Trafficking of Ganglioside GD3 to Mitochondria by Tumor Necrosis Factor-α

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The interaction of mitochondria with proapoptotic proteins activates apoptosis pathways. Previous findings have identified ganglioside GD3 (GD3) as an emerging apoptotic lipid intermediate that targets mitochondria in response to death signals. Using immunoelectron and laser scanning confocal microscopy, we characterize the trafficking of GD3 to mitochondria in response to tumor necrosis factor-α (TNF-α) in rat hepatocytes. In control hepatocytes, GD3 is present predominantly at the plasma membrane as well as in the endosomal/Golgi network, as verified by its colocalization with the asialoglycoprotein receptor. Following TNF-α exposure, GD3 undergoes a rapid cellular redistribution with a gradual loss from the plasma membrane before its colocalization with mitochondria. This process is mimicked by acidic sphingomyelinase and ionizing radiation but not by neutral sphingomyelinase or staurosporin. TNF-α stimulated the colocalization of GD3 with early and late endosomal markers, Rab 5 and Rab 7, whereas perturbation of plasma membrane cholesterol or actin cytoskeleton prevented the trafficking of GD3 to mitochondria in response to TNF-α signaling. This occurs through actin cytoskeleton vesicular trafficking and contributes to TNF-α-mediated hepatocellular death.

Apoptosis, a genetically controlled form of cell death, fulfills an important physiological role essential for tissue development, although its dysregulation is involved in many disorders and degenerative diseases (1–3). Current evidence positions mitochondria at the central stage of the death scene due to their recruitment to the apoptotic program. In this setting, a varied repertoire of proteins, including bcl-2 family members (Bax, Bid, Bad, Bim), signaling enzymes (JNK/SAPK, protein kinase C δ), transcription factors (p53, TR3), or viral-encoded proteins (VpR), interact with mitochondria, transducing upstream stimuli to apoptosis pathways (4–8). The specific interaction of these proapoptotic proteins with mitochondrial components stimulates the release of mitochondrial factors, normally concealed in the intact organelle, that set in motion apoptosis pathways leading to the demise of the cell (3–5). The mitochondrial downstream process that activates the caspases responsible for cell death involves the interaction of cytochrome c with apaf-1, a pre-existing cytosolic component, resulting in the proteolytic activation of procaspase 9, which then triggers caspase 3 activation (9, 10). Furthermore, mitochondria can also regulate cell death by releasing several other proapoptotic factors, including caspases, the apoptosis-inducing factor, and Smac/Diablo (11–14).

In addition to the proapoptotic function of proteins, ceramide is a pleiotropic signaling lipid that regulates multiple cellular processes including apoptosis (15, 16). In this regard, it has been shown that the levels of ceramide increase before the onset of cell death (17). Moreover, it was shown that ceramide stimulated reactive oxygen species generation, mitochondrial permeability transition, and caspase activation in isolated mitochondria (18–21). Recently, it has been shown that the mitochondrial generation of ceramide by enforced mitochondrial targeting of bacterial sphingomyelinase induced apoptosis in MCF7 cells (22).

In addition to this role in apoptosis, ceramide provides the carbon backbone for the synthesis of complex glycosphingolipids within the Golgi network (23). In particular, ganglioside GD3 (GD3), a sialic acid-containing glycosphingolipid, has attracted considerable attention due to its emerging role as a cell death effector. As with ceramide, the cellular levels of GD3 increase in response to Fas or TNF-α (24, 25), whereas the down-regulation of GD3 synthase, the enzyme responsible for GD3 synthesis from its precursor ganglioside GM3, prevents Fas- or β-amyloid-induced cell death (24, 26, 27). Recent studies have indicated the ability of GD3 to reproduce the previously described effects of cell-permeable ceramide with isolated mitochondria, stimulating reactive oxygen species formation,
after 4 hr fincubation with TNF-α, staining of GD3 using anti-GD3 antibody and the corresponding secondary gold-labeled (10 nm) antibody. Similar results were observed after 4 h of incubation with TNF-α or ASMase. Representative photomicrographs of at least five independent experiments per condition performed are shown. Bar = 500 nm.

