Upregulation of forkhead box O3 transcription is involved in C2-ceramide induced apoptosis and autophagy in ovarian cancer cells in vitro

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Abstract. Ceramide is a bioactive lipid which functions as a tumor suppressor, mediating processes such as apoptosis, growth arrest, senescence and differentiation. The effects of ceramide in ovarian cancers have not been well established. The objective of the present study was to investigate the effects of C2-ceramide treatment in A2780 ovarian cancer cells and its possible molecular mechanism. C2-ceramide-induced proliferation inhibition was analyzed using an MTT assay and Trypan blue test. Flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling were used to identify the induction of apoptosis. Transmission electron microscopy was used to confirm the formation of autophagosomes. Quantitative polymerase chain reaction was performed to analyze the messenger RNA expression of the autophagy and cell death associated genes and western blotting was used to analyze the protein expression of beclin 1, LC3, Akt, forkhead box O3 (FOXO3) and adenosine monophosphate-activated protein kinase in ovarian cancer cells. It was found that C2-ceramide inhibited A2780 cell proliferation in a time- and dose-dependent manner and C2-ceramide induced A2780 cell apoptosis and autophagy. However, C2-ceramide-induced autophagy did not result in cell death, but instead protected ovarian cancer cells from apoptosis. Akt inhibition and FOXO3 activation were implicated in C2-ceramide-treated ovarian cancer cells. Furthermore, FOXO3 target genes, which were associated with autophagy (MAP1LC3, GABARAP and GABARAPL1) and cell death (BNIP3, BNIP3L, BIM and PUMA), were upregulated. The present study has shown that C2-ceramide induced apoptosis and autophagy in ovarian cancer cells. FOXO3 transcription was upregulated, which may contribute to C2-ceramide-induced apoptosis and autophagy.

Introduction

Ovarian cancer is one of the most common three malignant tumors of the female reproductive system. Ovarian cancer has been recently reported as the leading cause of mortality in gynecological cancers in the United States (1) and other countries around the world. There have been pooled studies investigating the pathogenesis of ovarian cancer in order to acquire new approaches for alternative treatment. However, there have been minor changes in the survival of patients with ovarian cancer since platinum-based anticancer drugs were introduced more than three decades ago (2). Therefore, novel therapeutic agents are required in order to improve the prognosis of ovarian cancer.

Ceramide, a derivative of sphingolipid breakdown products, was initially identified as a regulator of apoptosis (3) and cellular senescence (4) in the 1990s and has since become a well-established mediator of cell death. Dysregulated ceramide has been documented in cancer development and prevention, since ceramide acts as a tumor-suppressor lipid in numerous tumor cells. However, a recent study, which investigated the effects of ceramide on the biomechanical properties of murine ovarian cancer cells, has not indicated that suppression results from exogenous ceramide administration (5). Paradoxically, paclitaxel and ceramide co-administration effectively inhibited the growth of multidrug resistant ovarian cancer cell xenografts (6). Exogenous ceramide administration has been shown to successfully suppress tumor growth in both sensitive and resistant ovarian cancer xenograft models. An increase in apoptosis was observed in both the ceramide-only and combined treatment groups, not only in sensitive tumors but also in multidrug resistant tumors. These data have raised the question as to whether ceramide induces ovarian cancer cell death. In the present study, exogenous C2-ceramide was used to explore the possible response and related mechanism in A2780 ovarian cancer cells.
The purpose of the present study was to understand the effects of exogenous C2-ceramide on ovarian cancer cells and to establish the possible therapeutic value of using C2-ceramide in treating this disease. Experiments were designed to reveal whether C2-ceramide induced programmed cell death in ovarian cancer cells. Furthermore, the death mechanism was investigated, to establish whether C2-ceramide could initiate apoptosis as well as cause autophagy.

Materials and methods

Reagents and antibodies. C2-ceramide and 3-methyladrenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the former was prepared as a 50 mm stock solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The final concentration of DMSO in the culture medium was <0.2% volume. Forkhead box O3 (FOXO3), phosphorylated (P)-FOXO3, adenosine monophosphate-activated protein kinase (AMPK) and P-AMPK (Thr172) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Akt and P-Akt (Ser473) antibodies were purchased from Abcam (Cambridge, MA, USA). Beclin 1 and microtubule-associated protein 1 light chain 3 (LC3) antibodies were both purchased from Novus Biologicals LLC (Littleton, CO, USA). GAPDH antibody and horseradish peroxidase-secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Crystal Biotech Company, (Northborough, MA, USA), respectively.

