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Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells

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ABSTRACT

The role of ceramide neo-genesis in cellular stress response signaling is gaining increasing attention with recent progress in elucidating the novel roles and biochemical properties of the ceramide synthase (CerS) enzymes. Selective tissue and subcellular distribution of the six mammalian CerS isoforms, combined with distinct fatty acyl chain length substrate preferences, implicate differential functions of specific ceramide species in cellular signaling. We report here that ionizing radiation (IR) induces *de novo* synthesis of ceramide to influence HeLa cell apoptosis by specifically activating CerS isoforms 2, 5, and 6 that generate opposing anti- and pro-apoptotic ceramides in mitochondrial membranes. Overexpression of CerS2 resulted in partial protection from IR-induced apoptosis whereas overexpression of CerS5 increased apoptosis in HeLa cells. Knockdown studies determined that CerS2 is responsible for all observable IR-induced $C_{24:0}$ CerS activity, and while CerS5 and CerS6 each confer ~50% of the $C_{16:0}$ CerS baseline synthetic activity, both are required for IR-induced activity. Additionally, co-immunoprecipitation studies suggest that CerS2, 5, and 6 might exist as heterocomplexes in HeLa cells, providing further insight into the regulation of CerS proteins. These data add to the growing body of evidence demonstrating interplay among the CerS proteins in a stress stimulus-, cell type- and subcellular compartment-specific manner.

1. Introduction

Diverse cellular and environmental stresses (e.g. chemotherapeutics [1], heat shock [2], ischemia-reperfusion [3], ultraviolet radiation [4], and ionizing radiation (IR) [5], to list a few) stimulate cells to generate ceramide, an established second messenger in apoptotic signaling pathways [6–8]. Ceramide (N-acyl-D-*erythro*-sphingosine) can be generated *via* two major pathways: by hydrolysis of sphingomyelin *via* sphingomyelinases, or by ceramide synthase (CerS)-mediated synthesis, either *via de novo* acylation of the sphingoid base sphinganine with fatty acyl-CoAs of varying chain length from C_{14} to C_{26} to yield (dihydro)ceramides, followed by oxidation of sphinganine to sphingosine to yield ceramide, or *via* a salvage (or recycling) pathway where ceramide is deacylated by ceramidases to form sphingosine, which is reutilized by CerS to regenerate ceramide [9]. The sphinganine analogue, fumonisin B_1 (FB_1), is a competitive inhibitor of CerS activity [10].

IR-induced CerS-mediated ceramide generation, and subsequent apoptosis, occurs in a cell-type specific manner. Unlike the fast generation of ceramide at the plasma membrane (seconds to minutes) *via* sphingomyelinases, engagement of CerS and ceramide neo-genesis is delayed (hours to days) in almost every system defined to date [1,11]. Furthermore, it was recently found that IR activates CerS to generate ceramide *de novo* in *C. elegans* germ cell mitochondrial membranes [12], implicating involvement of ceramide in the commitment step of the mitochondrial death pathway. In mammals a pathway analogous to that in *C. elegans*, termed the mitochondrial death (also known as intrinsic) pathway is the main pathway for apoptotic death. In this pathway, signals, instigated by pro-apoptotic stimuli, converge on mitochondria to induce mitochondrial outer membrane permeabilization (MOMP), the commitment step in this apoptotic process. MOMP results in release of apoptogenic factors, such as cytochrome *c*, to trigger activation of caspases, key effector components of apoptosis [13,14]. MOMP is regulated by pro- and anti-apoptotic B-cell lymphoma 2 (Bcl-2) family members [15]. Relevant to this study, Bcl-xL, an anti-apoptotic Bcl-2 protein, protects numerous cell types from apoptosis by preventing

Abbreviations: CerS, ceramide synthase; IR, ionizing radiation; Bcl-2, B-cell lymphoma 2; MOMP, mitochondrial outer membrane permeabilization; MAM, mitochondria-associated-membrane; ER, endoplasmic reticulum; FB_1, fumonisin B_1; shRNA, short hairpin RNA; MT, mitochondria.

