Arch Pharm Res Vol 28, No 9, 1057-1064, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Pathophysiological Implication of Ganglioside GM3 in Early Mouse Embryonic Development through Apoptosis

Eun-Jin Ju, Dong-Hoon Kwak, Dae-Hoon Lee, Sung-Min Kim, Ji-Su Kim, Sun-Mi Kim, Han-Gil Choi, Kyu-Yong Jung[1], Seo-ul Lee[1], Su-Il Do[2], Yong-Il Park[3], and Young-Kug Choo

Department of Biological Science, Wonkwang University, Iksan, Jeonbuk 570-749, Korea, [1]Department of Pharmacology, Wonkwang University School of Medicine, Iksan, Jeonbuk 570-749, Korea, [2]Department of Life Science, Ajou University, Suwon, Kyunggi-do 443-749, Korea, and [3]Department of Biotechnology, Catholic University, Bucheon, Kyunggi-do 420-749, Korea

(Received April 22, 2005)

Apoptosis may occur in early embryos where the execution of essential developmental events has failed, and gangliosides, sialic acid-conjugated glycosphingolipids, are proposed to regulate cell differentiation and growth. To evaluate the regulatory roles of ganglioside GM3 in early embryonic development, this study examined its expressional patterns in apoptotic cells during early embryonic development in mice. Pre-implanted embryos were obtained by *in vitro* fertilization, which were treated at the 4-cell stage with three the apoptosis inducers, actinomycin D, camptothecin and cycloheximide, for 15 h. All three inducers significantly increased the percentage of apoptotic cells, as measured using a TUNEL method, but remarkably reduced the total cell numbers. The numbers of morula and blastocyst stages were significantly decreased by treatment of the embryos with the three apoptosis inducers compared with the control, with a similar result also observed in the number of blastomeres. Staining of early embryos with Hoechst 33342 revealed a significant percentage of apoptotic nuclei. Prominent immunofluorescence microscopy revealed a significant difference in the ganglioside GM3 expression in apoptotic embryos compared with the control, and RT-PCR also demonstrated a dramatic increase in ganglioside GM3 synthase mRNA in the apoptotic embryos. These results suggest that ganglioside GM3 may be pathophysiologically implicated in the regulation of early embryonic development through an apoptotic mechanism.

Key words: Ganglioside GM3, Apoptosis, Early embryonic development

INTRODUCTION

The programmed cell death process, apoptosis, is involved in the regulation of cell populations and lineages of all major mammals. Increased apoptosis may be related to embryo losses that occur during the first weeks of development and the lower developmental competence of *in vitro* fertilized and cultured embryos. Human embryos generated from *in vitro* fertilization (IVF) also exhibit varying degrees of cytoplasmic fragmentation, with abundant evidence demonstrating that cytoplasmic fragmentation in human embryos arises due to apoptosis (Keefe *et al.*, 2005). Blastocysts with reduced cell numbers has are believed to have a high and variable incidence of DNA fragmentation, with cell death beyond a certain threshold being detrimental to further embryo development, which eliminates nonviable offspring (Betts and King, 2001; Byrne *et al.*, 1999; Hardy, 1997). Murine oocyte fragmentation *in vitro* is dependent upon the functional expression of several genes that wake up the evolutionarily conserved apoptotic cell death program. Thus, substantial evidence indicates that fragmentation and death of ovulated murine oocytes *in vitro* is an unequivocal example of apoptosis (Perez *et al.*, 1999).

