (Table 1), consistent with the formation of GD3 from GM3 upon the addition of a second sialic acid residue. Indeed, these findings with HT-29 cells are in agreement with previous findings in F-11 cells transfected with antisense GD3 synthase [37]. Of broader significance was the fact that under these circumstances TNF failed to enhance the levels of GD3 in cells transfected with antisense GD3 synthase, yet, as expected TNF did increase the levels of LactCer and gangliosides GM1 and GM3 (Table 1). Finally, we next examined the susceptibility of the clones expressing the antisense GD3 synthase towards TNF-induced apoptosis. Compared with controls HT-29 cells transfected with vector alone, HT-29 clones expressing antisense GD3 synthase displayed a marked resistance to TNF-mediated ap-

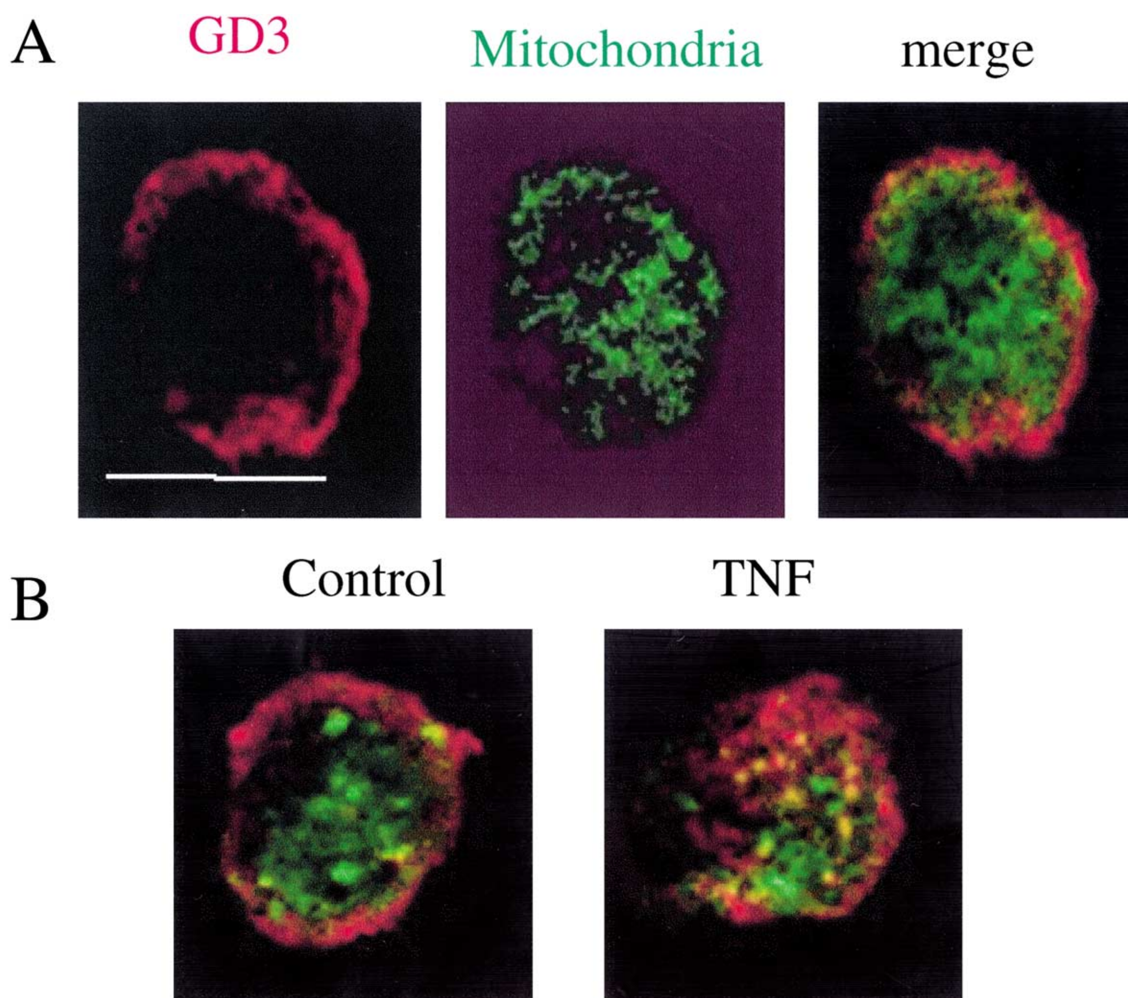

Fig. 5. Cellular distribution of GD3 by TNF. A: HT-29 cells were immunostained with antibodies anti-GD3 and anti-mitochondrial, followed by appropriate secondary antibodies. Merged images of red (GD3) and green (mitochondria) fluorescence were collected in a confocal microscope. B: HT-29 cells were treated with TNF (280 ng/ml), for 8 h and then cells were fixed and immunostained for GD3 and mitochondria localization. At least 100 cells per condition were examined. Less than 10% of control cells displayed merged fluorescence, while those exhibiting co-localization by TNF were observed in more than 85% of the cells examined. Representative images of four independent experiments showing similar results. Bar is 10 μm.

optosis (Fig. 4A). Furthermore, the loss of susceptibility towards TNF was seen after initial sensitization by actinomycin D as well (Fig. 4B), further supporting the protection of GD3 synthase downregulation against TNF-induced cytotoxicity. Although these data pinpoint the specific role of GD3 depletion in apoptosis signaling by TNF, the putative role of other enzymes involved in the metabolism of GM3 remains to be addressed. These findings, however, suggest a role for GD3 as a putative apoptotic effector contributing to TNF-induced apoptosis. Indeed, our findings are in line with previous studies reporting that the downregulation of GD3 synthase protects different cell types against apoptotic stimuli such as Fas or β-amyloid [31,40].

3.4. Redistribution and mitochondrial co-localization of GD3 induced by TNF

