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Ceramide modulates pre-mRNA splicing to restore the expression of wild-type tumor suppressor p53 in deletion-mutant cancer cells

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ABSTRACT

Mutants of tumor suppressor p53 not only lose the activity in genome stabilizing and in tumor suppression, but also exhibit oncogenic function in cancer cells. Most efforts in restoring p53 biological activity focus on either altering mutant-protein conformation or introducing an exogenous p53 gene into cells to eliminate p53-mutant cancer cells. Being different from these, we report that ceramide can restore the expression of wild-type p53 and induce p53-dependent apoptosis in deletion-mutant cancer cells. We show that endogenous long-carbon chain ceramide species (C_{16}- to C_{24}-ceramides) and exogenous C_6-ceramide, rather than other sphingolipids, restore wild-type mRNA (intact exon-5), phosphorylated protein (Ser15 in exon-5) of p53, and p53-responsive proteins, including p21 and Bax, in ovarian cancer cells, which predominantly express a deleted exon-5 of p53 mutant before treatments. Consequently, the restored p53 sensitizes these p53-mutant cancer cells to DNA damage-induced growth arrest and apoptosis. Furthermore, we elucidate that ceramide activates protein phosphatase-1, and then the dephosphorylated serine/arginine-rich splicing-factor 1 (SRSF1) is translocated to the nucleus, thus promoting pre-mRNA splicing preferentially to wild-type p53 expression. These findings disclose an unrecognized mechanism that pre-mRNA splicing dysfunction can result in p53 deletion-mutants. Ceramide through SRSF1 restores wild-type p53 expression *versus* deletion-mutant and leads cancer cells to apoptosis. This suggests that heterozygous deletion-mutants of p53 can be restored in posttranscriptional level by using epigenetic approaches.

1. Introduction

The p53 protein, encoded by human gene *TP53*, is a key tumor suppressor that stabilizes the genome, preventing tumorigenesis and cancer progression [1]. As an essential transcription factor, p53 activates the expression of *p21*, *Bax*, *Puma*, *Fas* and other p53-responsive genes, consequently promoting cell division-arrest, apoptosis, DNA repair and cell differentiation [2]. Mutants of p53, which are detected more frequently than any other gene, compromise its functions [3,4]. p53 mutants not only lose their activities in suppressing tumor, but they also confer dominant-negative activity and oncogenic function in cancer cells [2,3]. These changes promote tumor progression and result in drug resistance; therefore, p53 mutants have become the most common prognostic indicator both for tumor recurrence and for cancer death [2,5]. Most tumors that exhibit disrupted p53-signaling pathways remain addicted to p53 mutants, and p53 mutants have emerged as perhaps the most important target to improve cancer treatments [2,5]. Current approaches targeting p53 mutants mainly focus on replacing wild-type p53 by introducing an exogenous p53 gene, reactivating p53 mutants by altering mutant-protein conformation and augmenting wild-type p53 by inhibiting MDM2-mediated degradation [6–8]. In cancers, the dominant-negative activity and gain-of-function of p53 mutants potentially compromise the efficacy of these approaches [8,9]. To develop consistently effective approaches targeting p53 mutants, greater promise would seem to rest in the possibility of regulating the expression of wild-type p53 *versus* mutants in cancer cells, which are mostly heterozygous for the p53 gene.

Ceramide is the central metabolite of sphingolipids, and has myriad effects on cell function, including cell growth arrest, senescence,

Abbreviations: SRSF1, serine/arginine-rich splicing factor 1; PP1, protein phosphatase 1; GCS, glucosylceramide synthase; MBO-asGCS, mixed backbone oligonucleotide against glucosylceramide synthase; PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; SMase, sphingomyelinase; FB1, fumonisin 1; Cal A, calyculin A; siRNA, small interfering RNA; RT-PCR, reverse transcription polymerase chain reaction; ESI/MS/MS, electrospray ionization-tandem mass spectrometry

