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Determination of Ceramide-Induced Apoptotic Cell Death in Mouse Granulosa Cell Cultured *In Vitro*

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생쥐 난소 과립세포의 체외배양중 세라마이드에 의한 자연세포사의 측정

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ABSTRACT : In mammalian ovary, major portion (>99%) of ovarian follicles undergo atresia. Recent studies have shown that this phenomenon is mediated via GC apoptosis. Ceramide, a product of sphingomyelin hydrolysis, has been proposed as a novel lipid second messenger with specific roles in mediating antiproliferative responses including apoptosis and cell cycle arrest. In the present study, we have examined the effect of ceramide on apoptotic cell death of GC *in vitro*. GCs were harvested by squeezing the antral follicles from the immature mice (3~4 weeks) and cultured in MEM medium with 10% fetal bovine serum. The cells were treated with various concentrations of ceramide (0 to 50 μM) and cultured up to 24 h. Cell death was determined by MTT cell viability assay and apoptosis was examined by acridine orange staining, *in situ* 3'-end labeling (TUNEL), and flow cytometry. Ceramide treatment induced apoptotic cell death of GC in a time- and a dose-dependent manner. Results of flow cytometric analysis showed that ceramide-induced cell death was mostly confined to the G0/G1 cells. These results provide an evidence for ceramide as a lipid second messenger of apoptosis in mouse GC.

Key words: Ovary, Granulosa cell, Apoptosis, Ceramide, Mouse.

요약 : 본 연구에서는 자연세포사 (apoptosis)를 유발시키는 것으로 알려진 ceramide를 배양 중인 생쥐 과립세포에 처리한 뒤 형광염색, *in-situ* 3'-end labeling (ISEL), 그리고 flow cytometry 기법을 이용하여, 자연세포사 및 세포주기에 미치는 ceramide의 영향을 조사하였다. Ceramide를 처리하지 않은 대조군에 비하여, ceramide를 처리한 실험군에서 세포의 생존율은 농도에 비례하여 유의하게 감소하였다. 또한 acridine orange에 의한 형광염색결과, 자연세포사의 양상을 보이는 핵을 갖는 세포의 수가 ceramide의 농도가 증가됨에 따라 현저하게 증가되었다. 또한 ISEL을 실시해 본 결과, 자연세포사가 ceramide의 처리농도가 증가됨에 따라 점차적으로 증가되었다. 한편, ceramide를 처리한 과립세포의 세포주기 분석을 위한 flow cytometry 결과도 자연세포사가 일어난 A_0기에 있는 세포들의 비율이 대조군에 비하여 농도 의존적으로 증가하였으며, G_0/G_1기에 있는 세포들의 비율은 현저하게 감소됨을 관찰할 수 있었다. 위의 결과로 보아 ceramide는 생쥐 과립세포의 G_0/G_1기에 특이적으로 작용하여 자연세포사를 유발하며, 난포의 폐쇄시 과립세포의 자연세포사를 유발할 것으로 사료된다.

INTRODUCTION

Apoptosis and necrosis constitute two distinct forms of cell death. Necrosis includes membrane disruption, hypoxia, membrane collapse, cell swelling and rupture in pathologic tissue. In contrast, programmed cell death (PCD) or apoptosis is a process by which cells die in response to specific physiological and toxicological signals. This genetically programmed form of cellular suicide is intimately involved in many biological processes, especially in ovarian germ cell endowment and depletion (Tilly & Tilly, 1991).

In mammalian ovary, the major portion of ovarian follicles is not ovulated but removed through the process of follicular atresia. However, the exact mechanisms have not been elucidated yet. Recent studies have shown that this phenomenon occurs by apoptosis of granulosa cells (Billig et al., 1994; Yang et al., 1997).

