Activation of a p53-independent, Sphingolipid-mediated Cytolytic Pathway in p53-negative Mouse Fibroblast Cells Treated with N-Methyl-N-nitro-N-nitrosoguanidine*

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Sphingolipids such as ceramide are important mediators of apoptosis and growth arrest triggered by ligands such as tumor necrosis factor and Fas-L binding to their receptors. When LM (expressing p53) and LME6 (lacking p53) cells were exposed to the genotoxin N-methyl-N-nitro-N-nitrosoguanidine (MNNG), both cell lines underwent cytostasis in a very similar manner, suggesting the presence of a p53-independent apoptotic response to this genotoxic stress. To determine whether sphingolipids such as ceramide might serve as mediators in this system, the responses of these cells to exogenous sphingolipids as well as their changes in endogenous sphingolipid levels after DNA damage were examined. Treatment with exogenous C2-ceramide and sphingosine led to cell death in both LM and LME6, and treatment of the LME6 cells with MNNG resulted in a transient increase in intracellular ceramide of ~50% over a period of 3 h. Finally, treatment with the de novo inhibitor of ceramide synthesis ISP-1 protected LME6 cells from MNNG-triggered cell death. This MNNG-triggered induction of ceramide was not observed in the p53-expressing LM cells, suggesting that it may be down-regulated by p53. Although ceramide-mediated cell death can proceed in the absence of p53, exogenously added C2-ceramide increased the cellular p53 level in LM cells, suggesting that the two pathways do interact.

Sphingolipids are emerging as an important group of signaling molecules involved in the cellular responses to stress (reviewed in Refs. 1–3). Recent research on sphingolipids has shown that they are involved in signal transduction pathways that mediate cell growth, differentiation, and death. For example, sphingosine and sphingosine 1-phosphate modulate cellular calcium homeostasis and cell proliferation (4–6), and sphingosine induces cell cycle arrest and apoptosis (7–10). Other studies have shown that ceramide, the structural backbone of sphingolipids, has multiple biological activities that are predominantly growth-suppressive. For example, exposure to short-chain, membrane-permeable ceramide analogs can cause cell cycle arrest or apoptosis (11–14). A number of extra-cellular stimuli and reagents, including tumor necrosis factor, Fas ligand, interleukin-1, ionizing radiation, and chemotherapeutic drugs can cause an increase in the cellular ceramide level, resulting in either cell cycle arrest or apoptosis (15–22). In addition, ceramide can activate the stress-activated protein kinase/Jun-N-terminal kinase cascade, which is thought to play a central role in various stress responses (23–26).

One kind of stress frequently encountered by cells is DNA damage. Damage can be induced by exposure to UV- or γ-irradiation, environmental chemicals, or therapeutic reagents. Several signal transduction pathways involved in the cellular response to genotoxic stress have been identified, one of which is mediated by the tumor suppressor protein p53 (reviewed in Refs. 27–29). After genotoxic stress, the cellular level of p53 is increased (30–32). This protein then functions as a transcriptional modulator to activate or repress specific gene expression. One possible outcome is G1/S arrest, which allows repair of damaged DNA before replication, whereas a second possible outcome is apoptosis. In either case, replication of damaged DNA is prevented. The mechanism leading to this increased p53 protein level is stabilization of the protein, which is caused by changes in the phosphorylation and acetylation of p53 through the activation of several pathways, including the Jun-N-terminal kinase/stress-activated protein kinase pathways (33–36). It has been proposed that many, if not all, genotoxic response pathways converge on p53, allowing it to serve as the “universal sensor of genotoxic stress” (37). However, there are also studies indicating the presence of a p53-independent pathway in the genotoxic stress response (38).

Recently, a role for ceramide in the genotoxic response has been proposed (1, 2). For example, Santana et al. (39) showed that acid sphingomyelinase-deficient human lymphoblasts and mice were unable to generate ceramide and undergo apoptosis after exposure to ionizing radiation; these responses were restored after transfecting the cells with cDNA encoding human acid sphingomyelinase. Other reports also suggest that radiation can affect ceramide levels (24, 40, 41). However, whether this induction of ceramide is a general response to genotoxic stress or whether it is limited to ionizing or UV radiation has not been demonstrated.