MPT, and cytochrome c release (24–26, 28, 29). Furthermore, the N-fatty acyl-sphingosine moiety, common for both ceramide and GD3, is required for the mitochondrial interacting function of sphingolipids (30). However, despite the available evidence in support of the mitochondrial-dependent apoptosome activation by GD3, the role of glycolipids in apoptosis is controversial as the inhibition of glycolipid synthesis enhances apoptosis, reversing multidrug resistance in cancer cells (31, 32).

Recent findings in CEM cells have indicated the redistribution of GD3 by Fas or ceramide, inducing the colocalization of GD3 with ezrin (33) or mitochondria (28), respectively, by an uncharacterized mechanism. Therefore, the present study was undertaken to partially characterize the dynamic redistribution of GD3 and its mitochondrial targeting during TNF-α apoptotic signaling in rat hepatocytes.

**MATERIALS AND METHODS**

**Cell Isolation, Incubations, and Antibodies—**Hepatocytes were prepared by collagenase perfusion and cultured on rat tail collagen-coated dishes as described previously (34, 35). Hepatocytes were incubated with recombinant human TNF-α (Promega, 15–280 ng/ml) or exogenous sphingomyelinases from Bacillus cereus (in phosphate-buffered saline) or human placenta (in 50% glycerol, 25 mM potassium phosphate, pH 4.5, 0.1% Triton X-100, and 0.05 mM phenylmethylsulfonyl fluoride) (Sigma) as described before (35). In some instances, cells were irradiated (4 gray) in a linear accelerator (KDS Siemens) at room temperature as described before (36). Cells were treated with d-threo-PDMP, filipin, or latrunculin A before TNF-α exposure. Mouse anti-GD3 monoclonal antibody, clone R24 (Matreya, Pleasant Gap, PA), generated as described originally (37), displays a specificity for GD3 as characterized previously by compositional, partial structural analyses, immunostaining in thin layer chromatography plates, and immunoelectron microscopy (24, 26, 37). The human anti-mitochondrial antibody, a gift from Dr. A. Serrano (Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas, Madrid, Spain), obtained from the serum of a patient with primary biliary cirrhosis, has been characterized previously and shown to recognize the E2 polypeptide of the mammalian mitochondrial pyruvate dehydrogenase complex (38, 39). Antibody anti-ASGPR was generated and characterized as described previously in our laboratory (40). Rabbit polyclonal anti-Rab5 antibody and goat polyclonal anti-Rab7 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Laser Confocal Microscopy—**Treated cultured rat hepatocytes 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. Cells were incubated for 1 h with primary antibodies, mouse anti-GD3 antibody (1:500), human anti-mitochondrial serum (1:2000), rabbit anti-Rab5 polyclonal antibody (1:100), goat anti-Rab7 polyclonal antibody (1:500), or rabbit anti-

**Immunoelectron Microscopy—**Primary hepatocyte monolayers grown on Petri dishes were fixed at room temperature in 3% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M Sorensen’s phosphate buffer for 1 h. Cells were scraped, pelleted, and collected into cold fixative. Pellets were then washed in phosphate buffer and postfixed with 1% OsO4 for 1 h at 4 °C and dehydrated with increasing concentrations of ethanol up to 80% ethanol. Resin infiltration was with LR White:ethanol (1:2 and then 2:1 at −20 °C for 1 h each) and then with undiluted LR White. For polymerization, each pellet was transferred to a gelatin capsule containing LR White for 48 h at 40 °C. Ultrathin sections were cut 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). The number of cells observed per field was at least 70–100 per condition.

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**Selective Depletion of Mitochondrial GSH—**HP was synthesized as described previously (41) and used to selectively deplete hepatocellular mGSH levels (42). Hepatocytes were incubated with 1 mM HP for 5 min and then washed and fractionated into cytosol and mitochondria by Percoll centrifugation (43). Succinate dehydrogenase and lactate dehydrogenase activities were determined in either fraction to check for cross-contamination. GSH was determined in either fraction by high performance liquid chromatography.