Intensive investigation, particularly in the past decade, has firmly established the role of membrane glycerolipids in transmembrane signal transduction. The binding of growth factor to cell-surface receptors initiates the multiple casca-

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des of intracellular signals that culminate in DNA synthesis and cell division (Ulrich & Schlessinger, 1990). Some of the signaling networks have been studied intensively, such as the pathways that utilize diacylglycerol (DAG), inositol 1,4, 5-triphosphate ($InsP_3$)[2] and other glycerolipid metabolites (Nishizuka, 1992; Berridge, 1993). However, the sphingolipids have only recently begun to be appreciated. Recent discoveries have revealed that these sphingolipids may play important roles in cell growth regulation. These include ceramides (Hannun, 1994; Kolensnick & Golde, 1994), sphingosine (Heller & Kronke, 1994), sphingosine 1-phosphate (Mattie et al., 1994) and sphingosine phosphorylcholine (Desai et al., 1993).

Especially, ceramide, an immediate product of sphingomyelin hydrolysis, is known as a mediator of apoptosis and stimulation of cells with nerve growth factor (NGF) or tumor necrosis factor α (TNFα) activates a sphingomyelinase which generates an intracellular ceramide as a mediator of apoptosis. As with other agonist-induced PCD, effects of ceramide on DNA fragmentation were inhibited by zinc, suggesting an involvement of a Ca^{2+}-dependent endonuclease. Additional studies suggest that ceramide may also participate in apoptosis induced by dexamethasone (Quintans et al., 1994), the Fas ligand or serum withdrawal (Venable et al., 1994; Tepper et al., 1995). All of these agents are associated with significant changes in the intracellular levels of ceramide. Thus, data are beginning to implicate ceramide as a generalized mediator of apoptosis.

Recent study revealed that TNFα or its second messenger, ceramide, stimulates apoptosis of early antral follicles in culture and suggested potential role for TNFα as an intraovarian regulator of follicle atresia by acting through the ceramide signaling pathway (Kaipia et al., 1996). Based upon these results, ceramide is assumed to be a mediator of apoptosis in the follicular atresia. Therefore, we investigated the possibilities that ceramide could induce the apoptotic changes in granulosa cells cultured *in vitro*.

MATERIALS AND METHODS

1. Animals

Immature (21-day-old) female mice (ICR strain) were purchased from Animal Breeding Center in Seoul National University. Animals were housed in a temperature-controlled room with a 14 h light-10 h dark cycle and allowed free access to food and water. The animals were treated with single i. p. injection of 5 IU PMSG (Sigma) in 0.2 ml PBS, pH 7.4, and ovaries were removed 24 h later and used for all experiments.

2. Granulosa cell cultures

Granulosa cells (GC) were collected in sterile MEM culture medium by a nonenzymatic needle (26-gauge) puncture technique. Cells were washed twice by centrifugation (250× g; 3 min) and viability was determined by trypan blue exclusion test. GC were inoculated into 60-mm culture dishes (Falcon) at a density of 1×10^6 cells/dish and cultured in 3ml MEM medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate and fetal bovine serum (Gibco) at 37℃ in a humidified 95% air-5% CO_2 atmosphere. For cell viability test, cell were plated in 4-well multidishes (Nunc) at a density of 2×10^5 cell/dish.

For the experiment to evaluate the effect of ceramide on apoptotic cell death, GCs were plated overnight and adherent cells were washed twice and cultured with 0 (ethanol vehicle), 12.5, 25 or 50 μM C6-ceramide for up to 24 h.

All experimental agents were freshly diluted in ethanol to give final concentrations of less than 0.01% diluent in the working solutions

3. MTT cell viability assay

Cell viability was determined by a modification of the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Sigma) reduction method (Mossman, 1983) and expressed as a percentage of the corresponding control. Briefly, at the end of the culture, GC was carefully washed and incubated with 500 μl MTT (0.05% in MEM) solution for another 3 h at 37℃. After MTT was removed, formazan crystals were solubilized in 200 μl DMSO (dimethyl sulfoxide; Sigma). The absorbance of aliquots from each well was measured using ELISA reader (EL340, BIO-TEK) at wavelength 570 nm and a reference wavelength of 620 nm.

