Journal of Biochemistry and Molecular Biology, Vol. 31, No. 4, July 1998, pp. 333–338

JOURNAL OF
Biochemistry
and
Molecular Biology
© BSRK & Springer-Verlag 1998

Ceramide-Mediated Cell Death Was Accompanied with Changes of c-Myc and Rb Protein

Soon-Ok Moon* and Jin-Woo Lee
Division of Life Science, The University of Suwon, Suwon 445-743, Korea

Received 26 January 1998
Revised 6 April 1998

The sphingomyelin cycle and ceramide generation have been recognized as potential growth suppression signals in mammalian cells. Ceramide has been shown to induce differentiation, cell growth arrest, senescence, and apoptosis. Although the intracelluar target for the action of ceramide remains unknown, recent studies have demonstrated the role of cytosolic ceramide-activated protein phosphatase(CAPP). In this study, the cytotoxic effect of C2-ceramide, a synthetic cell-permeable ceramide analog, on HEp-2 cells and the mechanism by which ceramide induces cell death were investigated. The addition of exogenous C2-ceramide resulted in a concentration dependent cell death. Okadaic acid, a potent inhibitor of CAPP, enhanced ceramide-mediated cell death, which suggests that CAPP is not involved in this process. To understand the mechanism of action of ceramide, we studied the relationship between ceramide and c-Myc and pRb which are defined components of cell growth regulation. Western blot analyses revealed that C2-ceramide (10 μM) induced c-Myc down-regulation, but there were no significant changes in pRb. However, treatment of okadaic acid (10 nM) enhanced c-Myc and pRb down-regulation. Reduction of the amount of c-Myc and pRb occurred during HEp-2 cell death. These results suggest that the cytotoxic effect of ceramide in HEp-2 cells may not be mediated through the action of CAPP and that the downstream target for ceramide is c-Myc and pRb.

Keywords: Cell death, Ceramide, c-*myc*, Okadaic acid, pRb.

* To whom correspondence should be addressed.
Tel: 82-331-220-2289; Fax: 82-331-220-2289
E-mail: somun@mail.suwon.ac.kr

Introduction

Although the molecular and biochemical understanding of mitogenic pathways have been established in the last twenty years, growth suppressor and cell death pathways are only now being developed. Recently, the sphingomyelin cycle has emerged as an important pathway of antiproliferation and apoptosis. The sphingomyelin cycle was initially described by Okazaki *et al.* (1990) in human HL-60 cells. Various extracellular agents, including transforming growth factor α, γ-interferon, dexamethasone, complement, vit.D3, interleukin-1, nerve growth factor, and brefeldin A, stimulate membrane sphingomyelinase (see a review by Obeid and Hannum, 1995). Sphingomyelinase hydrolyzes membrane sphingomyelin, generating phosphorylcholine and ceramide. Ceramide serves as a second messenger which mediates the effects of extracellular agents on cell growth, differentiation, and apoptosis (Okazaki *et al.*, 1990; Obeid *et al.*, 1993; Jarvis *et al.*, 1994; Kim *et al.*, 1994). Ceramide also regulates cell senescence (Venable *et al.*, 1995). Endogenous levels of ceramide increased as the cell entered the senescent phase, and the addition of exogenous ceramide to WI-38 human diploid fibroblast inhibited DNA synthesis and induced a senescent phenotype. Recently, multiple intracellular targets for the action of ceramide have been identified. These include the membrane ceramide-activated protein kinase (CAPK) (Mathias *et al.*, 1991), cytosolic ceramide-activated protein phosphatase (CAPP) (Dobrowsky and Hannum, 1992), and the zeta isoform of protein kinase C (PKC) (Lozano *et al.*, 1994). Thus, it is being elucidated that ceramide may induce apoptosis and growth arrest by stimulating a distinct set of phosphatases and kinases which transduce signals to downstream effectors. Downstream effects of ceramide include c-*myc* down-regulation (Kim *et al.*, 1991) and dephosphorylation of pRb (Chao *et al.*, 1992), activation of nuclear localization of NF-κB (Yang *et al.*, 1993), increase of cyclooxygenase,

and decrease of phospholipase D (Ballou *et al.*, 1992; Gomez-Munoz *et al.*, 1994). Among these downstream targets, c-Myc and pRb have been known to be important regulators of cell growth control and oncogenesis.

