Ceramide Induces Apoptosis and Growth Arrest of Human Glioblastoma Cells by Inhibiting Akt Signaling Pathways

Eun Chang Lee¹,a, Young Seok Lee¹,a, Nahee Park¹, Kwang Sup So¹, Young-Jin Chun¹,* and Mie Young Kim¹,*

¹College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea

Abstract
Ceramide is an important lipid mediator of extracellular signals that control various cellular functions, including apoptosis. In this study, we showed that ceramide induced apoptosis in U373MG human glioblastoma cells associated with G1 cell cycle arrest. Treatment of cells with ceramide increased proapoptotic Bax expression and inhibited the expression of antiapoptotic Bcl-2 and Bcl-xL. Ceramide also downregulated cyclin E, cyclin D1, cdk 2, and cdk4 which are involved in regulating cell cycle. In addition, ceramide suppressed phosphorylation of Akt, Bad, p70 S6 kinase, and 4E-BP1, suggesting the involvement of Akt/mTOR signaling pathway. Additionally, okadaic acid, an inhibitor of protein phosphatase 2A, partially blocked the ceramide mediated inhibition of phosphorylation of Akt and 4E-BP1. These results suggest that ceramide induces apoptosis in U373MG glioblastoma cells by regulating multiple signaling pathways that involve cell cycle arrest associated with Akt signaling pathway.

Key Words: Akt, 4E-BP1, Glioblastoma cells

INTRODUCTION
Ceramide has long been recognized as a lipid mediator of cell death (Obeid et al., 1993; Hannun, 1996). Many anticancer drugs such as doxorubicin, vincristine, etoposide, and paclitaxel exert their antitumor effect against cancer cells by inducing apoptosis associated with an increase of cellular ceramide (Jaffrezou et al., 1996; Herr et al., 1997; Cabot et al., 1999; Selzner et al., 2001). Aberrant or decreased ceramide signaling has been implicated in contributing to tumor progression and resistance to therapy (Selzner et al., 2001; Schenckov et al., 2001; Struckhoff et al., 2004).

Ceramide activates proapoptotic mechanisms in response to various stress stimuli, predominantly by acting ceramide-activated phosphatases and kinases, which in turn regulate PKC, Akt, c-Jun, and Bcl-2 family proteins (Hannun and Obeid, 2002). Akt inhibits apoptosis by inactivating proapoptotic proteins such as Bad, prosapase-9, and forkhead and by activating antiapoptotic proteins such as NFκB and cyclic adenosine monophosphate (cAMP)-response element binding protein (Kennedy et al., 1997; Brunet et al., 1999; Wendel et al., 2004). Recently, PI3-Kinase/Akt/mTOR pathways have emerged as an important cancer therapeutic target (Park et al., 2002; Sun et al., 2005; Armengol et al., 2007). p70 S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) are downstream effectors of PI3K/Akt/mTOR and frequently activated in many human cancer cells. 4E-BP1 has been suggested as a prognostic factor in ovarian cancer, in which increased levels of phosphorylated 4E-BP1 are associated with high-grade tumors and a poor prognosis (Wendel et al., 2004; Armengol et al., 2007). Recent studies have shown that inhibition of 4E-BP1 phosphorylation activates apoptosis (Hu et al., 2007; Barnhart et al., 2008). Hypophosphorylated form of 4E-BP1 induces apoptosis through its ability to sequester eIF-4E from a translationally active complex and reducing the rate of protein synthesis.

Malignant gliomas are the most frequent primary brain tumors in adults and the prognosis of patients remains poor (Vescovi et al., 2006; Nandi et al., 2008). Among the deregulated signaling pathways in glioblastoma, aberrant activation of the PI3K/Akt plays a critical role in the tumorigenesis of glioblastoma (Giussani et al., 2009). It has previously shown that ceramide induced apoptosis associated with inhibition of PI3-Kinase/Akt pathway in certain cancer cells including glioblastoma cells (Kim et al., 2008; Giussani et al., 2009). However, the mechanism by which ceramide regulates Akt signaling...
pathways in glioblastoma cells have not been elucidated yet. In this study, we demonstrated that ceramide induces apoptosis and cell cycle arrest in U373MG human glioblastoma cells through regulation of Akt and its downstream targets, 4E-BP1 and S6K. Moreover, our results show that PP2A plays an important role in mediating ceramide-induced inactivation of Akt-mediated signaling pathway.