4. Fluorescent staining of apoptotic nuclei

For morphological evaluation of the degree of apoptosis, GC was stained with acridine orange and visualized by fluorescence microscopy (Diaphot 300) (Yang et al., 1997). After culture, GC was treated with mild hypertonic solution (distilled water:PBS = 1:1) for 10 min, and prefixed in 50% solution of fixative (methanol:acetic acid = 3:1) for 5 min. After fixation, the cells were stained with acridine orange (1mg/ml) for 10 min, and then observed with fluorescence microscope.

5. *In situ* detection for GC apoptosis

After culture, the cells were harvested by non-enzymatic cell dissociation solution (Sigma) and centrifuged onto the slides. The cells were then subjected to 3'-end labeling of free DNA ends by *in situ* TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling) method to identify the specific cells undergoing DNA fragmentation. *In situ* apoptosis detection was performed using the ApopTag kit according to the protocol recommended by the manufacturer (Oncor).

6. Flow cytometric analysis

GC was prepared for flow cytometry as previously described for thymocyte (Telford et al., 1991). After culture, the cells were harvested, centrifuged at 400×g for 5 min, and chilled to 4℃. And then, they were fixed at a concentration of 1-2×10^6 cells/ml in 80% ethanol at 4℃ for 30 min. The fixed cells were resuspended by gentle vortexing at room temperature in 1 ml of propidium iodide (PI, 50 μg/ml; Sigma) in modified HBSS (Hank's balanced salt solution; pH 7.4, Sigma) containing 0.1% Trition X-100 (Sigma), 0.1 mM EDTA ($Na)_2$, and 50 μg/ml (50 U/mg) of RNase (Boehringer-Manheim). The percentage of GC with degraded DNA was determined using a flow cytometer (Hialeah). PI nuclear stain was exited through use of the 488 nm line of an argon laser. Cell cycle histograms were obtained from minimum of 10,000 cells. The profile Ⅱ histogram analysis option was used to set up analysis cursor for data acquisition in the A_0 (subpopulation of cells with degraded DNA and with lower DNA fluorescence than G_0/G_1 cells), G_0/G_1, S, and G_2/M regions of the DNA histogram.

7. Data analysis

Each experiment was repeated at least three times. Quantitative data represent the mean±S.D. of at least five cultures expressed as percent change as compared with control samples incubated without ceramide treatment. statistical differences were assessed by ANOVA followed by student's t test, and $P<0.05$ was considered to be significant.

RESULTS

1. Effects of ceramide on cell viability

To test whether ceramide induces a cell death in GC, various concentrations of ceramide were treated for 24 h. Fig. 1 depicts that GC viability decreased in a dose-dependent manner. Cell viability decreased significantly from 17% (12.5 μM) to 85% (50 μM) compared to control.

2. Characterization of ceramide-induced cell death as an apoptosis

In order to determine whether ceramide-mediated death of GC occurred by apoptosis, cells incubated with various

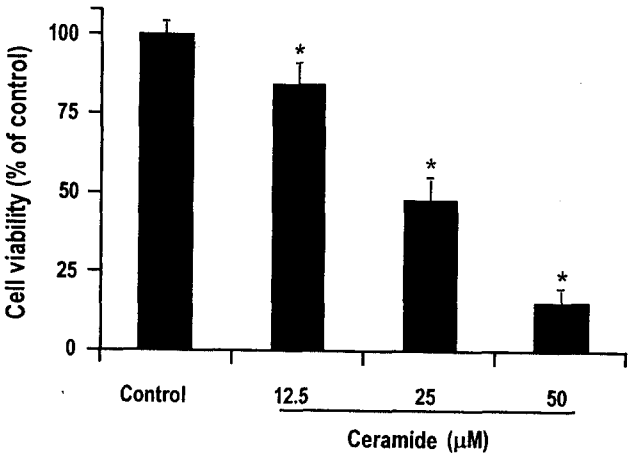

Fig. 1. Cytotoxic effect of ceramide on mouse granulosa cells.
Cells were cultured for 24 h with ethanol vehicle (control) or various concentrations of ceramide, as indicated. Cell viability was determined by the MTT assay and expressed as a percentage of the values of controls. Data are the mean ± S.D. of 5 different determinations. *, $p<0.01$ versus control.