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MOMP [16–18]. The biologic significance of CerS in mediating mammalian apoptosis has been confirmed *in vivo*, as CerS transactivates disease pathogenesis in several experimental models of human disease [19–24].

There are six identified mammalian CerS isoforms, termed CerS/LASS1-6, characterized by a conserved lag1 domain. Although these six CerS isoforms all have a similar K_m value towards sphinganine in the low micromolar range [25], they differ in their fatty acyl-CoA substrate chain length preference. For example, CerS1 uses exclusively long chain $C_{18:0}$-fatty acyl-CoA [26], CerS5 and CerS6 prefer $C_{16:0}$ [27,28], whereas CerS2 utilizes very-long-chain species such as $C_{24:0}$ and $C_{24:1}$ [25,28]. CerS family members also exhibit different tissue-specific expression patterns. CerS1 is largely confined to tissues of the nervous system [28] and CerS3 expression is predominant in testis [29] and keratinocytes [30]. CerS2, CerS4, CerS5, and CerS6 appear to have a broader tissue distribution [27,28,31], which corresponds to evidence showing that $C_{24:1}$-, $C_{24:0}$-, and $C_{16:0}$-ceramides are the most abundant species in cells of many tissues, including epithelial cells, fibroblasts, and cells of the immune system [32,33]. The interplay between long chain $C_{16:0}$-ceramide and very long chain $C_{24:1}$- and $C_{24:0}$-ceramides has come into recent spotlight regarding their roles in maintaining cellular homeostasis [34–36], as $C_{16:0}$-ceramide species is most often pro-apoptotic, whereas C_{24}-ceramides do not appear to display this propensity. There is also evidence of intracellular differences in CerS distribution. CerS activity was initially localized to the cytoplasmic leaflet of the endoplasmic reticulum [37,38], but increasing evidence reveals that CerS have additional subcellular localizations, such as perinuclear membranes [39] and the mitochondria-associated-membrane (MAM) [40], where distinct ceramides may confer discrete, and at times even opposing, signaling endpoints, including apoptosis and cell survival [39,41,42].

The mitochondrial-associated-membrane (MAM) is a compartment comprising the physical interaction between the endoplasmic reticulum (ER) and mitochondria (reviewed in [43]), enriched in lipid synthetic and transfer proteins, including CerS [40]. Recently, it was shown that long-chain ceramide generated *de novo* is rapidly transferred from MAM to mitochondria, likely catalyzed by a not yet identified transfer protein, resulting in MOMP [44]. Our laboratory also recently identified the MAM fraction of HeLa human cervical carcinoma cells as the site of IR-induced CerS activity, and the mitochondria as the predominant site of IR-induced ceramide elevation (Lee and Kolesnick, submitted). Based on this information, here we report that IR induces *de novo* ceramide synthesis to influence HeLa cell apoptosis, specifically activating CerS2, 5, and 6 in the MAM, generating opposing anti- and pro-apoptotic mitochondrial ceramides.

2. Materials and methods

2.1. Cell culture, transfection, FB_1-treatment, and irradiation

HeLa cells were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/ml), streptomycin (50 μg/ml) and 2 mM glutamine. Cells were transfected using Fugene 6 transfection reagent (Roche) in antibiotic-free culture media according to the manufacturer's protocol. Medium containing DNA and reagent was replaced 6 h after transfection with complete culture medium. FB_1 (Biomol) was solubilized in 1X PBS at a concentration of 5 mM and added to cells at a final concentration of 75 μM. Note commercially available FB_1 is a biologic product isolated from *Fusarium verticillioides* and *Fusarium proliferatum* that displays batch-to-batch variation. Hence, effectiveness of each batch must be tested empirically. Irradiation was carried out at 22 °C using a Cs-137 irradiator (Shepherd Mark-I, model 68, SN 643) at a dose rate of 240 cGy/min.

