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Potentiation of Ceramide-Induced Apoptosis by $p27^{kip1}$ Overexpression

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The cyclin-dependent kinase inhibitor $p27^{kip1}$ (p27) has been implicated in the regulation of cell cycle and apoptosis. Recently, we have demonstrated that ceramide induces apoptotic cell death associated with increase in the level of p27 in HL-60 cells. In the present study, we showed that overexpression of p27 increases ceramide-induced apoptotic cell death in HL-60 cells. Furthermore, overexpression of p27 accelerated DNA fragmentation, PARP cleavage and cytochrome c release induced by ceramide. In addition, ceramide induced Bax expression independent of p27. These findings indicate that enhanced effect on apoptosis by p27 is associated with mitochondrial signaling which involves cytochrome c release.

Key words: Ceramide, p27, Apoptosis, Cytochrome c release

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 $p15^{INK4B}$ and $p16^{INK4A}$ specifically bind cdk4 and cdk6 and inhibit cyclin D association. The $p21^{waf1}$ (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. $p27^{kip1}$ (p27), a member of a family of proteins that includes p21 and $p57^{kip2}$, is a universal cdk inhibitor that negatively regulates G1 cdks. 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.

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MATERIALS AND METHODS

Materials

C_6-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% CO_2 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.

Transfection of p27 in HL-60 cells

The full-length human p27 cDNA insert was released by digesting pBluescript II SK-hp27 with *Eco*RI (Promega, Madison, USA) and was subcloned into the *Eco*RI site in pcDNA3. Transfection of HL-60 cells was carried out with the use of LipofecAMINE reagent (Invitrogen, USA) as described previously (Hawley-Nelson *et al.*, 1993). After transfection, cultures were maintained in serum-depleted medium for 15-17 h prior to ceramide treatment.

Subcellular fractionation

The basic methodology for the preparation of mitochondria and cytosol fractions was modified from a previous report (Kim *et al.*, 2001). Briefly, HL-60 cells (3×10^6) at the end of the treatment were harvested and washed with ice-cold PBS. Cells were resuspended in 500 μL of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, and 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin, and 1 μg/mL chymostatin). To lyse the cells, the cell suspension was passed five times through a 26-gauge needle fitted to a syringe. Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at 1,000×g at 4°C for 10 min. The resulting supernatant was subjected to 10,000×g centrifugation at 4°C for 20 min. The pellet fraction (i.e., mitochondria) was first washed with the above buffer A containing sucrose and then solubilized in 50 μL of TNC buffer (10 mM Tris-acetate, pH 8.0, 0.5% NP-40, 5 mM $CaCl_2$). The supernatant was recentrifuged at 100,000×g at 4°C for 1 h to generate cytosol.

Western blot analysis

Cells were solubilized with ice-cold lysis buffer containing 1% Triton X-100, 50 μM 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 increase of 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.

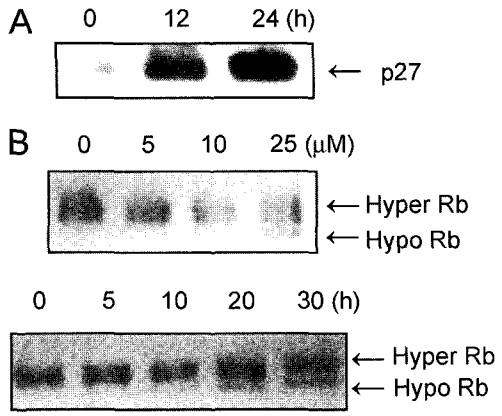

Fig. 1. Ceramide induces p27 expression (A) and pRb dephosphorylation (B). HL-60 cells were treated with 20 μM C_6-ceramide for the indicated time points, or treated with varying doses as indicated. Equal amounts of cell lysates were separated by SDS-PAGE, followed by Western blot with anti-p27 antibody or pRb-specific antibody.