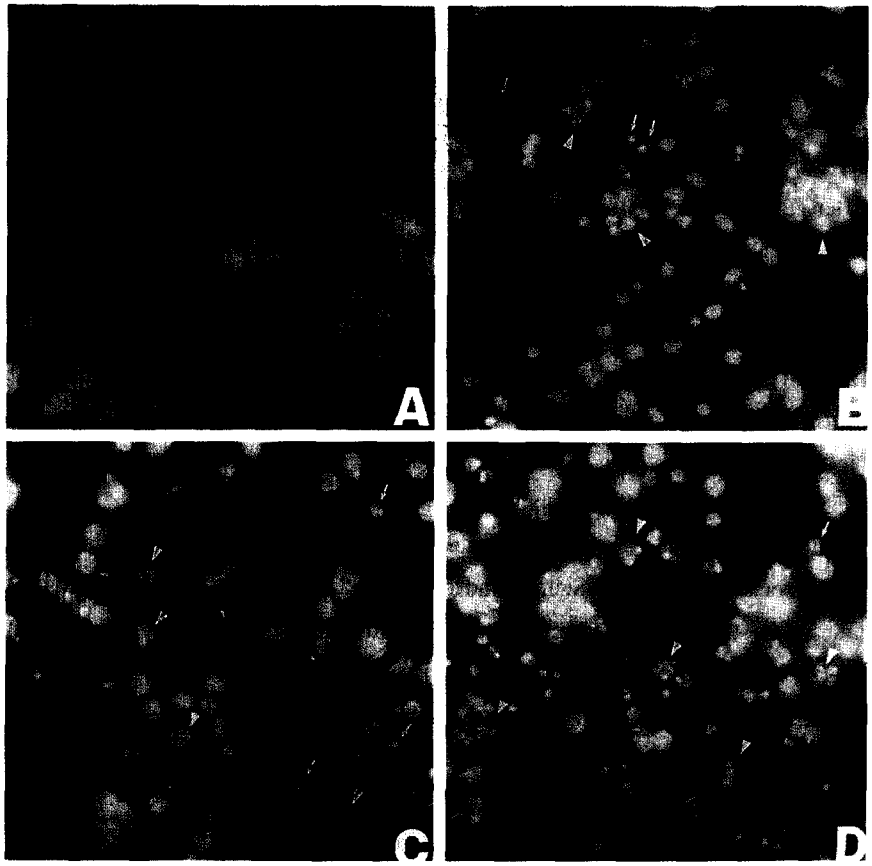

Fig. 2. Fluorescence in the apoptotic nuclei of granulosa cells treated with various concentrations of ceramide.
Fluorescent staining of cells with acridine orange was performed on the cultured dishes after fixing with methanol: acetic acid. Arrows indicate typical pyknotic nuclei and white arrow-head points to apoptotic nuclei. (A, control; B, 12.5 μM; C, 25 μM; D, 50 μM). Magnification, ×400.