Cell culture and culture conditions. The A2780 human ovarian cancer cell line was purchased from Wuhan University (Wuhan, China). Cells were maintained in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), and incubated at 37°C with 5% CO2.

MTT assay and trypan blue exclusion test. For the MTT assay, A2780 cells were plated (1x10^4 cells/well) in 96-wells. Following 24 h of incubation, the cells were treated with C2-ceramide at concentrations ranging from 0 to 100 µmol/l and grown over a 24-h period. DMSO was used as a control. Cell viability was measured using an MTT assay according to the manufacturer's instructions. For the trypan blue exclusion assay, A2780 cells were plated onto 6-well plates (1x10^4 cells/well) and then treated with 25 µmol/l C2-ceramide at different time points. 3-MA, at concentration of 2 mmol/l, was added 1 h prior to C2-ceramide treatment. Cells were then collected and mixed 1:1 (w/v) with trypan blue dye. Cells that excluded the dye were counted. Data are presented as the means ± standard error of the mean, derived from triplicate samples of three independent experiments.

Analysis of apoptosis. A2780 cells were plated, and after 12 h were treated with vehicle control (DMSO) or C2-ceramide at the indicated concentrations. Following 24 h incubation, the cells were washed twice in phosphate-buffered saline (PBS) and then resuspended in 500 µl binding buffer and stained with 5 µl Annexin V-fluorescein isothiocyanate (FITC) and 5 µl propidium iodide for 15 min at room temperature in the dark. Samples were measured using a FACScan flow cytometer within 60 min following staining. The rate of apoptosis was analyzed using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

In addition to Annexin V-FITC staining, an in situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany) was used to confirm the induction of apoptosis according to the manufacturer's instructions. A2780 cells were plated onto 60 mm tissue culture plates (4x10^5 cells/plate) and treated with a vehicle control (DMSO) or C2-ceramide at the indicated concentrations for 24 h. Adherent cells were harvested by trypsin treatment and were washed with PBS once, added to polylysine treated glass slides, and then fixed with 4% paraformaldehyde before permeabilization with 0.2% Triton X-100. The cells were then treated with terminal deoxynucleotidyl transferase (TUNEL) in the presence of fluorescein-labeled nucleotide polymers. TUNEL-positive cells were analyzed by fluorescence microscopy to quantify the number of apoptotic cells.

Transmission electron microscopy. A2780 cells were plated in 6-well plates and incubated overnight. The cells were then treated with C2-ceramide for 24 h. DMSO was used as a control. Samples were fixed in 4% glutaraldehyde for 1 h at room temperature and then treated with 1% osmium tetroxide (OsO4) for 1 h. The samples were then dehydrated with increasing concentrations of ethanol, and gradually infiltrated with araldite resin. Ultrathin sections were obtained using an ultramicrotome (Leica, Mannheim, Germany). Sections were stained with uranyl acetate and lead citrate, and examined using a Tecnai™ G2 20 transmission electron microscope (FEI, Hillsboro, OR, USA).

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from cells by using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA) reagent, according to the manufacturer’s instructions. The reverse transcription reaction was performed with ReverTra Ace (Toyobo, Osaka, Japan). Oligo(dT)20, RNase inhibitor, 5X RT buffer and dNTP mixture (Invitrogen Life Technologies). The PCR primers were designed using Premier Primer 5.0 software (Premier Biosoft International, Palo Alton, CA, USA). Primer sequences are shown in Table I. PCR amplification of cDNA was performed by using SYBR green I (Biotium, Hayward, CA, USA) and ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Relative quantification of the targets was normalized with an endogenous housekeeping gene (GAPDH) and data analysis was performed by using the comparative (ΔΔCt) method.

Western blot analysis. A2780 cells were seeded in 6 well plates and incubated overnight, followed by the addition of 25 µmol/l ceramide and incubation for the indicated time points. The cells were then lysed in lysis buffer (50 mm Tris, 150 mm NaCl, 1% NP-40, EDTA, β-glycerophosphate and protease inhibitor cocktail). The protein extracts were quantified using a bovine serum albumin protein assay kit. Equal amounts of protein (50 µg) were separated by 10% SDS-PAGE (beclin 1, Akt, P-Akt, FOXO3, P-FOXO3, AMPK, and P-AMPK) and 15% SDS-PAGE (LC3), respectively.