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apoptosis and autophagy [10]. Furthermore, several reports have shown that ceramide is involved in regulating gene expression [11–14]. For example, ceramide upregulates the expression of p21 [11,12], cyclooxygenase-2 [15], and glucosylceramide synthase (GCS) [14]; it down-regulates the expression of c-*myc* [11] and human telomerase reverse transcriptase (hTERT) [16]. By activation of Sp1 binding to the promoter, ceramide increases the expression of GCS [14]; conversely, ceramide decreases hTERT promoter activity by rapid proteolysis of the ubiquitin-conjugated c-myc [16]. Interestingly, ceramide can modulate alternative pre-mRNA splicing process and allow cells to express apoptotic isoforms of bcl-x and caspase-9 [17,18]. Our previous report shows that suppression of ceramide glycosylation restores the expression of wild-type p53 protein in p53-mutant cells [19]. Current study examines whether or not ceramide modulates pre-mRNA splicing to regulate the expression of wild-type p53 protein in p53-mutant cells.

2. Materials and methods

2.1. Cell culture and treatments

Human NCI/ADR-RES ovary cancer cell line, which presents multidrug resistance and a 7-amino acid deletion in exon-5 of tumor suppressor p53 [20], was kindly provided by Dr. Kenneth Cowan (UNMC Eppley Cancer Center, Omaha, NE, USA) and Dr. Merrill Goldsmith (National Cancer Institute, Bethesda, MD, USA). Cells were cultured in RPMI-1640 medium containing 10% fetus bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 584 mg/l L-glutamine. Cells were maintained in an incubator humidified with 95% air and 5% CO_2 at 37 °C.

For treatments, cells (3×10^6/100-mm dish; 4000 cells/well in 96-well plate) were grown in 10% FBS RPMI-1640 medium overnight and then treated with C_6-ceramide (C_6-Cer, 5 μM), C_6-dihydroceramide (C_6-diH-Cer, 5 μM), D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, 10 μM), okadaic acid (OA, 10 nM) and calyculin A (Cal A, 5 nM) in Opti-MEM reduced-serum medium for 4 h and then cultured in 5% FBS medium containing 2.5 μM doxorubicin (Dox) for additional 48 h, as described previously [14]. In combination groups, cells were pretreated with OA (10 nM) or Cal A (5 nM) in Opti-MEM reduced-serum medium for 4 h, and then treated with C_6-Cer (5 μM) in 5% FBS medium for additional 48 h. C_6-ceramide and C_6-dihydroceramide were purchased from Biomol (Plymouth Meeting, PA). D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol HCl (PDMP) was purchased from Matreya (Pleasant Gap, PA). Sphingomyelinase (acidic, human placenta, 100 units/mg proteins) and doxorubicin hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Calyculin A (Cal A) and okadaic acid (OA) were purchased from Cell Signaling Technology Inc. (Danvers, MA).

For Geimsa staining, cells in 35-mm dishes were fixed with ice-cold methanol, and then stained with Karyomax® Giemsa stain improved R66 solution (Invitrogen, Carlsbad, CA) at room temperature for 2 min. Following wash with deionized water, cells were photomicrographed (×100 magnification) under Nikon Eclipse TS-100 microscope equipped with a digital camera.