pRb is a nuclear phosphoprotein that has been proposed to play a role in regulation of the cell cycle (Goodrich *et al.*, 1991). The hypophosphorylated form of pRb is an active form in growth inhibition (Weinberg, 1991). Growth arrest and senescence were accompanied by dephosphorylation of pRb (Dbaibo *et al.*, 1995; Venable *et al.*, 1995). The addition of exogenous ceramide to leukemia cell lines such as Molt-4 resulted in cell cycle arrest which was accompanied by dephosphorylation of pRb (Dbaibo *et al.*, 1995). However, this pathway was independent of ceramide-induced apoptosis. Moreover, the addition of exogenous diacylglycerol and phorbol 12-myristate 13-acetate (PMA) prevented ceramide-induced apoptosis but not ceramide-induced cell cycle arrest (Jarvis *et al.*, 1994; Obeid and Hannum, 1995), which suggests that PKC blocks the ceramide-induced apoptosis but not the cell cycle arrest. Recent evidence showed that pRb suppresses ceramide-induced apoptosis in human bladder tumor cells (McConkey *et al.*, 1996). These studies imply that ceramide regulates the cell cycle arrest and apoptosis by distinct intracellular pathways.

In various cell types, ceramide down-regulates c-*myc* (Kim *et al.*, 1991; Wolff *et al.*, 1994; Obeid and Hannum, 1995). Even though the direct molecular target for c-*myc* down-regulation has not been identified, studies have begun to implicate CAPP as a potential mediator of ceramide. In cells, ceramide caused c-*myc* down-regulation which was inhibited by okadaic acid (Wolff *et al.*, 1994) which is the specific inhibitor of CAPP (Dobrowsky and Hannum, 1992). However, the addition of okadaic acid to cells treated with PMA appeared to enhance c-*myc* down-regulation. These results show that activation of CAPP is a distinct signaling pathway for ceramide (Wolff *et al.*, 1994).

To elucidate the mechanism of action of ceramide, it is important to define the relationship between ceramide and other transduction molecules which are involved in specific cellular processes such as apoptosis, proliferation, and differentiation. In this study, we attempted to investigate the effect of ceramide on HEp-2 cells and to identify the relationship between ceramide and c-Myc protein and pRb by using a cell permeable analog of ceramide. Our results show that ceramide induced HEp-2 cell death, which was accompanied by the decrease of c-Myc protein and pRb, and this process was enhanced by okadaic acid.

Materials and Methods

Cell culture The human epidermoid carcinoma HEp-2 cell line was obtained from American Type Culture Collection (ATCC) and cultured using minimum essential alpha medium supplemented with 10% heat inactivated fetal bovine serum (Gibco, Grand Island, USA), penicillin G (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator, containing 5% CO_2 in air at 37°C.

Cell viability Cell viability was determined by XTT (sodium 3′-(1-(phenylamino-carbonyl)-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay as described in the manufacturer's manual (Boehringer Mannheim, Mannheim, Germany). XTT assay was developed as a assay for measurement of cell viability and cytotoxicity. Tetrazolium XTT is useful for the quantitation of metabolically active cells because XTT is metabolized by mitochondrial dehydrogenases to form a formazan dye which can be measured by spectrometrical absorbance at 490 nm. When cells were treated with C2-ceramide (Amersham, Arlington Heights, USA) and okadaic acid (Boehringer Mannheim), cells were seeded at densities of 5×10^5/ml in serum-free medium supplemented with insulin, transferrin, and Selenium A (Gibco). After 24 h, cells were treated with C2-ceramide or okadaic acid and incubated for 18 h, and XTT assay was performed. The assay was followed according to instructions provided by the manufacturer (Boehringer Mannheim, cell proliferation kit II XTT)