**TNF-α-mediated Hepatocellular Apoptosis—**The survival of HP-treated hepatocytes following TNF-α (28–280 ng/ml) exposure was quantitated by measurement of lactic dehydrogenase (LDH) in culture.
supernatants and in the remaining cell monolayer after lysis with 0.1% Triton X-100, expressing the percentage of LDH release in the medium as the fraction of total LDH (medium plus cells). 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. In some cases, hepatocytes were pretreated with cyclosporin A before exposure to TNF-α. Hydrogen peroxide and mitochondrial membrane potential were determined using dichlorofluorescein diacetate and tetramethylrhodamine methylster from Molecular Probes (Eugene, OR), respectively. Cells were incubated with the corresponding fluorescent probe, analyzing the fluorescence and side light scatter (SSC, 90° angle) in 10,000 events/test using a FACStar flow cytometer (BD PharMingen) as described previously (18, 25).

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

RESULTS

Mitochondrial Targeting of GD3—Recent findings in T cell lymphoma CEM cells reported the colocalization of GD3 with mitochondria induced by cell-permeable ceramide (26). However, the characterization of the trafficking of GD3 to mitochondria was not further pursued. To assess whether the mitochondrial targeting of GD3 occurs in response to death ligands, we monitored the mitochondrial colocalization of GD3 in rat hepatocytes following TNF-α treatment by immunoelectron microscopy using an antibody anti-GD3 whose specificity toward GD3 has been documented previously (24, 26, 37) and a gold-labeled secondary antibody. As seen in Fig. 1, TNF-α stimulated the presence of gold particles within mitochondria from hepatocytes treated with TNF-α. The stimulated trafficking of GD3 to mitochondria by TNF-α was observed after 4 h post-incubation (not shown). To follow the dynamic trafficking of GD3 to mitochondria, we monitored the distribution of GD3 in relation to different cellular markers. In basal conditions, most of GD3 localization was located at the plasma membrane and in the Golgi/endosomal region, as verified by the colocalization of GD3 with AS-GPR (44) (Fig. 2, A and B). In response to TNF-α, GD3 underwent a striking redistribution to intracellular compartments accompanied by its virtual disappearance from the plasma membrane (Fig. 2B). Furthermore, to assess the dynamic targeting of GD3 to mitochondria during TNF-α signaling, we analyzed the localization of GD3 with respect to mitochondria at different time points after TNF-α treatment. Hepatocytes...
were double immunostained using the anti-GD3 antibody and an anti-mitochondrial antibody that recognizes the E2 polypeptide of the mammalian pyruvate dehydrogenase complex (38, 39). In control hepatocytes, GD3 was predominantly located at the plasma membrane without colocalization with mitochondria (Fig. 3A). TNF-α incubation induced a time-dependent redistribution of GD3; at 30 min after TNF treatment, part of GD3 was lost from the cell surface without mitochondrial colocalization (Fig. 3B). However, by 1 h, the loss of GD3 from the plasma membrane progressed further with evident colocalization of GD3 with mitochondria as detected by the presence of yellow structures from the merged fluorescence of both channels (Fig. 3B). These processes were completed by 3 h after incubation (Fig. 3B). In comparing the redistribution of GD3 in relation to mitochondria over time, the disappearance of GD3 from the plasma membrane preceded its trafficking to mitochondria. The localization of GD3 with mitochondria by TNF-α has been detected in other cell types, such as human colon carcinoma cells HT-29, suggesting that the targeting of GD3 to mitochondria by TNF-α is not a cell type-dependent event.