N-methyl-N-nitro-N-nitrosoguanidine (MNNG), 1 a substance found in cigarette smoke, is a monofunctional alkylating agent. These chemicals target the cellular DNA and induce severe genotoxic stress to the cell that can result in chromo--

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1 The abbreviations used are: MNNG, N-methyl-N-nitro-N-nitrosoguanidine; Cer, C2-ceramide; DHCer, C2-dihydroceramide; ISP-1, myriocin; So, sphingosine; Sa, sphinganine; MEM, minimal essential medium; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography.
somalian aberrations, sister chromatid exchanges, point mutations, and cell death. Therefore, most such alkylating agents, including MNNG, are considered genotoxic mutagens and carcinogens (42). MNNG treatment also induces apoptosis in cell culture, and this process can be inhibited by the Bcl-2 protein (43). Furthermore, the Jun-N-terminal kinase/stress-activated protein kinases and p38 kinase are activated in MNNG-treated cells (44), and our laboratory has previously shown that treatment with MNNG can activate the p53 pathway (32).

Because of the ability of sphingolipids to mediate growth arrest and apoptosis and the growing evidence suggesting their possible role in responses to DNA damage, we wished to investigate whether sphingolipids such as ceramide might mediate cellular responses to DNA damage induced by genotoxins such as MNNG. We also wished to determine whether the sphingolipid- and p53-mediated pathways, both of which can lead to growth arrest and apoptosis, might interact with each other.

**EXPERIMENTAL PROCEDURES**

**Reagents—**C2-ceramide (Cer), C2-dihydroceramide (DH Cer), ISP-1 (myriocin), sphingosine (So), sphinganine (Sa), and sphingosine 1-phosphate were obtained from Biomol (Plymouth Meeting, PA) and prepared following the manufacturer’s instructions (19). Briefly, Cer was dissolved in dimethyl sulfoxide (Me2SO) as a 60 mM stock solution; So was dissolved in ethanol as a 100 mM stock solution; DH Cer was dissolved in methanol as a 15 mM stock solution; Sa was dissolved in Me2SO as a 10 mM stock solution; and sphingosine 1-phosphate was dissolved in Me2SO as a 5 mM stock solution. MNNG was obtained from Aldrich (Milwaukee, WI).

**Cell Culture and Treatment—**LM mouse fibroblast cells were cultivated in minimum essential medium (MEM) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). LME6 cells were derived from LM cells that were cotransfected with pSV2neo and the human papilloma virus 16 E6 gene, which mediates the rapid degradation of p53 through the ubiquitin pathway. They express E6 mRNA, and their level of p53 is undetectable even after the rapid degradation of p53 (Ref. 46 and references therein). They were transfected with adenovirus vectors that encode wild-type p53 (Ad5/p53) (22) or vector control (Ad5/Control) (22) and grown in 3 ml of media. After treatment, cells were harvested with 0.25% trypsin and the number of Trypan blue-excluding cells was determined using a hemacytometer.

**Apoptosis Measurement—**The CellTiter 96Aqueous One Solution cell proliferation assay (Promega, Madison, WI) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. It contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), which is bioreduced by viable cells into a colored formazan product that is soluble in tissue culture medium. It was performed according to the manufacturer’s instruction. Briefly, 1 × 10⁴ cells were seeded into a 96-well culture plate (Corning Glass, Corning, NY) and treated with the indicated concentrations of sphingolipids or genotoxins for the specified times. 20 μl of the CellTiter solution was added directly to each culture well and incubated at 37 °C for 2 h, and the absorbance at 490 nm was determined in a Bio-Rad microplate reader.

**Cytotoxicity Measurement—**The CellTiter 96Aqueous One Solution cell proliferation assay (Promega, Madison, WI) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. It contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), which is bioreduced by viable cells into a colored formazan product that is soluble in tissue culture medium. It was performed according to the manufacturer’s instruction. Briefly, 1 × 10⁴ cells were seeded into a 96-well culture plate (Corning Glass, Corning, NY) and treated with the indicated concentrations of sphingolipids or genotoxins for the specified times. 20 μl of the CellTiter solution was added directly to each culture well and incubated at 37 °C for 2 h, and the absorbance at 490 nm was determined in a Bio-Rad microplate reader.