2.2. Gene silencing of GCS and SRSF1

To silence GCS expression, mixed-backbone oligonucleotide against human GCS (MBO-asGCS, 0–200 nM) was introduced into cells (3×10^6/100-mm dish; 4000 cells/well in 96-well plate) after overnight growth, facilitating with Lipofectamine 2000 in Opti-MEM reduced-serum medium (Invitrogen) for 4 h. The cells continuously grew in 5% FBS medium containing 2.5 μM Dox for an additional 48 h, as described previously [21]. In combination groups, cells were pretreated with FB1 (100 μM) or Cal A (5 nM) and OA (10 nM) in Opti-MEM medium for 4 h, and then transfected with MBO-asGCS (100 nM), and further grown in 5% FBS medium containing 2.5 μM Dox for an additional 48 h. To silence serine/arginine splicing factor 1 (SRSF1), cells were transfected with siRNA targeting SRSF1 (siSRSF1, 100 nM) or scrambled control (siRNA-SC, 100 nM) in Opti-MEM medium and grew in 5% FBS medium containing 2.5 μM Dox for an additional 48 h. To co-silence GCS and SRSF1, cells were transfected with both MBO-asGCS (100 nM) and siSRSF1 (100 nM) or siRNA-SC simultaneously. Mixed-backbone oligonucleotide against human GCS (MBO-asGCS) and scrambled control (MBO-SC) [21] were purchased from Integrated DNA Technologies (Coralville, IA). siRNA targeting human SRSF1 (a pool of 3 target-specific 20–25 nt siRNA, sc-38319) [22] and its scrambled control (siRNA-SC, sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. SRSF1 transfection

pSRSF1-EGFP plasmid (previously named as EGFP-SF2/ASF), which was generated by Tom Misteli [23] inserting human SRSF1 into the *Hind*III and *Pst*I restriction sites of pEGFP-C1, was generously provided by Addgene (#17990; Cambridge, MA). The pEGFP-C1 was purchased from Clontech Laboratories (Mountain View, CA). Four micrograms of each plasmid was introduced into NCI/ADR-RES cells (3×10^6/100-mm dish) after overnight growth, facilitating with Lipofectamine 2000 in Opti-MEM reduced-serum medium (Invitrogen). After 4 h transfection, cells were cultured in 10% FBS RPMI medium, and geneticin G418 (400 μg/ml) was added into culture after overnight growth. Transfected SRSF1 cells were treated with indicated agents as described above, and the cells transfected with pEGFP-C1 were used as mock control.

2.4. ESI/MS/MS analysis of sphingolipids

Endogenous sphingomyelin molecular species were performed on a Thermo-Fisher TSQ Quantum triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring (MRM) positive ionization mode, as described previously [24,25]. Total cells, fortified with internal standards, were extracted with ethyl acetate/iso-propanol/water (60/30/10 v/v), evaporated to dryness and reconstituted in 100 μl of methanol. The reconstituted samples were injected on the Surveyor/TSQ Quantum LC/MS system and gradient eluted from the BDS Hypersil C8 column (150 × 3.2 mm, 3 μm particle size) with 1.0 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. The peaks for the target analytes and internal standards were collected and processed using the Xcalibur software. Calibration curves were constructed by plotting peak area ratios of synthetic standards, representing each target analyte, to the corresponding internal standard. The target analyte peak area ratios from the samples were similarly normalized to their respective internal standards and compared with the calibration curves using a linear regression model. The levels of sphingolipids of samples were normalized against cellular protein, and expressed as pmol/μg protein.

2.5. RNA extraction and reverse transcription-polymerase chain reaction

After treatment, total RNA was extracted from NCI/ADR-RES cells using a SV total RNA isolation kit (Promega, Madison, WI). Equal amounts of total RNA (500 ng) were used to synthesize first strand DNA using the SuperSrcriptR III kit (Invitrogen) and 5 μl of the first strand DNA reaction from each sample was amplified by using the Platinum® Blue PCR SuperMix kit (Invitrogen) [14,19]. To detect the deletion in exon-5 of p53, a 400-bp fragment in the region of human TP53 mRNA (ORF 113–512; accession number BC003596.1) was generated by using the upstream primer (5′-TCACTGCCATGGAGGAG-3′) and downstream primer (5′-TTGAGGGCAGGGGAG-3′). This 400-bp product that includes the deleted region of exon-5 (codons 126–133) would be absent in the p53 deletion mutant. Another 393-bp p53 fragment was generated in the region of the TP53 mRNA (223–490) to detect the expression of p53 mRNA using the upstream primer (5′-TTGCCGTCCCAA