Overexpression of p27 potentiates ceramide-induced apoptotic cell death

To examine a possible role of p27 in ceramide-mediated apoptosis, we have established HL-60/p27 cells and compared the sensitivity of HL-60 and HL-60/p27 cells to ceramide treatment. The extent of p27 expression of HL-60/p27 cells was confirmed by Western blot (Fig. 2A). As shown in Fig. 2B, ceramide induced cell death in a dose-dependent manner, with a concentration of 20 μM ceramide resulting in death of 50% of cell population. Overexpression of p27 resulted in 40% increase of cell death at the same concentration of ceramide. Ceramide-induced DNA fragmentation was also significantly enhanced in HL-60/p27 cells compared with the control HL-60 cells (Fig. 3A). Proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) by caspase is a predominant biochemical hallmark of apoptosis. To further analyze apoptosis, PARP cleavage by ceramide during the course of apoptosis was examined. Within 24 h of ceramide treatment, cleavage of PARP into an 89 KDa fragment was observed in HL-60 cells (Fig 3B). In contrast, PARP cleavage was evident at 6 h after ceramide treatment in HL-60/p27 cells.

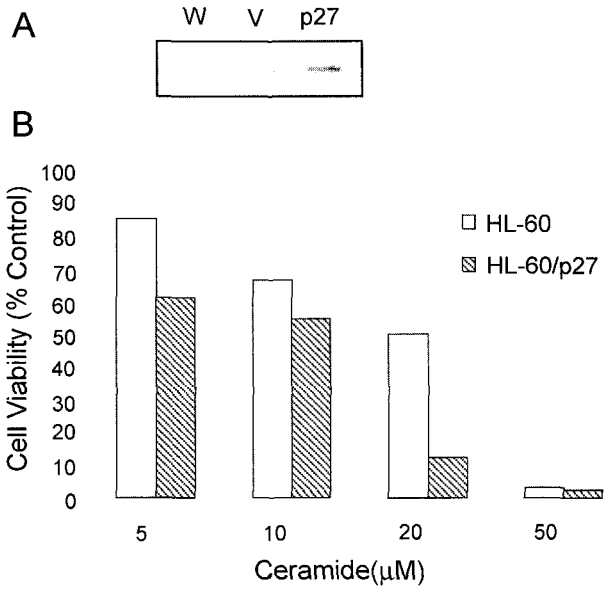

Fig. 2. Overexpression of p27 accelerates cell death induced by ceramide. A. p27 protein levels in wild- type (W), vector-(V) and p27-transfected HL-60 cells were determined by Western blot. B. Cells were treated with varying doses as indicated and cell viability was measured by trypan blue exclusion method.

Overexpression of p27 accelerates ceramide-induced cytochrome c release

Release of cytochrome c from mitochondria into cytosol is an important event of apoptosis signaling. It has been reported that levels of cytosolic cytochrome c are increased in response to various apoptotic stimuli such as staurosporine, etoposide, and ionizing radiation (Kluck *et*