**Measurement of Cellular Ceramide Content—**Determination and quantification of intracellular ceramide content were performed as described (22) with some modifications. 1 × 10⁴ cells were cultured in MEM (10% fetal bovine serum) with 10 μg/ml of the halphenol (Sigma) for 16–24 h before the addition of MNNG. After further incubation for the times indicated (allowing the labeled palmitate to remain in the culture medium), cells were harvested by scraping into a glass tube and pelleted by centrifugation. Lipid Cer was extracted as described (47). Briefly, CH3OH (2 ml) and CHCl3 (0.5 ml) were added to the cells, followed by H2O (3 ml), and the mixture was vortexed and centrifuged to separate the phases. After transfer of the organic phase to a clean tube, residual proteins were removed by an additional extraction with H2O, then the organic phase was transferred to a sodium sulfate column to remove residual salt. The eluate was dried and resuspended in 40 μl of chloroform:CH3OH:CHCl3 (20:1, v/v) for analysis. Half was used to determine the total counts of the labeled lipids, whereas the other half was analyzed by thin layer chromatography (TLC) (Panvera, Madison, WI) using chloroform:acetone:methanol:acetic acid:H2O (50:20:15:10:5) as the solvent system. Lipids were visualized by iodine vapor, and the radioactive spots corresponding to ceramide were scraped off and quantitated by liquid scintillation counting. The internal standard used was obtained from Matreya, Inc. (Pleasant Gap, PA).

**High Performance Liquid Chromatography (HPLC)—**Determination and quantification of intracellular sphinganine and sphingosine content was performed as described (47). Briefly, lipids were extracted as above except that the cells were not radiolabeled. The acylglycerolipids were cleaved by incubation with 0.1 M KOH in CH3OH and chloroform (2:1, v/v). The free long-chain bases were then recovered in the chloroform phase and derivatized with o-phthalaldehyde and derivatized with o-phthalaldehyde (Sigma).

The HPLC analyses were performed using an Iasco (Lincoln, NE) model 2300 pump with a Waters (Milford, MA) Nova Pak C18 column (5 μm, 3.9 × 150) and a Nova Pak C18 guard column (Waters). Sphingosine and C20-sphingosine standards (Matreya, Inc.) were prepared by dissolving them in EtOH to make 5 mM stocks. They were then serially diluted to 10 μM. 10 μl of each of the standards was derivatized with o-phthalaldehyde, and the final volume was made up to 1 ml with CH3OH, 5 mM potassium phosphate, pH 7.0 (90:10, v/v), yielding a final standard concentration of 100 μM.

**RESULTS**

**MNNG Treatment Causes Cell Death in Cells Lacking p53—**LM is a mouse fibroblast cell line that expresses p53, whereas LME6 is a cell line derived from LM cells transfected with the human papilloma virus 16 E6 protein. In contrast to the parental LM cells, LME6 cells express no detectable p53 either before or after genotoxin treatment (46). They are there-
Sphingolipid-mediated Responses to Genotoxic Damage

De Novo Synthesis Is Required for the Accumulation of Ceramide after MNNG Treatment—Ceramide can be generated by de novo synthesis or by sphingomyelin hydrolysis (3). ISP-1 is a potent inhibitor of serine palmitoyltransferase, an enzyme in the de novo biosynthetic pathway of sphingolipids.

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If the ceramide accumulation in LME6 cells after MNNG treatment were due to de novo synthesis, blockage of this pathway by the addition of ISP-1 would be expected to abrogate this induction. LME6 cells were pretreated with ISP-1 for 2 h, then treated with MNNG. As shown in Fig. 6, ISP-1 pretreatment completely eliminated the induction of ceramide by MNNG (compare with Fig. 4), indicating that the accumulated ceramide is derived primarily from de novo synthesis. Similar results were obtained with the ceramide synthase inhibitor fumonisin B1, although the results were more transitory (data not shown).