2.2. Cloning pCMV2B-CerS1, 2, 5, and 6, pcDNA3-HA-CerS2, and pCMV3B-CerS6

We cloned full length human *CerS1*, *2*, *5* and *6* into the pCMV2B plasmid vector (Stratagene) as described previously [25]. We cloned full length human *CerS2* into the pcDNA3 plasmid vector (Invitrogen), containing an N-terminal HA tag, using a human liver tissue library (Clontech). The genes were inserted using the following primers flanked with HinDIII and EcoRI restriction sites (Gene Link, Inc.): 5′ ggaattcctccagaccttgtatgattac′3 and 5′cgaagcttgggagcggggtagttccttggc′ 3. The PCR products and pcDNA3-HA were digested with EcoR1-HinDIII, ligated, transformed into *E. coli DH5α* (Invitrogen), and directly sequenced. Full length human *CerS6* was inserted into the pCMV3B plasmid vector (Stratagene), containing an N-terminal myc tag, by BamHI and EcoRI restriction digest of pCMV2B-CerS6 plasmid, subsequent ligation of the *CerS6* insert into BamHI and EcoRI-digested pCMV3B, transformation, and direct sequencing.

2.3. Cloning pSUPER-CerS2, 5 and 6

Sense and antisense shRNA constructs flanked by HindIII restriction enzyme sites for each CerS isoform were annealed and ligated into the pSUPER expression vector (OligoEngine, Seattle, WA) as follows:

CerS2 (NM_181746):
sense 5′gatccccggatatcccatacagagcattcaagagatgctctgtatgggatatccttttta;
antisense 5′agcttaaaaaggatatcccatacagagcatctcttgaatgctctgtatgggatatccggg.
CerS5 (NM_147190.2):
sense 5′gatccccgactgcaaggcactgaggattcaagagatcctcagtgccttgcagtcttttta;
antisense 5′agcttaaaaagactgcaaggcactgaggatctcttgaatcctcagtgccttgcagtcggg.
CerS6 (NM_203463.1)
sense 5′gatcccctgaactgcttctggtcttattcaagagataagaccagaagcagttcatttta;
antisense 5′agcttaaaaatgaactgcttctggtcttatctcttgaataagaccagaagcagttcaggg.

Vectors were transformed into *E. coli DH5α* (Invitrogen) and inserts verified by direct sequencing. shRNA expression vectors were screened for gene specific mRNA knockdown by real-time qPCR, and for protein knockdown by western blotting for CerS2 and CerS6.

2.4. Reverse transcriptase (RT)-PCR

Total RNA was isolated from cultured HeLa cells using an RNeasy Mini Kit (Qiagen), converted to cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen), and PCR was performed using Fast Start *Taq* DNA Polymerase Kit (Roche) according to the manufacturers' protocols. PCR conditions were as follows: initial denaturation for 4 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. A final elongation step followed for 10 min at 72 °C. PCR products were separated by electrophoresis in 1% agarose gels. Primers are as follows: *CerS1*- 5′cgtcgcggcctggctgagcacg and 5′gccgatggtaggagccgccgc. *CerS2*- 5′ctccagaccttgtatgattact and 5′ ggccacatggtggatgatctg; *CerS3*- 5′ggactggcaaagaagtg and 5′cagggtgttacaggtct; *CerS4*- 5′ggaaccaggatcgacc and 5′ggactcgtagtatgtgg; *CerS5*- 5′ ggagggcctgtcaaagc and 5′cctagtcgtgtaacc; *CerS6*- 5′cgacaaagacgcaatcagg and 5′cggcaaacataacaaacagg; *β-actin*- 5′gctcgtcgtcgacaacggctc and 5′caaacatgatctgggtcatcttctc. Results were confirmed using previously published *CerS1-6* primers [30].