**MATERIALS AND METHODS**

**Materials**

Fetal bovine serum was purchased from Welgene (Daegu, Korea), and ECL kit from GE Healthcare (Piscataway, NJ, USA). Antibodies to 4E-BP1 and phospho-70S6K (Thr 389) were from Cell Signaling Technology (Danvers, MA, USA), and antibodies to cyclin D1, cyclin E, CDK2, CDK4, p70S6K, Bcl-2, Bcl-xL, Bax, Bad, phospho-Bad (Ser136), Akt, phospho-Akt (Ser473), or GAPDH and HRP-conjugated secondary antibody were Santa Cruz Biotechnology (Santa Cruz, CA, USA). C6-ceramide was purchased from Sigma (St. Louis, MO, USA). Antibodies to 4E-BP1 and phospho-70S6K (Thr 389) were from Cell Signaling Technology (Danvers, MA, USA), and antibodies to cyclin D1, cyclin E, CDK2, CDK4, p4E-BP1, Bcl-2, Bcl-xL, Bax, Bad, phospho-Bad (Ser136), Akt, phospho-Akt (Ser473), or GAPDH and HRP-conjugated secondary antibody were Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cytochrome c was purchased from Sigma (St. Louis, MO, USA).

**Cell culture**

U373MG human glioblastoma cells were purchased from Cell Line Bank (Seoul, Korea). U373MG cells were grown in RPMI 1640 medium containing with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

**Cell proliferation assay**

Cell proliferation assays were performed by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). U373 MG cells were plated at 96-well plates at 1×10⁴ cells per well and cultured in the RPMI growth medium. At the indicated time points, the cell numbers in triplicate wells were measured at the absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt).

**Apoptosis assay**

Cells were washed with serum-free RPMI. Ceramide or vehicle (DMSO) was diluted into serum-free RPMI at the indicated concentrations. Cells were maintained in serum-free RPMI for 2 h before experiments. To analyze apoptosis, cells were treated with ceramide for 48 h and resuspended in buffer containing 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂, and pelleted by centrifugation. Cell pellets were washed in ice-cold PBS, resuspended in binding buffer, and incubated with FITC-conjugated Annexin V and propidium iodide (Zymed Laboratories, South San Francisco, CA) for 10 min at room temperature in the dark. Cells stained with Annexin V-FITC were washed with the binding buffer. Apoptotic cells were determined using fluorescence microscopy with excitation and emission wavelengths of 488 nm and 518 nm, respectively.

**Measurement of mitochondrial membrane potential (ΔΨm)**

To measure the ΔΨm of U373MG cells, the fluorescent probe JC-1 (5,5’6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazole carbocyanide iodide) was used. JC-1 exists as a monomer at low values of ΔΨm (green fluorescence), while it forms aggregates at a high ΔΨm (red fluorescence). Cells (6×10⁵) were collected by trypsinization, washed in warm phosphate buffered saline (PBS, pH 7.4) and incubated for 15 min at 37°C with 2 μM JC-1. Cells were pelleted at 1,000 rpm for 5 min, washed in warm PBS, resuspended with PBS, and analysed by flow cytometric analysis (Becton Dickinson FACScan, USA).

**Cell cycle analysis**

Cells were pelleted at 1,200 rpm and washed once with 1 ml of ice-cold PBS. The resulting pellets were resuspended in 1 ml of cold PBS, and ethanol (80%), pre-chilled at −20°C, were added with periodic vortexing. The resulting mixture was kept on ice for 60 min, and the cells were permeabilized in 0.5% Triton X-100, 20 μg/ml RNaseA, and 50 μg/ml propidium iodide in PBS. The samples were kept at 37°C for 30 min followed by flow cytometric analysis using the CellQuest program.

**Western blot analysis**

Proteins (30 μg/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, and were electrophoretically transferred onto Immobilon-P membrane. Blocking was performed in Tris-buffered saline containing 5% skimmed milk powder and 0.1% Tween-20. The membranes were probed with antibodies against p4E-BP1, p70 S6K, cyclin D1, cyclin E, CDK2, CDK4, Bcl-2, Bcl-xL, Bax, Bad, phospho-Bad, Akt, phospho-Akt, or GAPDH. Detection was performed with enhanced chemiluminescence (ECL) detection system (GE Healthcare). Protein content was determined with bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as a standard.

**RESULTS**

**Ceramide induces apoptotic cell death in U373MG glioblastoma cells**

The ability of ceramide to induce cell death was determined in the U373MG human glioblastoma cells. Cells were treated with different concentrations of ceramide for 24 h or 48 h, and cell viability was determined. Ceramide exerted a concentration-dependent inhibition of cell proliferation (Fig. 1A). Induction of apoptosis by ceramide was analyzed by detection of Annexin V-positive cells. Cells were treated with ceramide for 48 h and Annexin V-positive cells were determined using fluorescence microscopy. As shown in Fig. 1B, Annexin-positive cells were increased by treatment with ceramide, suggesting that a population of the cells underwent apoptotic cell death. To further investigate whether ceramide induces apoptosis, we evaluated reduction of mitochondrial membrane potential (ΔΨm). The opening of the permeabilization transition (PT) pore complex is thought to mediate release of proapoptotic proteins from the mitochondria (Green and Reed, 1998). One of the markers of the opening of PT pore is a decrease in mitochondrial membrane potential (ΔΨm). We used the cell-permeable JC-1 dye to monitor changes in mitochondrial membrane potential after ceramide treatment. In non-apoptotic cells with an intact membrane potential, the JC-1 dye accumulates and forms aggregates with a red fluorescence. In apoptotic and

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