Specificity of the Colocalization of GD3 with Mitochondria—To examine whether the redistribution and mitochondrial colocalization of GD3 occurs in response to other cytotoxic stimuli, we assessed the role of individual sphingomyelinases of relevance in TNF-α signaling (16, 45). Incubation of hepatocytes with the Mg2+-dependent, neutral pH optimum neutral sphingomyelinas (NSMase) from B. cereus neither altered the redistribution of GD3 from the plasma membrane nor induced its colocalization with mitochondria (Fig. 4). In contrast, the acidic pH optimum sphingomyelinas (SMase) from human placenta ASMase, first characterized by Landsmann et al. (46) and shown to enter cells by endocytosis becoming active in acidic compartment (35, 47), induced the loss of GD3 from the plasma membrane and its subsequent mitochondrial colocalization as determined by immunoelectron and confocal microcopy (Figs. 1 and 4). The mitochondrial targeting induced by the exogenous ASMase was blocked upon pretreatment of cells with monensin or mannose-6-phosphate (not shown). Furthermore, irradiated hepatocytes displayed a pattern of GD3 distribution similar to that caused by TNF-α and ASMase in which GD3 was lost from the cell surface and colocalized with mitochondria (Fig. 4). However, staurosporin failed to colocalize GD3 with mitochondria (not shown). These findings indicate that GD3 undergoes an intracellular redistribution targeting mitochondria in response to apoptosis stimuli other than TNF-α.

Trafficking of GD3 to Mitochondria by Actin-dependent Vesicles—To partially characterize whether the trafficking of GD3 stimulated by TNF-α was dependent on endocytic vesicles, we monitored the localization of GD3 with endocytic markers. TNF-α-stimulated the colocalization of GD3 with Rab 5, a plasma membrane/early endosomal marker, and Rab 7, a late endosomal marker, respectively (Fig. 5). Furthermore, since endocytic vesicles traffic from the plasma membrane to the intracellular compartment through actin filaments, we examined the effect of agents that disrupt the actin cytoskeleton organization or interfere with plasma membrane cholesterol. Pretreatment of hepatocytes with latrunculin A, an actin-disrupting agent, or filipin, a cholesterol binding antibiotic, prevented the loss of GD3 from the cell surface and its subsequent colocalization with mitochondria (Fig. 6). Moreover, pretreatment with nocodazole, a microtubule-disrupting agent, prevented the loss of GD3 from the plasma membrane and its mitochondrial targeting of GD3 induced by TNF-α. Analyses of the morphology of the actin cytoskeleton and tubulin filaments stained with antibodies anti-actin and anti-tubulin confirmed the depolymerization of actin filaments and microtubules after latrunculin A or nocodazole exposure (not shown).

To distinguish whether the disappearance of GD3 from the plasma membrane and its colocalization with mitochondria involves the trafficking of pre-existing and/or newly synthesized GD3, we examined the effect of blocking GD3 synthesis on GD3 distribution and mitochondrial targeting following TNF-α exposure. Cultured rat hepatocytes were treated with d-threo-PDMP before treatment with TNF-α to inhibit glucosylceramide synthetase, a Golgi resident enzyme responsible for the synthesis of glucosylceramide, the precursor of complex glycosphingolipids (23). Compared with the inactive enantiomer d-erythro-PDMP, d-threo-PDMP abolished the redistribution of GD3 and its colocalization with mitochondria (Fig. 6). As expected, d-threo-PDMP blocked the TNF-α-stimulated the levels of GD3 determined by high performance layer chromatography (not shown).

Mitochondrial GD3 Targeting and Hepatocellular Cell Death—Having shown that GD3 is targeted to mitochondria during TNF-α signaling, we next examined its functional consequences on the survival of hepatocytes. Since hepatocytes are resistant to TNF-α, to fully unmask the apoptotic potential of TNF-α, hepatocytes were sensitized to TNF-α by selective depletion of mitochondrial GSH with HP. HP generates a Michael acceptor within mitochondria, which is then conjugated at the expense of GSH levels (41, 42). As shown, HP induced a selective depletion of mitochondrial GSH levels with the sparing of the cytosolic pool of GSH (Fig. 7A). This approach by itself did not impair mitochondrial function, as shown by maintenance of mitochondrial membrane potential estimated from tetramethylrhodamine methylester fluorescence by flow cytometry (not shown). When HP-treated hepatocytes were challenged with TNF-α, HP-treated hepatocytes underwent a time-dependent loss of viability that was accompanied by altered chromatin integrity, indicative of apoptotic cell death (Fig. 7, B