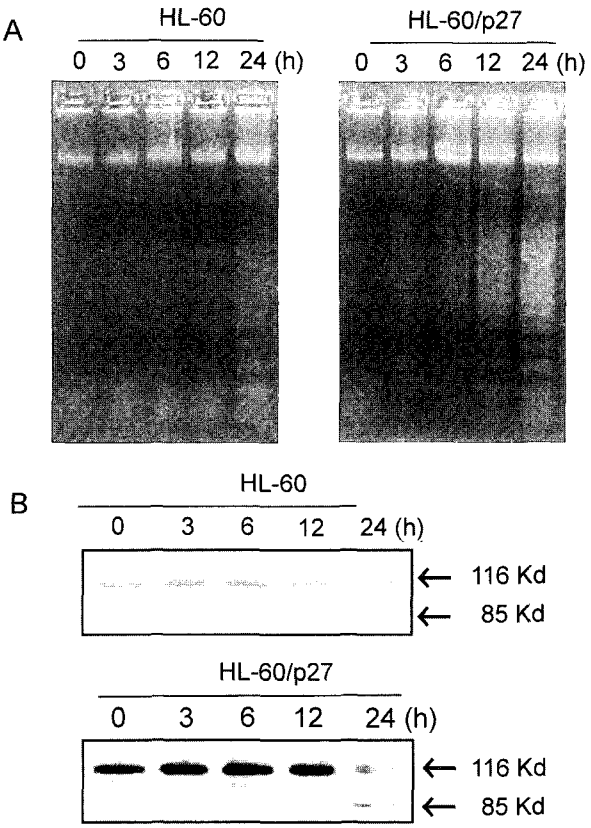

Fig. 3. Overexpression of p27 increases ceramide-induced apoptotic cell death. A. Cells were treated with 20 μM C_6-ceramide and harvested at the times indicated. Total genomic DNA was prepared, separated on agarose gel and visualized by ethidium bromide staining. B. Western blot analysis of PARP cleavage of HL-60 and HL-60/p27 cells. Cells were treated with 20 μM C_6-ceramide and harvested at the times indicated.

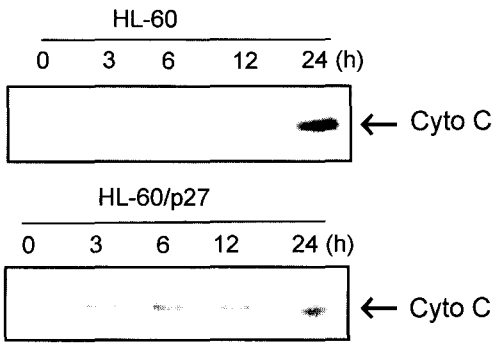

Fig. 4. Cytochrome c release induced by ceramide was accelerated in p27 overexpressed cells. Cells were treated with 20 μM C_6-ceramide. Cytosolic and mitochondrial fractions were prepared at the indicated times, separated by 12% SDS-PAGE, and immunoblotted with a mouse anti-cytochrome c antibody.

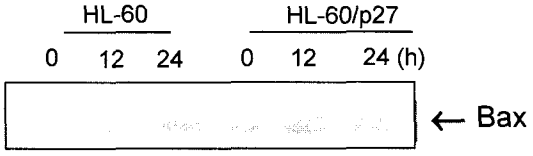

Fig. 5. p27 does not increase the expression of Bax protein. Cells were treated with 20 μM C_6-ceramide and harvested at the times indicated. Equal amounts of lysates were loaded, separated by SDS-PAGE and immunoblotted with anti-Bax antibody.

al., 1997; Li *et al.*, 1997). Therefore, we tested whether level of cytosolic cytochrome c is increased in response to ceramide, and determined the effect of p27 on cytochrome c release. Cytochrome c in the cytosol fraction was increased significantly after 24 h treatment of ceramide in HL-60 cells (Fig. 4). Ceramide-induced cytochrome c release was detected at earlier time in p27-transfected cells.

p27 does not increase the expression of Bax protein

The mitochondrial release of cytochrome c is regulated by Bcl-2 family proteins, Bcl-2 and Bax (Perlman *et al.*, 1999). To determine whether the effect of p27 on cytochrome c release could be mediated by the Bcl-2 family proteins, we compared the expression of Bax protein levels in HL-60 and HL-60/p27 cells. As shown in Fig. 5, the expression of the Bax protein level was increased by ceramide treatment in HL-60 cells and HL-60/p27cells. The expression of Bax protein levels was not potentiated over 24 h periods in ceramide-treated HL-60/p27 cells.