Measurement of DNA fragmentation DNA fragmentation was performed by the method described by Bissonnette *et al.*, (1994). HEp-2 cells were cultured in 100 × 20 mm tissue culture dishes as described above in the presence of 10 μM ceramide for 3 or 6 h. Cells were washed in 1 ml of phosphate buffered saline (PBS), lysed in 400 μl of lysis buffer (5 mM Tris-Cl pH 8.0, 10 mM EDTA, 0.5% triton X-100) for 15 min at room temperature, and centrifuged at 15,000 × g for 20 min. After incubation of lysate with 1 μl of RNase (10 mg/ml) for 2 h at 37°C and then 1 μl of proteinase K (20 mg/ml) plus 1% SDS for 2 h at 50°C, phenol extraction and ethanol precipitation followed. The pellet was dissolved in TE and DNA was fractionated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Western blot analysis After HEp-2 cells were treated with C2-ceramide or okadaic acid as described above on 100 × 20 mm tissue culture dishes, cells were lysed directly in SDS polyacrylamide gel loading buffer. Cell lysate was heated to 100°C for 5 min and protein was quantitated using the Bio-Rad microassay kit. After SDS-polyacryamide gel electrophoresis, proteins were electrophorytically transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, USA) by using an electrotransfer kit (Hoefer, San Francisco, USA). The membrane was blocked for 1 h with blocking buffer [0.2% (w/v) I-BlockTM, 1× PBS, 0.2% tween-20, Tropix, Bedford, USA] at room temperature and incubated for 1 h with primary antibody, which was rabbit polyclonal anti-pRb antibody (Ab-2, oncogene science) or rabbit polyclonal anti-c-Myc antibody (UBI). The membranes were washed in 0.1% (v/v) Tween-20/PBS and incubated with biotinylated secondary antibody in blocking buffer for 1 h at room temperature. After washing, membranes were incubated in alkaline phosphatase-conjugated streptoavidin containing blocking buffer for 30 min at room temperature. Bands were visualized by incubating the membrane in substrate solution containing CSPD (Tropix) and exposing it to X-ray film (Kodak, New York, USA).

Results and Discussion

Cytotoxic effect of C2-ceramide on HEp-2 cells To investigate the effect of ceramide on the HEp-2 cells, various concentrations of C2-ceramide (N-acetylsphingosine), a synthetic cell-permeable ceramide analog, was added to exponentially growing HEp-2 cells. The development of this cell-permeable analog of ceramide has allowed examination of the cellular activities of ceramide. Exogenously added C2-ceramide exerts a cytotoxic effect on HEp-2 cells (Fig. 1). Induction of cytotoxicity was dose-dependent with maximal response occurring at 40 μM C2-ceramide. Similar concentration of C2-ceramide has been shown to induce inhibition of DNA synthesis of WI-38 human fibroblast (Jarvis *et al.*, 1994), activate CAPP (Law and Rossie, 1995), CAPK (Westwick *et al.*, 1995), and down-regulate c-*myc* transcription (Wolff *et al.*, 1994). Recent evidences suggest that a significant component of the growth suppression induced by ceramide appears to be as a result of the induction of apoptosis. Ceramide induced apoptosis in various cell types (Obeid *et al.*, 1993; Jarvis *et al.*, 1994; Hannum and Obeid, 1995). These results imply that C2-ceramide may exert a cytotoxic effect on HEp-2 cells through apoptosis. To test this hypothesis, we performed a DNA fragmentation assay. Treatment of 10 μM C2-ceramide resulted in formation of a larger fragment than the internucleosomal fragment, which is considered as an early hallmark of apoptosis. Even though we failed to detect internucleosomal fragmentation, C2-ceramide may induce apoptosis in HEp-2 cells without oligonucleosomal DNA fragmentation, since it is now recognized that the generation of oligonucleosomal DNA fragmentation may not accompany apoptotic cell death in all cell types. For example, in MCF-7 breast adenocarcinoma cells, distinct 300–50 kbp DNA fragments have been demonstrated following exposure to antineoplastic agents, yet these cells do not exhibit oligonucleosomal DNA fragments (Oberhammer *et al.*, 1993). Okadaic acid-induced apoptosis in hepatocytes was not accompanied by oligonucleosomal DNA fragmentation (Boe *et al.*, 1991). To confirm whether or not ceramide induces apoptosis in HEp-2 cells, further studies including an observation of the morphological features are required.