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**FIG. 7. Regulation of TNF-α-mediated cell death.** In A, hepatocytes were pretreated with HP and then washed to remove excess HP. Cells were fractionated into cytosol, and mitochondrial and GSH levels were determined in either compartment. GSH levels were corrected by the recovery of LDH and succinate dehydrogenase in either fraction. LDH cross-contamination into succinate dehydrogenase was less than 0.5%, and succinate dehydrogenase was undetectable in cytosol fraction. In B, hepatocytes were then exposed to TNF-α (280 ng/ml) for the indicated periods of time, and cell survival was examined by LDH release. Results are the mean ± S.D. of five to seven individual experiments. *, p < 0.05 versus control; &, p < 0.05 versus TNF-α treated cells.

and C). This outcome of HP-pretreated hepatocytes exposed to TNF-α was preceded by an early generation of peroxides followed by the loss of mitochondrial membrane potential, indicating onset of MPT (not shown). The mitochondrial targeting of GD3 by TNF-α (Fig. 3) preceded the TNF-α-mediated cell death of sensitized hepatocytes. HP pretreatment of hepatocytes, however, did not perturb the relocation of GD3 to mitochondria induced by TNF-α (not shown). Cyclosporin A, an inhibitor of MPT, protected HP-treated hepatocytes to TNF-α exposure (Fig. 7D). Moreover, d-threo-PDMP, which abolished the mitochondrial targeting of GD3 and newly synthesized GD3, rescued HP-sensitized hepatocytes to TNF-α (Fig. 7D). As expected, filipin and latrunculin A, which perturb intracellular trafficking, protected HP-pretreated hepatocytes from TNF-α-mediated cell death.

**DISCUSSION**

Gangliosides are a family of sialic acid-containing glycosphingolipids that regulate multiple cellular and signal transduction pathways (48, 49). Recent evidence has identified GD3 as an emerging lipid entity involved in apoptosis activating the mitochondrial-dependent apoptosome through sequential MPT induction, cytochrome c release, and caspase activation (25, 28–30). Although most of the support for this novel function of GD3 has been derived from in vitro studies with isolated mitochondria, recent data showed the mitochondrial targeting of GD3 induced by death signals in lymphoma CEM cells (26). Since that particular report did not provide clues as to the potential mechanisms whereby GD3 targets mitochondria, the present study was undertaken to characterize the mitochondrial targeting of GD3. Our data show a dramatic redistribution of GD3 in response to TNF-α, leading to the physical interaction of GD3 with mitochondria. The relocation of GD3 in response to TNF-α involves two processes, its disappearance from the plasma membrane followed by its subsequent colocalization with mitochondria.

Gangliosides are synthesized from the precursor glucosylceramide within the Golgi network and are mainly located in the outer leaflet of plasma membranes (50). The synthesis is coupled to the exocytotic vesicle flow through the Golgi apparatus to the plasma membrane (50). We provide evidence for the colocalization of GD3 with ASGPR, indicating the predominant localization of GD3 at the plasma membrane and in the Golgi/endosomal network in resting hepatocytes. The kinetics of the redistribution of GD3 during TNF-α treatment show that the disappearance of GD3 from the plasma membrane precedes its mitochondrial colocalization, suggesting that the pool of GD3 that targets mitochondria derives from the plasma membrane. Two lines of evidence provide support for this possibility. First, the colocalization of GD3 with endosomal markers, Rab5 and Rab7, indicates that its mitochondrial targeting involves endosomal vesicle trafficking. Second, in the presence of latrunculin A, an actin-disrupting agent, GD3 remains at the cell surface, blocking the TNF-α-stimulated loss of GD3 from the plasma membrane and its trafficking to mitochondria. Our findings indicating a role for actin cytoskeleton in the targeting of GD3 to mitochondria are in line with recent observations in lymphoma CEM cells, in which Fas induced the redistribution of GD3 from the plasma membrane and its colocalization with ezrin, an actin cytoskeleton component (33). Together, these observations suggest that endosomal vesicles trafficking through actin cytoskeleton may be part of the TNF-α/Fas multicomponent signaling complex delivering death signals, e.g., GD3, to mitochondria. Although our observations favor the involvement of endosomal vesicle movement in the targeting of GD3 to mitochondria, we cannot discard at present a direct targeting of GD3 to mitochondria resulting from the continuity and contact between the Golgi/endoplasmic reticulum network with mitochondrial membranes (51, 52).