Blocking Ceramide Synthesis with ISP-1 Protects LME6 Cells from MNNG-induced Cell Death—If the de novo production of ceramide is a significant step in mediating apoptotic signals triggered by MNNG, blocking its accumulation in cells with ISP-1 should protect the cells. To test this prediction, LME6 cells were preincubated with ISP-1 for 2 h, then treated with MNNG. As shown in Fig. 7, ISP-1 pretreatment provided significant protection for the MNNG-treated LME6 cells, indicating a role for ceramide production in the transmission of genotoxic-triggered apoptotic signals in LME6 cells. This protection was most evident at 16 h post-treatment, although...
Cytotoxicity did become evident at later times. An additional dose of ISP-1 added midway through the experiment did not provide further protection of LME6 cells at 16 or 24 h, and LM cells were not protected by pretreatment with ISP-1 from cell death caused by MNNG (data not shown).

Ceramide Increases the p53 Level in LM Cells—The fact that ceramide can activate several signal transduction pathways involved in the regulation of p53 stability (including those mediated by Jun-N-terminal kinases and mitogen-activated protein kinases) suggested that the two pathways, although separable, might still interact with each other. Such interactions were also suggested by our demonstration that the LM cells differed in their ceramide response to MNNG treatment. To determine whether sphingolipids such as ceramide and sphingosine could affect the accumulation of p53, LM cells were treated with ceramide, and their cellular p53 protein levels were measured. As shown in Fig. 8, we found that the p53 protein level was increased in ceramide-treated LM cells compared with control cells and that this induction is time-dependent. On the other hand, sphingosine and sphingosine 1-phosphate did not affect the cellular p53 protein levels.

**DISCUSSION**

Because the ability of a cell to respond to genotoxic stress in an appropriate manner is critical to the health of an organism, multiple cellular pathways have developed to minimize the replication of damaged DNA. Developing a clear understanding of how these pathways interact and are regulated is crucial for understanding how cells respond to genotoxicity.

**FIG. 4.** Ceramide induction in cells after MNNG treatment. LM, LMD, and LME6 cells were treated with 10 μg/ml MNNG, and their cellular ceramide levels were measured at the times indicated. Each data point was measured in triplicate, and error bars represent the S.D. The asterisks denotes a significant increase compared with control (p ≤ 0.01, Student t test).

**FIG. 5.** Activation of the p53 pathway in LM cells after MNNG treatment. Each measurement was done in triplicate, and error bars represent the S.D. A, p53 ELISA for cells treated with 10 μg/ml MNNG for the times indicated. B, Western blot for Bax in cells treated with 10 μg/ml MNNG for the times indicated (25 μg of protein/lane). C, cell death ELISA for cells treated with 10 μg/ml MNNG for the times indicated.

**FIG. 6.** De novo synthesis is required for the accumulation of ceramide. LME6 cells were preincubated with 10 μM ISP-1 for 2 h and then treated with 10 μg/ml MNNG (added at time 0), and the cellular ceramide level was measured at the times indicated. Each point was measured in duplicate, and the error bars represent the S.D.

**FIG. 7.** Blocking ceramide synthesis protects LME6 cells from MNNG-induced cell death. LME6 cells were treated with MNNG (10 μg/ml) alone or preincubated with ISP-1 (10 μM) for 2 h, then treated with MNNG. Results were obtained using the cell death ELISA. Each data point was measured in triplicate, and error bars represent the S.D. The asterisk denotes a significant decrease in cell death (p ≤ 0.01, Student t test).

**FIG. 8.** p53 induction by sphingolipids. LM cells were treated with the indicated species of sphingolipid for 6 and 16 h, and the cellular p53 levels were measured by p53 ELISA. Each measurement was made in triplicate, and error bars represent the S.D. The asterisks denotes a p value ≤ 0.01 (Student t test). SPP, sphingosine 1-phosphate.
of the pathways involved in these responses and their relationships with each other is a rapidly growing area of current research. Numerous studies have implicated p53 as a central mediator in the cellular apoptotic response to genotoxins, leading to its description as the universal sensor of genotoxic stress (37). However, other studies have shown that apoptosis triggered by DNA damage can proceed in the absence of p53. For example, in a study conducted by Strasser et al. (38) it was shown that cycling T lymphoma cells and mitogenically activated T lymphocytes from p53−/− mice underwent apoptosis after irradiation or genotoxic drug treatment, suggesting that p53 is not the only mediator of apoptosis provoked by DNA damage. However, the mediator for this p53-independent pathway was not defined in this study. The possibility that ceramide might be involved in cellular responses to DNA damage was suggested by a study showing that acid sphingomyelinase-deficient human lymphoblasts or mice were unable to generate ceramide and undergo apoptosis upon ionizing radiation exposure (39). However, this study did not examine responses to chemical genotoxins, and no role was shown for de novo ceramide biosynthesis. In our present study, we have shown that LME6 cells, which display undetectable levels of p53, were as sensitive to MNNG treatment as the parental, p53-expressing LM cells (Fig. 1). Therefore, a p53-independent apoptotic mechanism triggered by MNNG operates in these fibroblasts as well as in the lymphocytes studied by Strasser et al. (38).

