Mass Spectrometric Identification of Increased C16 Ceramide Levels During Apoptosis*

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A variety of molecular changes occur during the process of apoptosis. Much of the recent work has focused on changes in critical cellular proteins, proteins necessary for the initiation and continuation of the apoptotic process. Given the fact that numerous membrane changes occur throughout the apoptotic process, we initiated an investigation aimed at determining the major lipid changes that occurred during programmed cell death. When ionizing radiation was used to initiate the apoptotic process in Jurkat cells, one of the major changes that occurred within 24 h was an increase in a species with a m/z of 572 as determined by negative ion electrospray mass spectrometry. This particular mass ion displayed high performance liquid chromatography characteristics of a neutral lipid species. Further analysis by collision-induced-dissociation tandem mass spectrometry indicated only one daughter species indicative of a C1 adduct and therefore a parent mass of 537. Comparison to a commercial C16 ceramide yielded identical spectra by mass spectrometry (MS) and MS/MS analysis in the negative ion mode. Increases in C16 ceramide levels occurred 2 h after initiation of apoptosis by ionizing radiation, and its accumulation paralleled apoptosis as determined by cellular morphology. Interestingly, radiation-sensitive Jurkat cells displayed increased levels of long term C16 ceramide accumulation, whereas radiation-resistant K562 cells did not. These findings were supported by increases in caspase-3 activity in Jurkat cells, whereas caspase-3 activity in K562 cells remained unchanged. C16 ceramide accumulation and sensitivity to ionizing radiation was investigated further in a melanoma cell line. Only those cells that were radiation sensitive (approximately 70–75%) displayed increases in long term ceramide accumulation. Taken together, these results indicated a correlation between increases in C16 ceramide accumulation and radiation sensitivity. Increases in long term C16 ceramide accumulation were also seen in Fas-induced apoptosis, which occurred at time points greater than 2 h. Analysis of mitochondrial modifications using the mitochondrial probe nonyl acridine orange (NAO) indicated that initial increases in C16 ceramide levels closely paralleled the decrease in mitochondrial mass during Fas or radiation-induced apoptosis. Taken together, these results support a role for C16 ceramide in the effector (mitochondrial) phase of apoptosis.

Sphingolipids and their metabolites comprise a family of lipid molecules which include the long chain bases, sphingosine and sphinganine, and their related phosphorylated and methylated derivatives, ceramide, gangliosides, neutral glycolipids, and sulfatides (1). A great deal of interest has been generated regarding this family of molecules because of their participation in cellular signaling and proliferation and their ability to alter a variety of other cellular functions. Sphingolipids inhibit protein kinase C (as well as other protein kinases) and phosphatidic acid phosphohydrolase activities, act as regulators of Ca2+ mobilization, modulate the phosphorylation and dephosphorylation of certain gene products, and stimulate cell proliferation as well as participate in the cell death pathway (2–4). In addition, α-galactosylceramides may serve as targets for NK-T cells when presented by the major histocompatibility complex-like CD1 molecule (2–4).

Recently, much attention has focused on ceramide, which has been implicated in coordinating a cell’s response to various forms of stress. Inducers of ceramide accumulation are varied and include ionizing radiation, Fas ligands, TNF-α,1 chemotherapeutic drugs, serum withdrawal, glucocorticoids, γ-interferon, retinoic acid, heat, nerve growth factor, 1,25-dihydroxyvitamin D3, and CD28 (5–16). The accumulation of ceramide is accomplished mainly through the action of the sphingomyelin cycle, although other pathways may contribute to the increased levels. The ultimate result is the accumulation of ceramide which varies from modest increases early on in the response to dramatic increases hours into the stress-induced response. Interestingly, many of the mediators that increase ceramide levels also initiate the apoptotic cascade. Ceramide itself induces apoptosis, whereas its dihydro analogue or closely related lipids fail to do so, indicating the stereospecificity of the response (17). One potential target for ceramide in the apoptotic process is the CPP32 protease, which has been shown to be directly activated by this lipid (18, 19).

