Potentiation of Ceramide-Induced Apoptosis by p27kip1 Overexpression

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INTRODUCTION

Ceramide is an important lipid mediator of the cell functions including apoptosis (Obeid et al., 1993; Hannun, 1996). Apoptosis induced by a variety of inducers such as tumor necrosis factor-α, Fas ligation and chemotherapeutic agents and environmental stresses is associated with the hydrolysis of sphingomyelin accompanied by the accumulation of ceramide (Kim et al., 1991; Hannun and Obeid, 1995; Herr et al., 1997; Jaffrezou et al., 1996). Moreover, exogenous cell permeable ceramide mimics the action of these inducers and induces apoptosis in many different cell types. Ceramide is therefore considered to be a common mediator of apoptotic mechanism.

Many lines of evidence indicate that apoptosis is linked to cell cycle arrest, and several cell cycle regulators might be involved in response to apoptotic stimuli. Recent studies have suggested that cyclin dependent kinase (cdk) inhibitors may play important roles in inducing apoptosis (El-Deiry et al., 1994; Sherr and Roberts, 1999; Cheng et al., 1999). The INK4 family members, which include p15INK4B and p16INK4A specifically bind cdk4 and cdk6 and inhibit cyclin D association. The p21^{wild} (p21) protein acts as a downstream mediator of the tumor suppressor p53 that functions as the G1 phase checkpoint, resulting in either G1 arrest or apoptosis. p27kip1 (p27), a member of a family of proteins that includes p21 and p57kip2 is a universal cdk inhibitor that negatively regulates G1 cdk's. p27 also acts as a putative tumor suppressor, and promoter of apoptosis (Katayose et al., 1997). However, the molecular mechanism on the role of p27 in the regulation of apoptosis is still not clear.

In addition to its role in apoptosis, ceramide has been shown to be involved in cell cycle arrest. Ceramide induces cell cycle arrest through the activation of the retinoblastoma gene product (pRb) in Molt-4 cells (Dbaibo et al., 1995), and also apoptosis through the p53-independent p21 induction in hepatocarcinoma cells (Oh et al., 1998). Previously, we have demonstrated that treatment of HL-60 cells with ceramide resulted in G1 phase elevation followed by apoptotic cleavage associated with increase in the level of cdk inhibitor p27, indicating that cell death and cell cycle pathways are connected in ceramide-mediated apoptotic process (Kim et al., 2000).

In this study, we examined the role of p27 in the induction of apoptosis by ceramide. We showed that overexpression of p27 enhanced cell death, DNA fragmentation and cytochrome c release from mitochondria in ceramide-treated HL-60 cells. These results show that p27 is the important component that is involved in the induction of apoptosis mediated by ceramide.
MATERIALS AND METHODS

Materials

C6-ceramide was obtained from Sigma (St. Louis, MO, USA). Lipofectamine was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal bovine serum was from Gibco BRL (Gaithersburg, MD, USA), ECL kit from Amersham Pharmacia Biotech. (Piscataway, NJ, USA), Antibody to cytochrome c was from Pharmingen (San Diego, CA, USA). Antibodies to p27, pRb, Bax, PARP and HRP-conjugated secondary antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell viability and internucleosomal DNA fragmentation

HL-60 promyelocytic leukemia cells were purchased from Korea Cell Line Bank (Seoul, Korea) and were maintained at 37°C in a 5% CO2 incubator. HL-60 cells were washed with serum-free RPMI. Ceramide or vehicle was diluted into serum-free RPMI at the indicated concentrations. Cell viability was determined by the trypan blue exclusion method at each time point. To assess DNA fragmentation, total genomic DNA was extracted using a lysis buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/mL proteinase K) at 50°C for 15 h. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation and then dissolved in a TE buffer (pH 8.0). After treatment with RNase A (0.1 mg/mL) for 1 h at 37°C, the fragmentation of genomic DNA was evaluated by 1.5% agarose gel electrophoresis and ethidium bromide staining.

Western blot analysis

Cells were solubilized with ice-cold lysis buffer containing 1% Triton X-100, 50 mM NaCl, 25 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin. Insoluble materials were removed by centrifugation at 10,000×g for 10 min. Extracted proteins (50 μg/well) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% 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 p27, PARP, cytochrome c, or Bax. Detection was performed with ECL system. Protein content was determined with Bradford method using bovine serum albumin as a standard.

RESULTS

Ceramide induces p27 protein expression and pRb dephosphorylation

It has been shown that p27 is a G1 Cdk inhibitor that may be functionally linked to induction of apoptosis induced by ceramide in HL-60 cells (Kim et al., 2000). pRb is a critical regulator of the Go/G1 cell cycle arrest, and since it has previously been shown that ceramide-induced cell cycle arrest can be associated with apoptosis, we analyzed the pRb phosphorylation status and p27 protein expression in ceramide treated HL-60 cells. Treatment with ceramide resulted in increased p27 protein level (Fig. 1A). In agreement with other cell lines (Dbaibo et al., 1995), ceramide induced pRb dephosphorylation in a time- and dose-dependent manner (Fig. 1B). pRb was decreased, and a higher proportion was in the dephosphorylated state when HL-60 cells were treated with ceramide. These observations suggest that p27 associates with cell cycle arrest in ceramide-induced apoptosis in HL-60 cells.