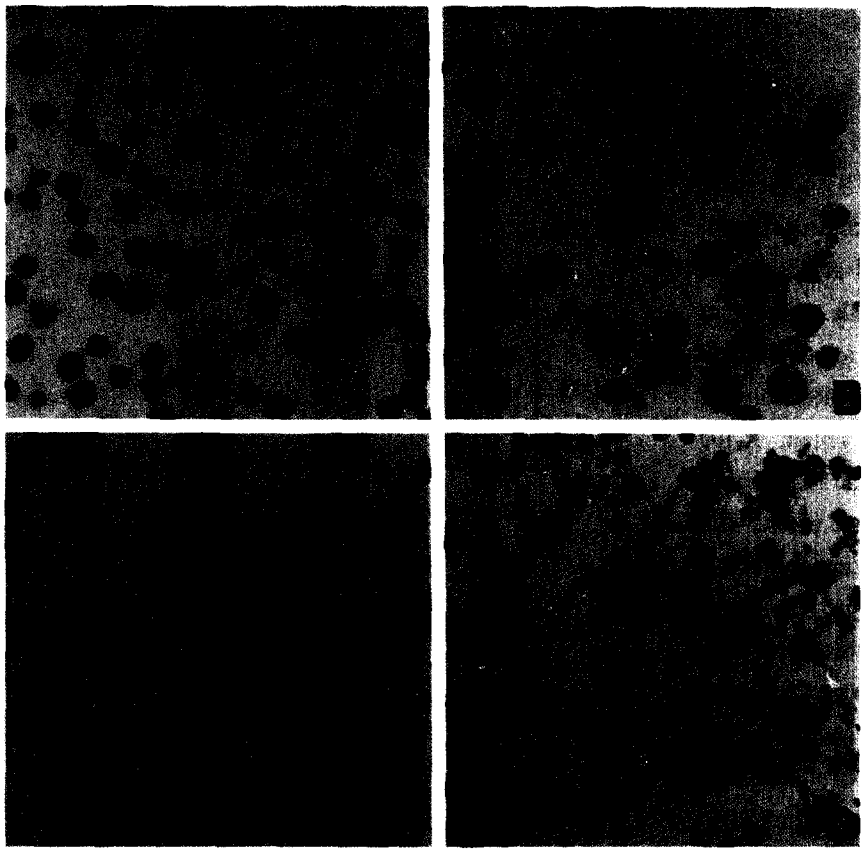

Fig. 3. *In situ* detection of DNA fragmentation of cultured granulosa cells using TUNEL method.
Cells were treated with ethanol vehicle(control, A) or ceramide(12.5 μM, B; 25 μM, C; 50 μM, D) for 24h and harvested by non-enzymatic technique. The cells were centrifuged onto slide and stained with *in situ* apoptosis detection kit. Arrows show apoptotic cells. Magnifcation, ×400.

concentration of ceramide were stained with acridine orange and analyzed by fluorescence microscopy (Fig. 2). In control culture (ethanol vehicle), nearly all cells could be observed as normal that there was not any apoptotic nucleus under fluorescence optics (Fig. 2A). On the contrary, cells treated with ceramide revealed a lot of apoptotic nuclei in a dose-dependent manner (Figs. 2B-2D).

The TUNEL technique was used to vizulize DNA fragmentation both in control and ceramide-treated cultures *in situ*. The nuclei of healthy GC were not labeled and showed the oval shape (Fig. 3A), while the nuclei in which DNA breakdown has occurred were darkly stained and irregularly shaped. When cells treated with ceramide were examined, intense 3'-end labeling was observed and the percentages of apoptotic bodies increased in a dose-dependent manner (Figs. 3B-3D).

3. Cell cycle analysis with flow cytometry of granulosa cell

Flow cytometric cell cycle histograms of PI-stained GC are shown in Fig. 4. In control the percentage of GC in A_0 phase, which represents characteristic of apoptotic cell, was 4.6% of total cells. However, when the cells were treated with various concentration of ceramide including 12.5 μM, 25 μM or 50 μM, the percentage of cells in A_0 phase (Region 1) were 20.6%, 44% and 59%, respectively. Furthermore, the number of cells in G_0/G_1 phase (Region 2) decreased significantly in a dose-dependent manner and similary the number of cells in G_2/M (Region 4) decreased, which, however, was not statistically significant.

DISCUSSION

The present study demonstrates that ceramide induces cell death of mouse GC cultured *in vitro* (Fig. 1) and that the cell death occurred by apoptosis (Fig. 2, 3, 4).