Furthermore, we have provided evidence that ceramide is an important mediator in this p53-independent pathway after MNNG treatment. First, ceramide induces apoptosis in both LM and LME6 cells. This indicates that the cellular machinery linking ceramide to apoptosis is intact and functional in this system. Secondly, we have shown that in the LME6 cells, which lack p53, treatment with the genotoxic MNNG causes an increase in the cellular level of ceramide. This response shows some specificity, as we were unable to detect increases in other sphingolipids such as sphingosine or sphinganine after MNNG treatment. Finally, we were able to demonstrate that treatment with ISP-1, an agent that blocks de novo ceramide synthesis, provided protection to LME6 cells from MNNG-induced cell death. These findings support a model in which cellular sphingolipids such as ceramide can be important mediators of genotoxin-induced cell death and underscore the need to consider the involvement of pathways other than those mediated by p53 during the induction of apoptosis.

One point to be considered is that the protection of cells after treatment with ISP-1, although significant, was not complete, and that the cells did die at later times (24 h). One possibility is that de novo synthesis of ceramide, although accounting for the majority of the apoptotic signal, may not account for the entire signal. That is, additional apoptotic signals may be generated either from the breakdown of sphingomyelin, generating ceramide, or from some other source. It should also be noted that soluble forms of ceramide, such as the C2-ceramide used in this study, although seeming to act in a manner very similar to endogenously produced ceramide, may not be completely representative of the endogenous form of the lipid.

Preincubation with ISP-1, an inhibitor of the de novo pathway for ceramide synthesis, prevented the increase in ceramide normally seen after treatment of LME6 cells with MNNG and, in fact, caused a decrease. These data indicate that the MNNG-induced accumulation of ceramide is largely a result of de novo synthesis. Although the usual mechanism of increasing intracellular ceramide levels is the activation of sphingomyelinases, as appears to be the case for the lymphocytes studied by Santana et al. (39), ceramide has also been shown to be synthesized from sphingosine de novo (reviewed in Ref. 2). For example, Xu et al. (22) show that de novo ceramide synthesis is involved in tumor necrosis factor/cycloheximide-induced cell death. Retinoic acid and daunorubicin have also been shown to stimulate de novo ceramide synthesis (55, 56).

Although the p53- and ceramide-mediated pathways are separable, they do appear to interact with each other. The p53-expressing LM and LMD cells, in contrast to the p53-deficient LME6 cells, do not increase their cellular ceramide levels in response to MNNG treatment, suggesting that operation of an intact p53 pathway down-regulates a potentially redundant ceramide pathway. Given that p53 acts at the level of gene regulation, it seems likely that activated p53 influences gene expression such as to abrogate this induction. Identification of the affected genes will be an important next step.

A second suggestion that the two pathways interact derives from the influence of ceramide on the level and stability of p53. p53 stability is known to be regulated by both its phosphorylation and acetylation patterns and its interaction with the MDM2 protein (57, 58). Several kinases are known to affect these patterns, including those in the MEKK1 (mitogen-activated protein kinase kinase kinase/Jun-N-terminal kinase) pathway (59, 60). Because p53 can be phosphorylated and stabilized by such kinases and ceramide is known to activate the stress-activated protein kinase/Jun-N-terminal kinase pathway (24, 26, 61), we investigated whether activation of the sphingolipid pathway could also increase cellular accumulation. We found that ceramide but not sphingosine did cause an increase in the cellular p53 level (Fig. 8), although each of these reagents shows a cytotoxic effect (Fig. 2). These results point to the possibility that some components in the sphingolipid and the p53 pathways may interact with the other pathway, thus allowing integration of these cellular events.

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