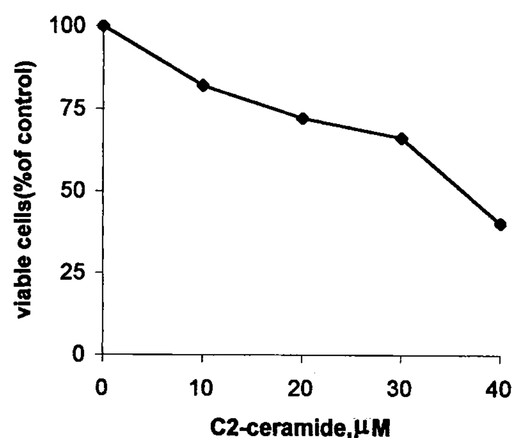

Fig. 1. Cytotoxic effect of C2-ceramide on HEp-2 cells. HEp-2 cells were treated with indicated concentrations of C2-ceramide for 18 h. XTT assay was performed as described in Material and Methods.

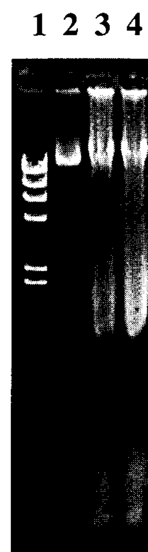

Fig. 2. Effect of C2-ceramide on DNA fragmentation. HEp-2 cells were treated with 10 μM C2-ceramide for 0 h (lane 2), 3 h (lane 3), or 6 h (lane 4). DNA was prepared and analyzed as described in Material and Methods. Lane 1 is the molecular weight marker, λ/*Hin*dIII.

Okadaic acid enhanced ceramide-induced cell death Previous studies show that ceramide induces apoptosis and growth arrest by stimulating CAPK (Mathias *et al.*, 1991) and CAPP (Dobrowsky and Hannum, 1992). To investigate whether C2-ceramide induced HEp-2 cell death by stimulating CAPP, the effect of okadaic acid, an inhibitor of CAPP, on the ceramide-mediated cell death was investigated. Okadaic acid (10 nM) was added to HEp-2 cells in the presence or absence of C2-ceramide (10 μM) (Fig. 3, panels a and b). Treatment of HEp-2 cells with ceramide or okadaic acid reduced cell viability by 18% and 54%, respectively (Fig. 3b). However, addition of okadaic acid with ceramide enhanced cell death up to 63%, which implies that CAPP may not play a role in ceramide-mediated cell death. We also observed cell morphology by phase contrast microscopy. The addition of okadaic acid and ceramide made cells round up and detached them from the substratum (Fig. 3a). This result is opposite to the previous study which demonstrated that okadaic acid blocked the effects of ceramide (Wolff *et al.*, 1994). Okadaic acid was also shown to prevent irradiation-induced DNA fragmentation in Burkitt lymphoma cells