The connection between ceramide and Fas/Fas ligand and other DISC family members in apoptotic signaling is unclear. Much debate has been centered around the timing and magnitude of the ceramide response. Some studies indicate early

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1 The abbreviations used are: TNF-α, tumor necrosis factor α; HPLC, high performance liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NAO, nonyl acridine orange; MS, mass spectrometry; TIC, total ion current; Z-DEVD-AFC, benzoylcarbonyl-DEVD-aminotrifuoromethylcoumarin.
changes in ceramide levels using ionizing radiation, Fas receptor, and TNF-α. However, these changes have been modest and have not been detected in other studies. Thus, it has been suggested that ceramide per se may not participate in the early phases of the apoptotic response. Reactive oxygen species have also been shown to play an

**FIG. 1.** Negative electrospray ionization-MS of Jurkat whole cell lipid extracts identifies multiple lipid species. Cells (5 x 10⁶) were exposed to ionizing radiation (100 Gy) and cultured for 6 h at 37 °C. Irradiated and control cells were then extracted as described under “Materials and Methods.” 5 x 10⁶ cell equivalents were injected into the mass spectrometer, and spectra were summed for 1 min over the mass range of 400–950 Da/e. A, control; B, irradiated cells. PG, phosphatidylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PC + Cl, phosphatidylcholine-chloride adduct; PE, phosphatidylethanolamine.
important role in the apoptotic process, perhaps by linking the induction phase, from a variety of private pathways, to the effector phase, or so-called common pathways of the apoptotic cascade. Recently, much attention has been focused on oxygen and lipid radicals as important mediators in a number of degenerative diseases as well as in the apoptotic process (20). The biological consequences of lipid oxidation include alterations in membrane fluidity and ion potentials, association with and modification of proteins, and protein function as well as the production of toxic mediators (20).

Given the potential role of lipids and their products as regulators and/or mediators in stress-induced responses, human disease, and in the apoptotic process, a mass spectrometric approach was undertaken to specifically define the predominant molecular changes that occur within lipid moieties in response to ionizing radiation and Fas ligation. Triple quadrupole mass spectrometric technology was used and was found to be superior to currently available techniques that measure lipid products on three levels: 1) total lipid profiles were able to be analyzed without any prior purification or chemical derivatization; 2) collision-induced dissociation (fragmentation) of the phospholipids allowed direct confirmation of their structure; and 3) changes in structure were able to be assessed directly at the molecular level. Using these approaches, we have identified a C16 ceramide as the predominant ceramide that is up-regulated during the later phases of apoptosis induced by ionizing radiation and Fas ligation in multiple cell types, appearing after 2 h and at time points that parallel mitochondrial changes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments—**The following human cell lines were used for these studies: Jurkat (T-cell leukemia), K562 (chronic myelogenous leukemia), and melanoma 526. Cells were cultured in RPMI medium (Life Technologies, Inc.) supplemented with penicillin/streptomycin, L-glutamine, and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and were maintained at 37 °C in a humidified atmosphere with 5% CO2. Cell viability was assessed by trypan blue exclusion. Cells were irradiated either in complete medium or phosphate-buffered saline with 4 gray/min for 6.25–25 min in a γ-irradiator (Gamma 1000 Elite, Nordion International Inc., Ontario, Canada) and incubated for varying times up to 24 h. Lipids were extracted at the indicated time points. For experiments involving Fas-induced apoptosis, Jurkat cells were incubated with 200 ng/ml of anti-Fas antibody (Upstate Biotechnology) for varying times in RPMI medium at 37 °C. Cells were processed for lipid extraction as described above.