Statistical analysis. The statistical significance of the differences were analyzed by t test in SPSS 13.0 (SPSS, Inc.,
Results

C2-ceramide inhibits ovarian cancer cell growth. To assess the effects of C2-ceramide on ovarian cancer cell growth, A2780 cells were treated with different concentrations (0, 12.5, 25, 50, 100 µmol/l) of C2-ceramide. Following 24 h treatment, the cells were analyzed by MTT assay. As shown in Fig. 1A, increasing concentrations of C2-ceramide significantly enhanced the inhibition of cell growth. The half maximal inhibitory concentration (IC50) of C2-ceramide was found to be 55.719 µmol/l. The live cell numbers were analyzed by trypan blue test in the presence of 25 µmol/l C2-ceramide, over a period of time. As shown in Fig. 1B, the number of live cells decreased over time following C2-ceramide treatment, whereas the number of live cells treated with DMSO remained relatively constant over time. The results suggested that C2-ceramide could inhibit ovarian cancer cell growth in a dose- and time-dependent manner.

C2-ceramide induces apoptosis in ovarian cancer cells. To understand the underlying mechanism of C2-ceramide cytotoxicity in A2780 cells, flow cytometric analysis and TUNEL assay were used to detect apoptosis. For the flow cytometric analysis, cells were cultured under standard conditions and treated with either DMSO as a control or the designated concentration of C2-ceramide, for 24 h. The basal early apoptotic rate in the DMSO group was 5.3%. The apoptotic rate was dose-dependent when the concentration of C2-ceramide was >25 µmol/l (Fig. 2A and B). In addition, the TUNEL assay confirmed the induction of apoptosis by C2-ceramide (Fig. 2C). These results indicated that C2-ceramide induced apoptosis in A2780 cells. On the basis of these results, 25 µmol/l C2-ceramide was used for the subsequent studies.

C2-ceramide induced autophagy in ovarian cancer cells. To determine whether C2-ceramide induced autophagy in ovarian cancer cells, transmission electron microscopy was used, which successfully confirmed that C2-ceramide induced the accumulation of autolysosome structures (Fig. 3A). The effects of C2-ceramide on beclin 1 and LC3 messenger (m)RNA and protein were analyzed by quantitative polymerase chain reaction and western blotting, respectively. As shown in Fig. 3B-F, C2-ceramide treatment increased beclin 1 and LC3 mRNA level in a time dependent manner (Fig. 3B and C). The same results were confirmed by western blotting (Fig. 3D-F). C2-ceramide

Table I. Primer sequences for quantitative polymerase chain reaction.

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<thead>
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<th>Human gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>AATGGGAAGCTGTTGCTCCTC</td>
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<td>CGATGACCAACGGTATGTTGAC</td>
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<td>CAAGTCCAGGTCTCCCATC</td>
<td>120</td>
</tr>
<tr>
<td>GABARAP</td>
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<td>CACTGTTGGTGAATGAC</td>
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</tr>
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<td>CACCACAGATCTAACGC</td>
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<td>142</td>
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</table>

bp, base pairs.

Figure 1. C2-ceramide inhibits A2780 cell growth in a dose- and time-dependent manner. (A) A2780 cells were plated in 96-well plates and treated with different concentrations of C2-ceramide (0-100 µmol/l) for 24 h and an MTT assay was performed. (B) At specified time points, a trypan blue test was performed in the presence of 25 µmol/l C2-ceramide to count the number of live cells. A DMSO-treated group was used as a control group. Values represent the means ± standard error. *P<0.05 as compared with the control. DMSO, dimethylsulfoxide.
Figure 2. C2-ceramide induces apoptosis in A2780 cells. (A and B) A2780 cells were treated with various concentrations of C2-ceramide for 24 h. Flow cytometry was used to quantify the apoptotic rate in A2780 cells. (C) A TUNEL assay was used to detect the apoptotic cells. The DMSO treated group was used as a control group. Values represent the means ± standard error. *P<0.05 as compared with the control. DMSO, dimethylsulfoxide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Figure 3. C2-ceramide induces autophagy in A2780 ovarian cancer cells. (A) Electron microscopy images of control and C2-ceramide treated A2780 cells. The arrows indicate autolysosome structures. The results represent three independent experiments. A2780 cells were plated onto 6-well plates and treated with 25 µmol/l C2-ceramide for various time periods (0, 3, 6, 18 h). The effects of C2-ceramide on (B) BECN1 and (C) MAP1LC3A mRNA were detected by quantitative polymerase chain reaction. *P<0.05 as compared with the 0 h time point sample. (D) A2780 cells were plated onto 6-well plates and treated with 25 µmol/l C2-ceramide for various time periods (3, 6, 18 h). Cells lysates from the treated or untreated A2780 cells were analyzed by western blot with Beclin1, LC3 and GAPDH antibodies. The relative expression levels of (E) Beclin1 and (F) LC3-II were normalized to GAPDH and compared with the control sample. The control group was defined as the group treated with DMSO for 18 h. The results were averaged from three independent studies. Values represent the means ± standard error. *P<0.05 as compared with the control. DMSO, dimethylsulfoxide.
increased both LC3-I and LC3-II protein in A2780 cells, while the LC3-II protein level was much higher than the LC3-I level. Thus, C2-ceramide induced typical autophagy in A2780 ovarian cancer cells.