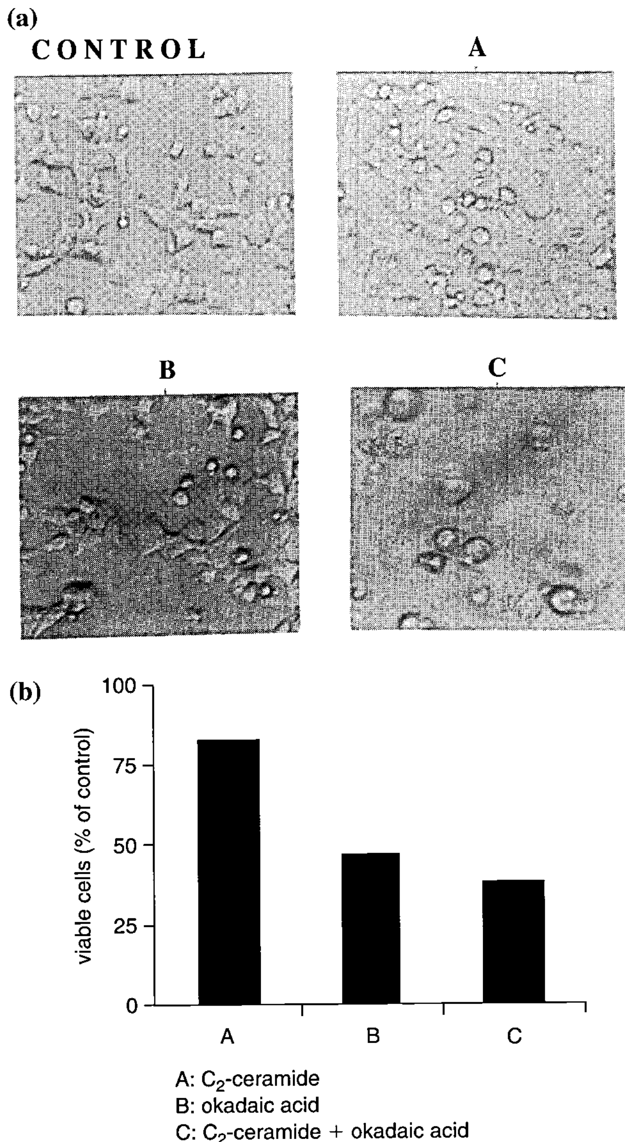

Fig. 3. Cytotoxic effect of C2-ceramide and okadaic acid on HEp-2 cells. HEp-2 cells were treated with 10 μM ceramide (A), 10 nM okadaic acid (B), or 10 μM ceramide plus 10 nM okadaic acid (C) for 18 h. (a) Morphological changes were observed by phase contrast microscope (×200). (b) XTT assay was performed.

(Song and Lavin, 1993). However, okadaic acid has also been shown to induce apoptosis in hepatocytes (Boe *et al.*, 1991) which is similar to our result. These results indicate that there are several signal transduction pathways in apoptosis which are specific for different cell lines.

Ceramide and okadaic acid down-regulate c-*myc* protein and pRb synergistically Recently, it has been shown that C2-ceramide is a potent and specific inducer of dephosphorylation of pRb in several cell types (Dbaibo *et al.*, 1995; Venable *et al.*, 1995) where ceramide also induced growth arrest. Therefore, it is suggested that pRb acts as a downstream target for a ceramide-dependent pathway of growth arrest. To investigate whether ceramide induces changes in the level of pRb during HEp-2 cell death, we performed a Western blot analysis. Figure 4 shows that neither C2-ceramide nor okadaic acid induces significant changes in pRb. However, addition of C2-ceramide and okadaic acid together synergistically reduced pRb. This result may reflect two distinct intracellular pathways induced by ceramide, such as cell cycle arrest and apoptosis which may be accompanied by dephosphorylation and reduction of the level of pRb, respectively. In our study, the degree of cell death is correlated with the reduction of pRb (Figs. 3 and 4). These results support the recent evidence that pRb suppressed ceramide-induced apoptosis in human bladder tumor cells (McConkey *et al.*, 1996) and suggest that down-regulation of pRb is a downstream target of ceramide plus okadaic acid-induced cell death.

One of the well known effects of ceramide was down-regulation of c-*myc* mRNA (Kim *et al.*, 1991; Wolff *et al.*, 1994). To investigate the relation between ceramide-induced cell death and c-*myc*, we performed a Western blot analysis using anti-c-Myc antibody. Figure 5 shows that C2-ceramide or okadaic acid down-regulates c-Myc and the treatment of C2-ceramide plus okadaic acid synergistically down-regulates c-*myc*. Down-regulation of c-*myc* by ceramide has been shown to be inhibited by okadaic acid (Wolff *et al.*, 1994), which provides evidence of a role of CAPP in this process. However, the addition of okadaic acid enhanced the ability of PMA to down-regulate c-*myc* transcript (Wolff *et al.*, 1994). These results demonstrate the existence of at least two distinct pathways in the regulation of c-*myc* expression. Although a direct molecular target for the action of ceramide in c-*myc* down-regulation is not known, our result implies that the activation of CAPP is not involved. However, from our result, we can suggest a possible role of the zeta isoform of PKC which is known as an intracellular target of ceramide (Lozano *et al.*, 1994) in this process, since the