**Extraction of Cellular Lipids—**Cellular lipids were extracted according to previously published procedures (21). Briefly, cells were washed three times in phosphate-buffered saline, the supernatant was removed, and cells were resuspended in methanol (0.5 ml) containing butylated hydroxytoluene (BHT, 0.1 mg, Sigma). One ml of chloroform was added, and the mixture was vortexed and kept on ice, under a nitrogen atmosphere, for 1 h in the dark. Three hundred μl of 0.15 M NaCl was added, and the chloroform layer was separated by centrifugation and dried under a stream of nitrogen. Extracts were dissolved in chloroform/methanol (1:2, 2.5 × 105 cell equivalents/μl) prior to analysis by mass spectrometry.
Mass Spectrometry—Lipids were analyzed by direct infusion into a Quattro II triple quadrupole mass spectrometer (Micromass, Inc., Manchester, UK) using a sheath flow of 5 $\mu$L/min consisting of chloroform/methanol (1:2, v/v). Sample injection was $5.0 \times 10^{6}$ cell equivalents and was infused via a 75-μm inside diameter capillary using a Beckman pump. The electrospray probe was operated at a voltage differential of $-3.10$ keV (negative ion mode) or 3.5 keV (positive ion mode). Mass spectra were obtained by scanning the range of 400–950 m/z every 1.6 s and summing individual spectra. Source temperature was maintained at 70 °C. Collision-induced dissociation spectra were obtained by selecting the (M-H)-ion of interest and performing daughter ion scanning in Q3 at 500 atomic mass unit/s using 3 millitesla argon in the collision chamber. The spectrometer was operated at unit resolution. We took advantage of the ability of ceramide to form a chlorine adduct and performed quantitation on a commercial C8 ceramide over the concentration range of $1.45 \times 10^{-2}$ to $1.18 \times 10^{-12}$ mol/μl. The commercial C8 ceramide was added to the extracted cellular lipids at a concentration of $1.3 \times 10^{-13}$ mol/μl prior to mass spectrometric analysis. External standardization was also performed with the commercial C16 ceramide. Excellent linearity was shown over the concentration range of $1.15 \times 10^{-14}$ to $9.30 \times 10^{-13}$ mol/μl.

Assays for Apoptosis—Cells were assayed for apoptosis by morphology and caspase-3 activity. To assess alterations in cellular morphology, cells were applied to cytocentrifuged slides and stained with 20% Wright-Giemsa stain. The percentage of cells undergoing apoptotic death were determined based on cellular architectural characteristics which included cell shrinkage, nuclear condensation, the formation of apoptotic bodies, and nuclear condensation. Caspase-3 activity was assessed through the use of the Fluorace-Apopain Assay Kit (Bio-Rad, Inc.). Briefly, $5 \times 10^{6}$ cells were lysed by 4–5 freeze-thaw cycles in the presence of pepstatin A, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, EDTA, and dithiothreitol in CHAPS detergent. All inhibitors and detergents were purchased from Sigma. Supernatants were isolated and caspase-3 activity determined at 37 °C in the presence of the fluorescent substrate Z-DEVD-AFC at various time points using a scanning fluorimeter. The change in caspase-3 activity was measured over time at 37 °C by detection of the fluorometric product (amino-4-trifluoromethyl coumarin) released from the substrate. Analysis of DNA fragmentation was performed by agarose gel electrophoresis. Briefly, pelleted cells were lysed with 0.5% Triton X-100 in 10 mM Tris-HCl/EDTA solution, pH 7.4. Lysates were treated with RNase A (1 h) and Proteinase K (1.5 h) at 37 °C. DNA fragments in the final extracts were resolved by electrophoresis at 120 V for 1.5 h on 1.5% agarose gels impregnated with ethidium bromide and were visualized under UV light. Decreases in mitochondrial mass were assessed by nonyl acridine orange (NAO) staining. Briefly, cells ($5 \times 10^{5}$/ml) were stained with 0.1 μM NAO in RPMI media for 15 min at 37 °C. Cells were washed and immediately analyzed by flow cytometry (35).
RESULTS