Previous studies have shown that GD3 activates the mitochondrial-dependent apoptosome through sequential mitochondrial permeability transition, cytochrome *c* release and caspase activation [26–31]. While most of the support for this novel function of GD3 has derived from in vitro studies with isolated mitochondria, recent data showed that GD3 undergoes an intracellular redistribution to intracellular compartments in CEM cells after Fas exposure [32]. Indeed, the physical interaction of GD3 with mitochondria has been recently shown in CEM cells following ceramide incubation [29]. Accordingly, we next examined whether GD3 co-localized with mitochondria in response to TNF. HT-29 cells were immunostained with primary antibodies anti-GD3 and anti-mitochondria and observed by laser confocal microscopy. In control, HT-29 cells, most of GD3 was localized in the plasma membrane without evidence of co-localization of GD3 with mitochondria (Fig. 5A). However, in response to TNF, GD3 underwent a redistribution from the cell surface to intracellular compartments including mitochondria, as revealed by the merged fluorescence (Fig. 5B). The present findings in HT-29 cells are similar to our recent results with cultured rat hepatocytes [41], and indicate that the interaction of GD3 with mitochondria is not a cell specific event. These studies in hepatocytes have partially characterized the trafficking of ganglioside GD3 to mitochondria through actin cytoskeleton vesicular movement sensitive to actin-disrupting agents [41]. Indeed, we observed that pretreatment of HT-29 cells with latrunculin A, an actin-disrupting agent which depolymerized actin filaments, protected HT-29 cells against TNF-mediated cell death (not shown). Together, these findings highlight the relevance of the trafficking of GD3 to mitochondria in TNF apoptosis signaling and imply that the regulation of the trafficking of endosomal vesicles loaded with GD3 to mitochondria emerges as a novel strategy in the modulation of apoptosis.

Thus, the present study adds further support for a role of GD3 in TNF-mediated apoptosis since its specific depletion through downregulation of GD3 synthase protects HT-29 cells against TNF-induced cell death. Our work documents for the first time the functional relation between ASMase activation and GD3 generation in TNF-induced apoptosis in human colon epithelial cells. The regulation of GD3 generation and/or its intracellular trafficking may prove useful in the modulation of cell death by death ligands. However, further work will be required to further characterize the intracellular movement of GD3 as well as the transcriptional regulation of GD3 synthase, which may be of relevance in the modulation of apoptosis induced by death ligands.

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