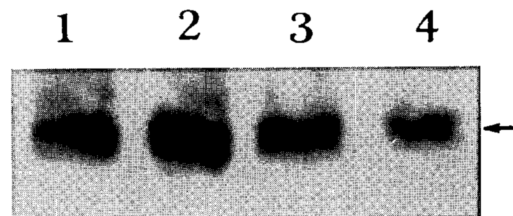

Fig. 4. Effect of C2-ceramide on pRb. HEp-2 cells were treated without (lane 1) or with 10 μM ceramide (lane 2), 10 μM okadaic acid (lane 3), or 10 μM ceramide plus 10 nM okadaic acid (lane 4) for 18 h. Western blot was performed with anti-pRb antibody as described in Material and Methods. The arrow indicates the 105 kDa pRb.

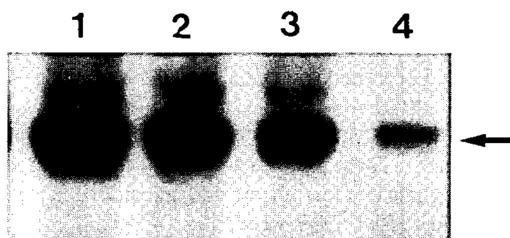

Fig. 5. Effect of C2-ceramide on c-Myc protein. HEp-2 cells were treated without (lane 1) or with 10 μM ceramide (lane 2), 10 nM okadaic acid (lane 3), or 10 μM ceramide plus 10 nM okadaic acid (lane 4) for 18 h. Western blot was performed with anti-c-Myc antibody as described in Material and Methods. The arrow indicates the 64 kDa c-Myc.

effect of okadaic acid on ceramide action was similar to that on PMA (Wolff *et al.*, 1994).

It becomes more important to define the relationship between ceramide and other defined components to understand the mechanism of action of ceramide. In this study, we showed the involvement of c-Myc and pRb down-regulation in ceramide- and okadaic acid-mediated cell death.

Acknowledgements This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Bioproducts Research Center at Yonsei University (94K4-0402-07-00-1).

References

Ballou, L. R., Chao, C. P., Holness, M. A., Barker, S. C. and Raghow, R. (1992) Interleukin-1-mediated PGE2 production and sphingomyelin metabolism. Evidence for the regulation of cyclooxygenase gene expression by sphingosine and ceramide. *J. Biol. Chem.* **267**, 20044–20050.

Bissonnette, R. P., Shi, Y., Mahboubi, A., Glynn, J. M. and Green, D. R. (1994) c-*myc* and apotosis; in *Apoptosis II: The Molecular Basis of Apoptosis in Disease*, pp. 327–356, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Boe, R., Gjertsen, B. T. G., Vintermyr, O. K., Houge, G., Lanotte, M. and Doskeland, S. O. (1991) The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. *Exp. Cell Res.* **195**, 237–246.

Chao, R., Khan, W. and Hannum, Y. A. (1992) Retinoblastoma protein dephosphorylation induced by D-erythro-sphingosine. *J. Biol. Chem.* **267**, 23459–23462.

Dbaibo, G. S., Pushkareva, M. Y., Jayadev, S., Schwarz, J. K., Horowitz, J. M., Obeid, L. M. and Hannum, Y. A. (1995) Retinoblastoma gene product as a downstream target for a ceramide-dependent pathway of growth arrest. *Proc. Natl. Acad. Sci. USA* **92**, 1347–1351.

Doborowsky, R. and Hannum, Y. A. (1992) Ceramide stimulates a cytosolic protein phosphatase. *J. Biol. Chem.* **267**, 5048–5051.