Previous studies have suggested that ceramide could be a major intracellular lipid mediating the cytotoxicity in response to a number of extracellular agents and insults including Fas agonist, TNFα (Obeid et al., 1993), radiation (Haimovitz-Friedman et al., 1994), and chemotherapeutic agents (Strum et al., 1994). Furthermore, in cultured rat granulosa cells, TNFα treatment was recently shown to increase ceramide levels, indicating that the TNFα signaling in GC was coupled to a sphingomyelin pathway (Santana et al., 1995).

In the present study, we evaluated the cytotoxicity of ceramide using MTT cell viability assay method, and the assay, which measures mitochondrial respiratory function, could detect the onset of cell death earlier than dye-exclusion-based method. From the results of our experiments, it was shown that ceramide strongly induced cell death and increasing concentrations of ceramide resulted in the increased cytotoxicity as expected.

Fluorescence staining of nuclei with acridine orange in dying cells obviously provided an evidence of apoptosis. Such analysis to detect the apoptotic nuclei has already been widely used in various cell types (Arends et al., 1990) including rat GC (Luciano et al., 1994). In our results, number of apoptotic fragmented nuclei increased in proportion to concentrations of ceramide, while, in control (ethanol vehicle), nearly all GC appeared to be normal as they exhibited healthy oval nucleus without signs of nuclear fragmentation. The *in situ* TUNEL method was also used to vizulize DNA fragmentation. Detection of apoptotic cells *in situ* with DNA 3'-end labeling using terminal transferase enzyme has been known as a powerful tool for the study of apoptosis (Chun et al., 1994). Nuclei of healthy cells were not labeled and were oval shape, while nuclei of which DNA breakdown has occurred was darkly stained and irregularly shaped. No sign of apoptotic signal was detected in control GC cultured for 24 h (Fig. 3A). In contrast, when GCs were treated with ceramide, an incresing level of apoptotic cells and apoptotic bodies were detected in a dose-dependent manner.

The phase of the cell cycle when cells are most sensitive to apoptosis may provide an important imformation to understand the mechanism of apoptosis in various cell types. It has been reported that a fungal cytotoxin, camptothecin causes immediate degeneration of nuclear DNA of HL-60 cells only in the S and G_2/M phases of the cell cycle with accumulation of apoptotic cells (Del Bino et al., 1990). In contrast, apoptosis induced by corticoid in rat thymocytes was selective to cells in the G_0/G_1 phase (Telford et al., 1991; Bruno et al., 1992). Recent results using porcine GC