Identification of the 572 Mass Ion in Irradiated Jurkat Cells—We examined the molecular lipid changes that occurred following ionizing radiation of Jurkat cells by negative ion electrospray mass spectrometry. Phospholipids were identified through collision-induced dissociation of individual mass ions and precursor ion scans and compared with MS and MS/MS profiles of phospholipid standards. The major change that was noted was an increase in intensity of the 572 mass ion which occurred within 2–24 h following irradiation (Fig. 1). Initial at-
tempts to fragment this particular mass ion in the negative ion mode yielded no evidence of fatty acid chains (not shown), typically seen with various phospholipids. This inability to fragment in the negative ion mode is characteristic of sphingomyelin or a sphingomyelin-like species. To identify this mass ion, whole cell lipid extracts from irradiated Jurkat cells were prepared 16–24 h after irradiation and subjected to high performance liquid chromatography to enrich the 572 species within the sample. Because our original fragmentation data suggested the presence of a sphingolipid-like species, a hexane/isopropyl alcohol/water solvent system was first used to remove any potential neutral lipid species. We screened each HPLC fraction for the presence of the 572 mass ion by negative ion electrospray ionization-MS (Fig. 2). The 572 mass ion eluted early on in the solvent program, supporting the presence of a neutral lipid species. Phospholipid species are normally retained on a silica gel column in this solvent system. Because it is known that noncovalent adducts can easily associate with lipids thus allowing negative ions to be formed, we again performed collision-induced dissociation on the 572 mass ion but started scanning in second set of quadrupoles (Q3) at the low end of the mass range. Under these conditions, collision-induced dissociation afforded only one daughter species at m/z 35, with an isotope mass at m/z 37, indicative of a chlorine adduct (Fig. 3). Loss of the chlorine species would indicate a parental mass of 537, well within the range of known ceramides. To confirm our findings, mass spectrometry was performed on a commercial C16 ceramide sample in both the positive and negative ionization mode. In the positive ionization mode (not shown), the C16 ceramide afforded a mass spectrum displaying the (M + H) + ion at 538, a sodium adduct (m/z 560), and a dehydro form (m/z 520), which has been recently shown by others (22). However, in the negative ionization mode, a single species with m/z 572 was evident (Fig. 4A). Upon collision-induced dissociation analysis, this species also yielded only one daughter ion (m/z 35, isotope mass at 37) as the experimental (Fig. 4B). Confirmation of a C16 ceramide was given by a comparison of negative EI spectra of the purified 572 from Jurkat cells with the commercial C16 ceramide (not shown).

**Time Course of C16 Ceramide Formation and Relationship to Caspase-3 Activation**—Jurkat cells were irradiated, and 5 × 10⁶ cell equivalents were extracted and processed for mass spectrometric lipid analysis and quantitation of C16 ceramide. Increases in intensity (ion counts) of the 572 mass ion were seen after 2–4 h. Maximum levels (6–12-fold increases) were seen at 8–24 after irradiation (Fig. 5A). The increases in C16 ceramide formation paralleled apoptosis as determined by cellular morphology (Fig. 5B). Interestingly, the radioresistant cell line K562 failed to display increases in ceramide levels (Fig. 6A) up to 48 h following irradiation. The cell line's resistance toward radiation-induced apoptosis was confirmed by its inability to induce caspase-3 activation (Fig. 6B). True increases in C16 ceramide levels induced by ionizing radiation were confirmed by plotting signal ratios of the C16 ceramide (experimental)/C8 ceramide (internal standard). Total ion counts of the internal standard remained constant, whereas the C16 ceramide levels increased (Fig. 6C).

**Radiation-resistant Melanoma Cells Fail to Increase Their C16 Ceramide Levels**—A melanoma cell line (526) was tested for its ability to undergo radiation-induced apoptosis. After irradiation, cells were returned to tissue culture flasks and cultured for an additional 6 h. After this time, cellular morphology was examined. Cells that were resistant to the treatment were easily identified by their ability to adhere to plastic, which is characteristic of this cell line, and by their lack of characteristic apoptotic morphology. In contrast, characteristic apoptotic morphology was already evident in melanoma cells...
that failed to re-adhere (approximately 70%), and in some cases, cell shrinkage had started to occur. Apoptotic and non-apoptotic cells were separated based on adherence, lipids were extracted as described above, and C16 ceramide levels were quantitated by mass spectrometry on equivalent numbers of cells. The radiation-sensitive melanoma cells displayed a 2-fold increase in C16 ceramide levels (a t 6 h post-irradiation), whereas the resistant (adherent) melanoma cells displayed levels slightly below control values (Fig. 7). These results confirm, within the same cell line, that radiation-resistant cells fail to show increased levels of C16 ceramide.