Gomez-Munoz, A., Martin, A., O'Brien, L. and Brindley, D. N. (1994) Cell permeable ceramides inhibit the stimulation of DNA synthesis and phospholipase D activity by phosphatidate and lysophosphatidate in rat fibroblasts. *J. Biol. Chem.* **269**, 8937–8943.

Goodrich, D. W., Wang, N. P., Qian, Y. W., Lee, E. Y.-H. P. and Lee H. (1991) The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* **67**, 293–302.

Hannum, Y. A. and Obeid, L. M. (1995) Ceramide: an intracellular signal for apoptosis. *TIBS* **20**, 73–77.

Jarvis, W. D., Fornari, F. A., Browning, J. L. and Gewirtz, D. A. (1994) Attenuation of ceramide-induced apoptosis by diacylglyceride in human myeloid leukemia cells. *J. Biol. Chem.* **269**, 31685–31692.

Kim, M. Y., Kim M. A., Moon, E. S. and Choi, K. H. (1994) Effects of sphingomyelin hydrolysis and ceramide on the inhibition of cell growth. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **27**, 271–274.

Kim, M. Y., Linardic, C., Obeid, L. and Hannum, Y. (1991) Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor and interferon. *J. Biol. Chem.* **266**, 484–489.

Law, B. and Rossie, S. (1995) The dimeric and catalytic subunit forms of protein phosphatase 2A from rat brain are stimulated by C2-ceramide. *J. Biol. Chem.* **270**, 12808–12831.

Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L. and Moscat, J. (1994) Protein Kinase C zeta isoform is critical for kappaB-dependent promotor activation by sphingomyelinase. *J. Biol. Chem.* **269**, 19200–19202.

Mathias, S., Dressler, K. A. and Kolesnick, R. N. (1991) Characterization of ceramide-activated protein kinase: stimulation by tumor necrosis factor a. *Proc. Natl. Acad. Sci. USA* **88**, 10009–10013.

McConkey, D. J., Goodrich, D. Bucana, C. and Klostergaar, J. (1996) The human retinoblastoma gene product suppresses ceramide-induced apoptosis in human bladder tumor cells. *Oncogene* **13**, 1693–1700.

Obeid, L. M. and Hannum, Y. A. (1995) Ceramide: A stress signal and mediator of growth suppression and apoptosis. *J. Cell. Biochem.* **58**, 191–198.

Obeid, L. M., Linardic, C. M., Karolak, L. A. and Hannum, Y. A. (1993) Programmed cell death induced by ceramide. *Science* **259**, 1769–1771.

Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R. and Sikorska, M. (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.* **12**, 3679–3684.

Okazaki, T., Bielawska, A., Bell, R. M. and Hannum, Y. A. (1990) Role of ceramide as a lipid mediator of 1α,25-dihydroxyvitamin D3-induced HL-60 cell differentiation. *J. Biol. Chem.* **265**, 15823–15831.

Song, Q. and Lavin, M. F. (1993) Calyculin A, a potent inhibitor of phosphatase-1 and phosphatase-2A, prevents apoptosis. *Biochem. Biophys. Res. Commun.* **190**, 47–55.

Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A. and Obeid, L. M. (1995) Role of ceramide in cellular senescence. *J. Biol.Chem.* **270**, 30701–30708.
Weinberg, R. A. (1991) Tumor suppressor genes. *Science* **254**, 1138–1146.
Westwick, J. K., Bielawska, A. E., Dbaibo, G., Hannum, Y. A. and Brenner, D. A. (1995) Ceramide activates the stress-activated protein kinases. *J. Biol. Chem.* **270**, 22689–22692.
Wolff, R. A., Dobrowsky, R. T., Bielawska, A., Obeid, L. M. and Hannum, Y. A. (1994) Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction. *J. Biol. Chem.* **269**, 19605–19609.
Yang, Z., Costanzo, M., Golde, D. W. and Kolesnick, R. N. (1993) Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor κB translocation in intact HL-60 cells. *J. Biol. Chem.* **268**, 20520–20523.