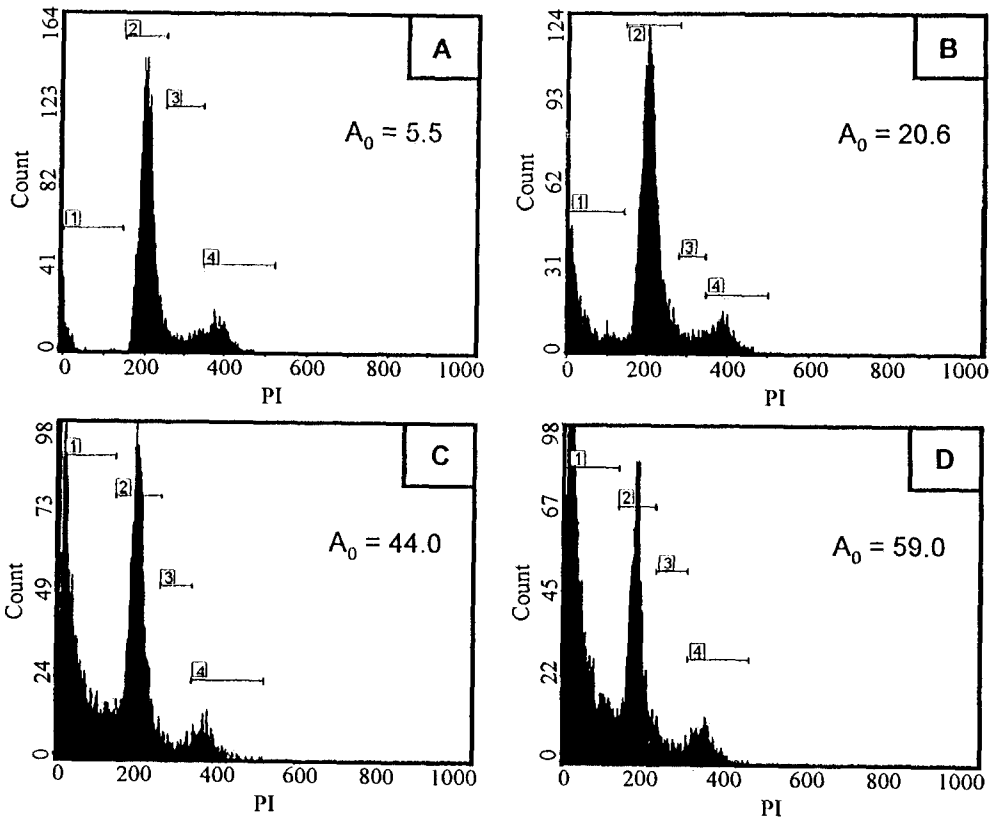

Fig. 4. DNA Histograms of DNA contents in cultured granulosa cells treated with various concentrations of ceramide using flow cytometry.

Cells were treated with ethanol vehicle (A), or ceramide (12.5 μM, B; 25 μM, C; 50 μM, D) for 24 h and harvested by non-enzymatic cell dissociation. The percentage of granulosa cells having sub-diploid amount of DNA (%A_0 cells) was determined by DNA fluorescence flow cytometry of PI-stained nuclei of ethanol-fixed cells. Region 1 represents the portion of apoptotic granulosa cell population having sub-diploid levels of DNA (A_0). Region 2, 3, and 4 represent the G_0/G_1, S, and G_2/M stages of the cell cycle, respectively.

also indicated that DNA degeneration in GC was largely specific to G_0/G_1 phase (Guthrie et al., 1994). In the present study, the percentage of cells in S and G_2/M phase decreased in a dose-dependent manner and the percentage of cells in G_0/G_1 phase was abruptly decreased and was inversely related to that of A_0. This result strongly provide an evidence that ceramide induced-apoptosis in GC was largely restricted to G_0/G_1 phase.

In summary, ceramide, a product of sphingomyelin hydrolysis, induced apoptotic cell death in mouse GC cultured *in vitro* and ceramide-induced cell death was mostly restricted to G_0/G_1 phase. There may be many gaps in understanding the regulatory mechanisms of hormone-responsive sphingomyelinase and downstream signaling pathways involved in mediating the actions of ceramide. Further investigation of the pathway will give an insight to the understanding of ovarian physiology including follicular atresia.

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REFERENCES

Arends ML, Morris RG, Wyllie AH (1990) Apoptosis. The role of the endonuclease. Am J Pathol 136:593-608.

Berridge MJ (1993) Inositol triphosphates and calcium sig-

nalling. Nature 361:315-32.

Billig H, Fukuta I, Hsueh AJW (1994) Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary:Biochemical and *in situ* detection of deoxyribonucleic acid fragmentation in granulosa cells. Endocrinology 134:245-250.

Bruno S, Lassota W, Giaretti W, Darzynkiewiez Z (1992) Apoptosis of rat thymocytes triggered by prednisolone, camptothecin, or teniposide is selective to G_0 cells and is prevented by inhibitors protease. Oncol Res 4:29-35.

Chun SY, Billig H, Tily JL, Furuta I, Tsafriri A, Hsueh AJW (1994) Gonadotropin suppression of apoptosis in cultured preovulatory follicles:mediatory role of endogenous insulin-like growth factor I. Endocrinology 135:1845-1853.