C2-ceramide does not induce autophagy-related cell death. Ceramide has been previously shown to induce autophagic cell death in malignant glioma cells (7). Since C2-ceramide treatment induces apoptosis and autophagy, it was examined...
whether C2-ceramide-dependent autophagy induced cell death in ovarian cancer cells. A2780 cells were treated with autophagy inhibitor 3-MA with or without C2-ceramide for 24 h. Live cells were then monitored by trypan blue test. As shown in Fig. 4, the presence of 3-MA reduced the number of live cells following C2-ceramide treatment. This suggested that in A2780 cells, autophagy may serve as a protective mechanism against C2-ceramide-induced apoptosis. A similar mechanism has been previously shown in MCF-7 cells treated with C6-ceramide (8).

FOXO3-dependent transcription is activated by C2-ceramide treatment. Ceramide has been shown to inhibit Akt phosphorylation (9). FOXO3 is phosphorylated and inactivated by activated Akt, and translocates from the nucleus thus decreasing the transcription of the target genes (10). In the present study, the Akt, P-Akt (Ser473), FOXO3, P-FOXO3 (Thr32) status was investigated following C2-ceramide treatment in A2780 cells. C2-ceramide treatment significantly inhibited Akt phosphorylation in a time dependent manner and FOXO3 phosphorylation was reduced, thus causing an increase in the unphosphorylated active pool of FOXO3 (Fig. 5A). In addition, a previous study has reported that AMPK was associated with FOXO3 phosphorylation (10). Given this data, the AMPK phosphorylation status was monitored. It was identified that AMPK was significantly phosphorylated in A2780 cells following C2-ceramide treatment. Given this, it was investigated whether FOXO3 target genes were modulated during C2-ceramide treatment. Consistently, the FOXO3 target genes, which were associated with autophagy and cell death, were significantly upregulated by C2-ceramide (Fig. 5C). GABARAP, GABARAPL1 and MAP1LC3 (Fig. 3C) belong to the ATG8 gene family and code for proteins involved in autophagosome biogenesis (11). Their expression was consistent with the formation of autophagic vesicles in C2-ceramide-treated A2780 cells (Fig. 3A). BNIP3, BNIPL3, BIM and PUMA are proapoptotic genes. Altogether, these data indicate that C2-ceramide promotes FOXO3 transcription in A2780 ovarian cancer cells.

Discussion

In the present study, it was identified that C2-ceramide inhibited the proliferation of A2780 ovarian cancer cells in vitro. C2-ceramide not only induced apoptosis in A2780 cells apoptosis, but also autophagy. However, C2-ceramide-associated autophagy did not induce cell death but protected cells from apoptosis. In addition, FOXO3 transcription was activated by C2-ceramide in A2780 ovarian cancer cells and its target genes were associated with apoptosis and autophagy.

Ovarian cancer is a particularly challenging disease attributed to late stage diagnosis and development of resistance to chemotherapy. Although the majority of the patients respond to the first-line chemotherapy, they will relapse and eventually succumb to the disease. The relapse is predominantly due to chemotherapeutic resistance (2). Therefore, identification of novel compounds that circumvent this resistance mechanism is required to improve the management of ovarian cancer.

The mechanism of ceramide-induced cell death through apoptosis is well established (12), inducing apoptosis through extrinsic and intrinsic pathways (13). The extrinsic apoptosis pathway is often triggered by the activation of tumor necrosis factor receptors, whereas the intrinsic pathway is initiated by mitochondrial dysfunction. Furthermore, ceramide has also been found to cause autophagy which results in either cell survival or death (14). Previous research has reported that ceramide caused COX-2-dependent apoptosis in ovarian cancer OVCAR-3 cells (15). In the present study, C2-ceramide did not only induce cell apoptosis, but also autophagy. C2-ceramide treatment of A2780 cells significantly enhanced the formation of autophagosomes and elevated the expression of LC3 and beclin 1 at both the mRNA and protein level.