Our data indicate that the disruption of plasma membrane cholesterol by filipin prevents the redistribution of GD3 from the plasma membrane and its colocalization with mitochondria. Since the presence of cholesterol at the plasma membrane is predominantly located in specific domains corresponding to caveolae (53), these observations suggest that the internalization and the mitochondrial targeting of GD3 may originate at the caveolae. This is consistent with the colocalization of GD3 and caveolin-1 reported in shed tumor cell membrane vesicles (54) and the role of caveolae in cell signaling. Whether or not caveolae of rat hepatocytes are enriched in GD3 remains to be established. This is of particular relevance since in other cell types, e.g., MDCK II or fibroblasts, caveolin-1 did not colocalize with monosialoganglioside GM3 (55).

To examine whether the disappearance of GD3 from the plasma membrane and its colocalization with mitochondria
involves the trafficking of pre-existing and/or newly synthesized GD3, we analyzed the effect of d-threo-PDMP, an inhibitor of glucosylceramide synthase. d-threo-PDMP would be expected to block the targeting of the newly synthesized GD3 to mitochondria, leaving intact the targeting via the endosomal/actin cytoskeleton trafficking. Unexpectedly, d-threo-PDMP blocked the TNF-α-induced redistribution of GD3, preserving its predominant localization to the cell surface, indistinguishable from basal hepatocytes. The exact mechanism whereby the newly synthesized GD3 recruits the preexisting GD3 at the plasma membrane to target mitochondria is presently unknown and will require further work. However, we have observed that nodocazol, a microtubule-disrupting agent, prevented the disappearance of GD3 from the plasma membrane and its colocalization with mitochondria caused by TNF-α. It may be conceivable that the coordinated function of secretory/endothelial vesicles allows the targeting of GD3 to mitochondria. These findings predict that only apoptotic stimuli that stimulate the neosynthesis of GD3 signal the mitochondrial targeting of GD3. Indeed, in addition to TNF-α, we have observed that ionizing radiation as well as ceramide generated specifically from ASMase stimulated the loss of GD3 from the plasma membrane and its targeting to mitochondria, whereas neutral bacterial sphingomyelinase or staurosporin did not.

An interesting aspect of the present work is that although GD3 reaches mitochondria in hepatocytes in response to TNF-α, the consequences of this interaction are controlled by mitochondrial GSH levels. The selective depletion of mitochondrial GSH by HP allows GD3 to activate a cell death cascade initiated by a stimulated burst of mitochondrial reactive oxygen species formation followed by MPT, cytochrome c release, and caspase activation (25, 28, 29). Our findings indicate that mitochondria constitute a predominant destination for GD3, although other organelles may be targeted as well by GD3. Recent findings reveal the targeting of GD3 to the nuclei by amiloid peptides in PC12 cells (27). However, despite that putative targeting of GD3 with other organelles, the interaction of GD3 with mitochondria is critical in the survival of hepatocytes. Indeed, our data indicate that cyclosporin A, an inhibitor of MPT, prevents TNF-α-mediated hepatocellular cell death. Furthermore, the modulation of intracellular trafficking through actin cytoskeleton emerges as a strategy to regulate cell death pathways, and accordingly, latrunculin A rescued cell sensitized hepatocytes to TNF-α-mediated cell death. Consistent with these findings, it has been reported the antiapoptotic role of gelsolin, an actin regulatory protein (56).

In summary, the present work reports novel findings on the signaling of TNF-α in rat hepatocytes. The targeting of GD3 to mitochondria via endosomal vesicular trafficking contributes to TNF-α-mediated cell death of sensitized hepatocytes.

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