DISSCUSION

It is known that p27 is recognized not only as a negative regulator of the cell cycle but also as an inducer of apoptosis. p27 mediates G1 arrest induced by TGF-β, growth factor deprivation, or contact inhibition (Polyak *et al.*,1994). p27 inhibits cdk universally but are preferential for cdk2 in cells arrested in G1 (Harvat *et al.*, 1997). It has been demonstrated that overexpression of p27 induces apoptosis in several different human cancer cell lines (Katayose *et al.*, 1997). In many human cancers, reduced p27 expression is frequently observed. The reduced expression of p27 is reported to correlate with tumor progression and poor prognosis in human breast and lung cancer (Lloyd *et al.*, l999). Thus, p27 may participate in tumor suppression by inhibiting abnormal cell-cycle progression. To clarify the role of p27 in the regulation of ceramide-induced apoptosis, we used p27-overexpressed cells. We have shown that ceramide-induced cell death was potentiated in p27-overexpressed HL-60 cells. We also found that p27 potentiated DNA fragmentation and PARP cleavage induced by ceramide, indicating that p27 is an important regulator in mediating ceramide-induced apoptosis. To further understand the molecular mechanism by which p27 induces apoptosis in HL-60 cells, we evaluated whether mitochondrial signaling is involved in the p27-regulated apoptosis. Mitochondria play a major role in the apoptotic process through the release of cytochrome c and other proapoptotic proteins that normally reside in the intermembrane space between the inner and outer mitochondrial membrane (Liu *et al.*, 1996; Li *et al.*, 1997). It is known that the release of cytochrome c from mitochondria is an important process during apoptosis induced by ceramide (Kim *et al.*, 2001). Therefore, we examined whether p27 may affect on the cytochrome c release induced by ceramide. As expected, p27 is found to influence the mitochondrial death signaling because overexpression of p27 enhanced the cytosolic cytochrome c level in ceramide-treated HL-60 cells.

It is reported that expression of p27 is associated with Bax expression in squamous cell carcinoma, suggesting the overexpression of p27 might induce apoptosis in cancer cells through elevation of Bax expression (Fujieda *et al.*, 1999). Bax is a proapoptotic member of the Bcl-2 family that resides in the cytosol and translocates to mitochondria upon induction of apoptosis (Pastorino *et al.*., 1998). In addition, Bax has been known to cause cytochrome c release from mitochondria and caspase activation in cell-free extracts and in cells treated with apoptosis-inducing agents (Finucane *et al.*, 1999; Kluck *et al.*, 1997). We have shown that the Bax protein levels were increased in both HL-60 and HL-60/p27 cells during a 24 h treatment with ceramide, but there is no significant difference on Bax expression between both cells, suggesting that Bax acts upstream or independent of p27.

Ceramide induces pRb hypophosphorylation and increase of p27 protein level. pRb is a multifunctional protein

that binds to transcription factors and kinases to regulate both cell growth and apoptosis (Bowen *et al.*, 2003). Unphosphorylated pRb binds the transcription factor E2F, while bound to pRb, E2F cannot promote transcription of a group of genes necessary for DNA synthesis, resulting in G1 cell cycle arrest. The pRb-E2F blocking mechanism is relieved when pRb is phosphorylated during late G1 by cyclin E-cdk2 and cyclin D-cdk4, allowing the cells to proceed from G1 to S phase. It was shown that ceramide inhibited the kinase activities of cdk2 and cdk4, which is associated with the increase of p27 protein level, resulting in G1 arrest of cells followed by apoptotic cleavage in HL-60 cells (Kim *et al.*, 2000). From these results, it is suggested that ceramide decreases pRb phosphorylation through the inhibition of kinases by p27 that is linked to the execution of apoptosis in HL-60 cells.

Regulation of cdk inhibitor expression, and particularly that of p27, has emerged as a critical aspect of growth control in normal mammalian cells and as a frequent point of dysregulation in a wide variety of tumors (Foster *et al.*, 2003). Recent reports suggest that p27 regulation occurs predominantly at the levels of translation and protein stability through ubiquitin/proteasome-dependent and -independent pathways (Foster *et al.*, 2003; Alkarain and Singerland, 2003). p27 protein expression is also regulated at the level of transcription. Little is known regarding the molecular mechanism by which ceramide induces p27 overexpression. Further studies will be required to identify the mechanism by which ceramide induces p27 upregulation.

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