Del Bino G, Skierski JS, Darzynkiewiez Z (1990) Diverse effects of camptothecin, an inhibitor of topoisomerase I, on the cell cycle of lymphocytic (L1210, MOLT-4) and myelogeneous (HL-60, KG1) leukemic cells. Cancer Res 50:5746-5750.

Desai NN, Carlson RO, Mattie ME, Olivera A, Buckely NE, Seki T, Brooker G, Spiegel S (1993) Signaling pathways for sphingosyl-phosphorylcholine mediated mitogenesis in Swiss 3T3 fibroblasts. J Cell Biol 21:1385-1895.

Guthrie HD, Welch GR, Cooper BS, Zakaria AD, Johnson LA (1994) Flow cytometric determination of degraded deoxyribonucleic acid in granulosa cells to identify atretic follicles during preovulatory maturation in the pig. Biol Reprod 50:1303-1311.

Haimovitz-Freidman A, Kan CC, Ehleiter D, Persand RS, Mclonghlin M, Fukc Z, Kolensnick RN (1994) Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J Exp Med 180:525-535.

Hannun YA (1994) The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269:3125-3128.

Heller RA, Kronke M (1994) Tumor necrosis factor receptor-mediated signaling pathways. J Cell Biol 26:5-9

Kaipia A, Chun SY, Eisenhauer K, Hsueh AJW (1996) Tumor necrosis factor-α and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. Endocrinology 137:4864-4870.

Kolensnick R, Golde DW (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell 77:325-328.

Luciano AM, Pappalardo A, Ray C, Pelluso JJ (1994) Epidermal growth factor inhibits large granulosa cell apoptosis by stimulating progesterone synthesis and regulating the distribution of intracellular free calcium. Biol Reprod 51:179-654.

Mattie AH, Brooker G, Spiegel S (1994) Sphingosin-1-phosphate, a putative second messenger, metabolizes calcium from internal stores via an inositol triphosphate-independent pathway. J Biol Chem 69:3181-3188.

Mossman T (1983) Rapid colorimetric assay for cellular growth and survival:Application to proliferation and cytotoxicity assays. J Immunol Method 65:55-63.

Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607-614.

Obeid LM, Linardic CM, Karolak LA, Hannun YA (1993) Programmed cell death induced by ceramide. Science 259:1769-1771.

Quintans J, Kilkus J, McShan CL, Gottschalk AR, Dawson G (1994) Ceramide mediates the apoptotic response of WEHI1231 cells to anti-immunoglobulin, corticosteroids and irradiation. Biochem Biophys Res Commun 202:710-714.

Santana P, Llanes L, Hernandez I, Gallardo G, Quintana J, Gonzalez J, Estevez F, Galarreta CR, Fanjul LF (1995) Ceramide mediates tumor necrosis factor effects on P450-aromatase activity in cultured granulosa cells. Endocrinology 136:2345-2348.

Strum JC, Small GM, Pauig SB, Daniel LW (1994) 1-b-Arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. J Biol Chem 269:15439-15497.

Telford WG, King LE, Fraker PJ (1991) Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flowcytometry. Cell Prolif 1133:275-285.

Tepper CG, Jayadev S, Liu B, Bielawska A, Wolff R,

Yonehara S, Hannun YA, Seldin MF (1995) Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. Proc Natl Acad Sci USA 92:8443-8447.

Tilly JL, Kowalski KI, Johnson AL, Hsueh AJW (1991) Involvement of apoptosis in ovarian follicular atresia and post ovulatory regression. Endocrinology 129:2799-2801.

Ulrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212.

Venable ME, Blobe GC, Obeid LM (1994) Identification of a defect in the phospholipase D/diacylglycerol pathway in cellular senescence. J Biol Chem 269:26040-26044.

Yang HW, Lee SJ, Yoon YD (1997) Determination of apoptosis in granulosa-luteal cells obtained from hyperstimulated human ovary. Kor J Dev Reprod 1:79-89.