On the other hand, gangliosides are sialic acid (NeuAc)-containing glycosphingolipids widely found in the plasma membranes of all vertebrate tissues, and are particularly abundant in the central nervous system (Svennerholm, 1980). They play important roles in a large variety of biological processes, such as cell-cell interactions, cell differentiation, growth and signal transduction (Ji *et al.*,

Correspondence to: Young-Kug Choo, Department of Biological Science, College of Natural Sciences, Wonkwang University, 344-2 Shinyong-dong, Iksan, Jeonbuk 570-749, Korea
Tel: 82-63-850-6087, Fax: 82-63-857-8837
E-mail: ykchoo@wonkwang.ac.kr

2000; Choo *et al.*, 1995; Varki, 1993; Hakomori, 1981). Gangliosides are synthesized *via* four primary biosynthetic pathways by a family of glycosyltransferase in the Golgi apparatus (Iber *et al.*, 1992; Fishman and Brady, 1976). Among the gangliosides, ganglioside GM3 (NeuAca3-Galβ4Glcβ1Cer) has the simplest carbohydrate structure, and its synthesis is the first step in ganglioside biosynthesis. Therefore, ganglioside GM3 synthase is generally considered a key regulatory enzyme for ganglioside biosynthesis, as it catalyzes the first committed step in the synthesis of nearly all gangliosides. In addition, accumulating evidence has also demonstrated that ganglioside GM3 is involved in the induction of HL-60 differentiation (Nojiri *et al.*, 1986), modulation of cell proliferation (Choo, 1999; Hakomori, 1990) and signal transduction (Hakomori *et al.*, 1998). With regard to the information above, it is our hypothesis that 'ganglioside GM3 might regulate the early embryonic development through an apoptotic mechanism'. To examine this hypothesis, this study evaluated a pathophysiological relationship between ganglioside GM3 expression and apoptosis in early embryonic development.

MATERIALS AND METHODS

Culture media and reagents

The medium used for fertilization and sperm culture was TYH (in mg/mL; 6.976 NaCl, 0.356 KCl, 0.19 $CaCl_2$, 0.162 KH_2PO_4, 0.293 $MgSO_4 \cdot 7H_2O$, 2.106 $NaHCO_3$, 1.0 glucose, 0.11 Na-pyruvate, 0.075 penicillin G, 0.05 streptomycin and 4 BSA). The medium used for embryo culture was MWM (in mg/mL; 6.4 NaCl, 0.356 KCl, 0.162 KH_2PO_4, 0.294 $MgSO_4 \cdot 7H_2O$, 1.9 $NaHCO_3$, 0.46 calcium lactate·5-hydrate, 1.0 glucose, 0.025 Na-pyruvate, 0.075 penicillin G, 0.05 streptomycin, 3 BSA, 50 μL of 20 μM β-mercaptoethanol and 50 μL of 100 mM EDTA). The actinomycin D, camptothecin and cycloheximide were obtained from the Sigma Chemical (St Louis, MO). All other chemicals were of the highest commercially available grades.

Superovulation

Superovulation was induced by the method of Summer *et al.* (2000), with slight modification. Mice, at 9 weeks of age, were intraperitoneally treated with 5 IU of hCG 48 h after the intraperitoneal administration of PMSG (5 IU). All animals were sacrificed by a cervical dislocation 15 h after the hCG treatment.

In vitro fertilization

In vitro fertilization was carried out in drops of MWM under paraffin oil. A preincubated, capacitated sperm suspension was gently added to the freshly ovulated oocytes, to a final motile sperm concentration of 1×10^6 cells/mL. The combined sperm-oocyte suspension was incubated for 5 h, the oocytes then washed via several changes of medium and finally incubated in 40 μL drops of medium under paraffin oil. Fertilization was confirmed by recording the number of two-cell embryos 24 h after completion of the *in vitro* fertilization (Kim *et al.*, 2004; Summers *et al.*, 2000).

Induction of apoptosis

Actinomycin D, camptothecin and cycloheximide were employed to induce apoptosis of embryonic cells. The 4-cell embryos were divided into four groups and washed three times. They were then transferred to 50 mL drops of MWM (embryo/mL), containing several concentrations of the three apoptosis inducers, and cultured at 37°C in a humidified 5.0% CO_2/95% air atmosphere for 15 h. After washing the embryos with MWM, they were further cultured with MWM to the blastocyst stage.