Irradiation Produces a Dose-dependent Increase in C16 Ceramide Levels—Jurkat cells were exposed to γ-irradiation for different lengths of time (varying from 5 to 25 min) and returned to culture for 16 h, after which cells were prepared for lipid extraction. C16 ceramide levels were assayed by mass spectrometry and are displayed as the sum of the total ion current (TIC) for the C16 ceramide (m/z 572) and its 37Cl isotope (m/z 574). In general, there was an increase in TIC for the C16 ceramide upon increasing radiation dosage (Fig. 8).

Anti-Fas-induced Apoptosis Produces Significant Long Term Increases (>2 h) in C16 Ceramide Levels in Jurkat Cells—Jurkat cells were treated with anti-Fas antibody for varying times ranging from 30 min to 2 h, after which cells were prepared for lipid extraction and analysis of C16 ceramide levels by mass spectrometry. Significant changes in C16 ceramide levels were seen at time points between 2 and 4 h (Fig. 9A). Six-fold increases in C16 ceramide levels were detected when cells were exposed to anti-Fas antibody for 6 h. In agreement with previously published data (23), no significant increases in ceramide were detected under 2 h after treatment with anti-Fas antibody. The kinetics of the apoptotic response, as determined by caspase-3 activity, appears to occur much...
Anti-Fas antibody induces late C16 ceramide accumulation in Jurkat cells. A, Jurkat cells (5 x 10^6) were treated with anti-Fas antibody (200 ng/ml) for 0.5–6 h, and the cells were harvested and processed for lipid extraction and C16 ceramide analysis by mass spectrometry. Results are expressed as the sum of the TIC of the 572 and 574 mass ions. B, time course of caspase-3 activity in anti-Fas treated and irradiated Jurkat cells. Results are expressed as means ± S.D. of three experiments.

FIG. 9. Anti-Fas antibody induces late C16 ceramide accumulation in Jurkat cells. A, Jurkat cells (5 x 10^6) were treated with anti-Fas antibody (200 ng/ml) for 0.5–6 h, and the cells were harvested and processed for lipid extraction and C16 ceramide analysis by mass spectrometry. Results are expressed as the sum of the TIC of the 572 and 574 mass ions. B, time course of caspase-3 activity in anti-Fas treated and irradiated Jurkat cells. Results are expressed as means ± S.D. of three experiments.

more rapidly in cells induced to undergo apoptosis by anti-Fas antibody as opposed to radiation-induced apoptosis (Fig. 9B). In fact, caspase-3 activation had reached its maximum within 60 min after the addition of anti-Fas antibody. Radiation-induced apoptosis, on the other hand, displayed a sigmoidal increase in caspase-3 activities which reached maximum levels at 5 h. These results argue against ceramide participating in the early events of apoptosis but supports a potential participating role of ceramide during the later stages of apoptosis.

Alterations in Nonyl Acridine Orange Staining in Jurkat Cells during Radiation-induced or Fas-induced Apoptosis—Cells were exposed to γ-irradiation (100 Gy) and incubated for an additional 2, 4, or 6 h at 37 °C. Cells were processed for NAO staining and flow cytometry as described above for the determination of loss of mitochondrial mass. Fig. 10A indicates that the loss in NAO staining parallels the increase in C16 ceramide accumulation (see Fig. 5). Using DNA fragmentation to assess the degradation phase of apoptosis, no significant fragmentation was seen at 2 h, but significant degradation was shown to occur at the 4- and 6-h time points post-irradiation (Fig. 10B). The data indicate that the initial increases in C16 ceramide accumulation closely parallels the effector phase of apoptosis. Similar results are shown for Fas-induced apoptosis, although the time frame is shifted. During Fas-induced apoptosis of Jurkat cells, losses in NAO staining occur within 1 h and increase steadily in a linear fashion (Fig. 10C). Again, increases in NAO staining closely paralleled C16 ceramide increases (see Fig. 9A). DNA fragmentation was near control levels at 1 h, but significant increases were seen at 2- and 3-h post-Fas ligation. Taken together, these data support a role for C16 ceramide during the effector phase of apoptosis.