Autophagy is a homeostatic and evolutionarily conserved process that regulates the cellular levels of long-lived proteins and organelles. Under several conditions, autophagy constitutes a stress adaptation that avoids cell death, whereas in other circumstances, autophagy constitutes an alternative pathway to cell death (16). LC3 (17) and mammalian homologue of yeast autophagy-related (ATG) gene 8 (ATG8), are associated with the autophagosome membranes. Beclin 1 (18), a mammalian homolog of the yeast ATG6, functions as a scaffold for the formation of the phosphoinositide 3-kinase (PI3K) complex, which is essential for the recruitment of other Atg proteins during the development of autophagosomes (19). Therefore, LC3-II together with beclin 1 was monitored in the present study as markers for autophagosomes. The expression of LC3 and beclin 1 in SKOV3 ovarian cancer cells has additionally been shown to inhibit proliferation and induce apoptosis (21). The decrease of autophagic capacity may be related to tumorigenesis and the development of epithelial ovarian cancer. Therefore, inducers of autophagy could be applied for the treatment of epithelial ovarian cancer (22). C2-ceramide-induced autophagy partially attenuated C2-ceramide-induced A2780 cell apoptosis in the present study. Mathew et al. (23) identified that reduced autophagy can promote chromosome instability, which associated with tumor progression and poor prognosis. It is possible that paclitaxel and ceramide co-administration could promote autophagy, to limit genome damage thus resulting in inhibition of the growth of multidrug resistant ovarian cancer cells (6). Further studies are needed to confirm whether C2-ceramide increases the chemosensitivity of ovarian cancers.

Interestingly, apoptosis and autophagy are two major opposing pathways that regulate cellular outcomes (24). They are often co-regulated by common upstream signaling components (16). FOXO3, one of the FOXO transcription factors, is the downstream target of the PI3K-Akt pathway, which functions in tumorigenesis and cancer progression (10). Inhibition of Akt causes translocation of FOXO3 to the nucleus, while Akt overexpression causes FOXO3 protein reduction in prostate cancer cells (25). FOXO3 acts as a suppressor of follicular activation at the earliest stages of follicular growth in mice (26). Low expression of FOXO3 has been significantly associated with poor prognosis in ovarian cancer patients (27). Detainment of FOXO3 could therefore be one option to treat ovarian cancer. In the present study, it was found that Akt activity was inhibited by C2-ceramide, accompanied by a decrease in FOXO3 phosphorylation and non-phosphorylated-FOXO3 accumulation. It has been recently shown that AMPK phosphorylation is associated with C6-ceramide-induced autophagy cell death.
in colorectal cancer cells (28), consistent with these results that AMPK was activated by C2-ceramide. It was shown that FOXO3 target genes, which were related to apoptosis (BNIP3, BNIP3L, BIM and PUMA) and autophagy (MAP1LC3, GABARAP and GABARAPL1) were upregulated by C2-ceramide in A2780 cells.

BNIP3, BNIP3L, BIM and PUMA are all members of the BH3-only subfamily which belongs to the Bcl-2 gene family (29). These proteins are well characterized pro-apoptotic proteins, that also regulate autophagy. BNIP3 is activated by C2-ceramide in malignant gliomas and functions in C2-ceramide-induced autophagic cell death (7). Beclin 1, BNIP3 and BNIP3L transcriptions were shown in the present study to be activated by C2-ceramide. However, C2-ceramide did not cause A2780 autophagic cell death. Interestingly, a previous study has shown that a hypoxic microenvironment promoted BNIP3 and BNIP3L competing with beclin-1-Bcl-2 and beclin 1-Bcl-X<sub>L</sub> complexes, releasing beclin-1 from the complex and enhancing autophagy (30). There is currently little known regarding the expression and activity of BIM and PUMA in ceramide treated cells, however they are known to function in mitochondrial apoptosis (31). It is therefore hypothesized that C2-ceramide may regulate apoptosis and autophagy through FOXO3 transcription in A2780 cells. The specific molecular mechanism for C2-ceramide regulating A2780 cells death and survival remains to be elucidated.

In summary, the present study has demonstrated that C2-ceramide can induce cell apoptosis and autophagy in ovarian cancer cells. FOXO3 target genes, which related to cell death and autophagy, were upregulated by C2-ceramide. These findings provide a novel concept to ceramide-induced cell death mechanism, as well as a potential role of ceramide in anti-ovarian cancer therapy.

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References