RT-PCR

To examine transcriptional changes of the ganglioside GM3 synthase gene, RT-PCR was conducted using the previously described method of Kawamura *et al.* (2001). Briefly, poly(A)$^+$ mRNAs, isolated from the blastocyst stage of embryos, were reversely transcribed into cDNAs. Exogenous rabbit-globin mRNA (Life Technologies, Inc., Rockville, MD) was added to each sample prior to the mRNA extraction to evaluate the efficiencies of the mRNA extraction and RT procedure. The amount of cDNA subjected to each PCR reaction was equivalent to the number of genomes. PCR thermocycling was performed using the following protocol: 94°C for 10 min, designated cycles of 94°C for 30 sec, 66.4°C for 30 sec and 68°C for 2 min, and then 72°C for 10 min before cooling to 4°C. The sense and antisense primers used in PCR were as follows: 5'-GCAAGCGCTGTGTGGTTGTTGGGAAC-3' and 5'-TGCCAGGCTGACTTCATCACACAGAT-3', respectively. The PCR products were separated by 1% agarose gel electrophoresis (Agarose-LE, Nacalai Tesque Inc., Kyoto, Japan), in the presence of ethidium bromide solution, and visualized with a UV transilluminator.

TUNEL assay

Pre-implanted mouse embryos were labeled with a terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP nick end-labeling assay (TUNEL) using the method of Byrne *et al.* (1999). Briefly, embryos were passed through three drops of PBS containing PVP (1 mg/mL) and then fixed in 4% paraformaldehyde in PBS. After fixation, the embryos were washed through four drops of PBS containing PVP (1 mg/mL) and permeabilized in 0.1% Triton X-100 for 2 min at 4°C. Embryos were washed three times with PBS, and TUNEL staining then

carried out according to the instruction manual supplied with the commercial kit (In Situ Cell Death Detection Kit; Roche Molecular Biochemicals, Germany).

Immunofluorescence staining

To evaluate the expression and distribution of ganglioside GM3 in pre-implanted mouse embryos, immunohistochemical study was conducted using the anti-ganglioside GM3 monoclonal antibody (Seikagaku Corporation, Tokyo, Japan, clone GMR6). The immunological characterization of GMR6 has been described in previous reports (Inoki *et al.*, 2000; Kotani *et al.*, 1992). Embryos were washed three times with PBS at room temperature, and then incubated with PBS, containing 5% BSA, for 15 min at room temperature. After washing three times with PBS, the embryos were incubated overnight with GMR6 diluted appropriately in PBS at 4°C. They were then washed three times with PBS and incubated at room temperature with FITC-conjugated goat anti-mouse IgM antibody for 1 h. After washing three times with PBS, the embryo nuclei were identified by incubation with DNA-specific fluorescent dye (Hoechst 33342), for 5 min at room temperature, followed by washing three times with PBS. Embryos were transferred to slide glasses and sealed with cover slips. All fluorescent samples were observed for FITC fluorescence, using a Carl Zeiss microscope, equipped with an epi-illuminator and a filter system.

Statistical analysis

All data are expressed as the mean ± S.D. The statistical significances were tested using a two way-analysis of variance (ANOVA), followed by the Scheffe's test for multiple comparisons. Two groups were compared by the unpaired Student's *t*-test. Only those differences where the *p* values were less than 0.05 were regarded as statistically significant.

RESULTS

Apoptosis inducers on the early embryonic development

To evaluate the pathophysiological relationship between apoptosis and early embryonic development, the effects of three apoptosis inducers, actinomycin D (Naora *et al.*, 1996), camptothecin (Yuan *et al.*, 2002) and cycloheximide (Calabrese, 2001), were examined on the early embryonic development in mice. As shown in Tables I, II, and III, all three apoptosis inducers markedly changed the early embryonic development (2-cell to blastocyst stage), in concentration-dependent manners (0.1 ng/mL-0.5 μg/mL). The apoptosis inducers at relatively low concentrations did not significantly change the numbers of morula and blastocyst stages in pre-implanted mouse embryos, whereas higher concentrations (≥0.005 μg/mL) markedly disturbed the early mouse embryonic development. The order of potency of the apoptosis inducers was actinomycin D > camptothecin cycloheximide.