DISCUSSION

The present study utilizes mass spectrometry to molecularly characterize the acute changes that occur in lipid species in cells stimulated to undergo apoptosis induced by ionizing radiation or by anti-Fas antibody. The major lipid change that occurred during Fas or radiation-induced apoptosis of Jurkat cells was an increase in a molecular species with a m/z of 572. Characterization of this mass ion through its enrichment by HPLC, and subsequent structural identification through the use of tandem mass spectrometry, indicated the presence of a C16 ceramide-chloride adduct. We took advantage of the ability to measure this species by negative ion mass spectrometry and were able to quantitate changes that occurred during radiation-induced apoptosis with the use of internal (C8 ceramide) and external (C16 ceramide) standards. We found no significant increases in the C16 ceramide within the early time points (0.5–2 h) following ionizing radiation or Fas ligation. However, later time points (>2 h) displayed significant (up to 6-fold) increases in C16 ceramide levels that were not due to differences in ionization efficiencies and appeared to parallel or lag slightly behind caspase-3 activation. It has recently been reported (23) that Jurkat cells fail to increase intracellular ceramide levels after treatment with Fas antibody (seconds to 2 h), contrary to many previously published studies (24–27). Using a similar assay system in this study, we found no significant increase in ceramide levels of Jurkat cells exposed to ionizing radiation (0.5–2 h) but show a clear increase in the later time points (>2 h). We have also confirmed the lack of significant ceramide increases within the first 2 h of anti-Fas antibody treatment of Jurkat cells.

Previous studies have indicated that the activation of the sphingomyelinase pathway by ionizing radiation is rapid (seconds to minutes) and that ceramide levels may return to near baseline values within 30 min, suggesting that early signal transducing events may have already occurred (24–27). It is also possible that the ceramide generated through the sphingomyelinase pathway is rapidly utilized by other sphingolipid biosynthetic pathways. Such pathways could produce sphingosine from ceramide through the action of a ceramidase or utilize ceramide for ganglioside synthesis because recent studies have indicated that ganglioside synthesis is necessary for Fas-induced apoptosis (28). However, we have failed to detect any significant increase in C16 ceramide in our system during the first few hours of apoptotic induction. Thus, it appears that the long and persistent phase of ceramide accumulation may be biologically relevant species. This sustained phase of ceramide accumulation may be generated from a distinct sphingomyelin pool (29), as previously suggested.

The present study focuses primarily on the long and persistent phase of ceramide accumulation in Jurkat cells following ionizing radiation or treatment with anti-Fas antibody. Using a mass spectrometrical-based approach, these particular apoptotic stimuli produced significant increases in C16 ceramide levels after 2 h, similar to previously published reports using different methods (3, 30). In addition, we failed to demonstrate increases in C16 ceramide levels in the radiation-resistant cell line K562 and the partially resistant melanoma 526 cell line. These results are in agreement with a recent study which indicated a resistance to radiation-induced apoptosis upon loss of ceramide production (31). Increases in C16 ceramide accumulation paralleled losses in NAO staining for cells exposed to γ-irradiation or anti-Fas antibody. Increases in C16 ceramide accumulation and losses in NAO staining clearly preceded DNA fragmentation in cells exposed to γ-irradiation, which
strongly suggests that C16 ceramide accumulation is critically linked to the effector phase of apoptosis. Several studies have already indicated critical links between ceramide action and components of the electron transport chain (32–33). Kinetics of induction of DNA fragmentation in Fas-induced apoptosis occurred somewhat more rapidly than radiation-induced apoptosis. Under these conditions, it becomes increasingly more difficult to definitively dissociate the effector phase from the later phases of apoptosis. However, during Fas-induced apoptosis, both mitochondrial independent and mitochondrial-dependent pathways may come into play when the death receptor Fas/FADD/procaspase-8 pathway is not sufficient (34), making it difficult to clearly distinguish the end of the effector phase from the beginning of the degradation phase. Taken together, these studies support an important role for C16 ceramide in radiation and Fas-induced apoptosis involved during the effector phase. Studies are underway to determine the source(s) of the long and persistent phase of ceramide accumulation, the levels of ceramide expressed in individual subcellular compartments, and potential ceramide/lipid changes induced by other means of apoptotic induction.

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