Ganglioside GM3 synthase mRNA expression

Since ganglioside GM3 synthase is a key regulatory

Table I. Effects of actinomycin D on the IVF and early mouse embryonic development

Actinomycin D (μg/mL)	No. of Oocyte	Fertilized Oocyte(%)	Percent (%) of embryos development to			
			2-cell	4cell	Morula	Blastocyst
Control	20±1.0	93.4±2.5	88.0±3.0	78.3±2.5	76.8±3.1	75.9±3.5
0.0005	23±2.0	95.6±0.4	94.3±2.6	73.8±2.3	69.4±2.7	69.4±2.7
0.005	25±1.5	92.9±1.7	80.3±3.8	71.9±4.9	21.9±4.6*	4.8±1.5**
0.05	26±2.5	91.8±2.9	87.5±2.1	72.7±2.3	3.7±2.9**	0.0±0.0***
0.5	22±2.0	94.7±3.4	77.8±6.4	74.8±7.4	0.0±0.0***	0.0±0.0***

*p<0.5, **p<0.01 and ***p<0.001 *vs.* control

Table II. Effects of camptothecin on the IVF and early mouse embryonic development

Camptothecin (μg/mL)	No. of Oocyte	Fertilized Oocyte(%)	Percent (%) of embryos development to			
			2-cell	4cell	Morula	Blastocyst
Control	24±2.9	94.5±1.7	92.0±3.8	80.4±2.3	72.4±1.9	72.4±1.9
0.0001	22±3.0	95.3±0.7	87.5±1.9	78.4±1.9	76.3±4.2	75.4±3.7
0.001	16±2.0	93.6±0.5	93.6±0.5	83.0±3.3	71.3±1.7	70.8±2.4
0.01	35±3.0	92.3±4.4	86.3±5.2	76.6±6.7	45.0±8.3**	15.7±5.9**
0.1	26±2.5	92.9±1.7	80.3±3.8*	71.9±4.9*	0.0±0.0***	0.0±0.0***

*p<0.5, **p<0.01 and ***p<0.001 *vs.* control

Table III. Effects of cycloheximide on the IVF and early mouse embryonic development

Cycloheximide (μg/mL)	No. of Oocyte	Fertilized Oocyte(%)	Percent (%) of embryos development to			
			2-cell	4cell	Morula	Blastocyst
Control	23±3.2	92.9±2.1	90.1±1.0	81.4±0.4	70.7±1.7	70.7±1.7
0.0001	17±0.6	94.2±0.1	74.3±2.1*	71.2±0.8*	65.4±1.2	65.4±1.2
0.001	22±2.6	95.4±0.5	89.2±3.2	75.5±4.3	68.3±1.4	63.7±1.4
0.01	28±4.2	89.3±3.0	84.5±5.2	82.0±4.5	28.7±3.2**	10.6±3.3 **
0.1	26±2.5	95.5±0.2	93.9 ±2.9	68.1±3.8*	0.0±0.0***	0.0±0.0 ***

*p<0.5, **p<0.01 and ***p<0.001 *vs.* control

enzyme for ganglioside biosynthesis (Fishman and Brady, 1976; Iber *et al.*, 1992), the expressional change of ganglioside GM3 synthase mRNA at the blastocyst stage of mouse embryos was examined. As shown in Fig. 1, transcriptional analysis by RT-PCR showed a marked change in the expression of ganglioside GM3 synthase mRNA in the apoptotic embryos. Actinomycin D, camptothecin and cycloheximide, at concentrations of 5 ng/mL, clearly elevated the expression of ganglioside GM3 synthase mRNA, with the highest increase was observed in the embryos treated with actinomycin D.

Triple staining of early developmental embryos

Immunohistochemical studies were carried out to clarify the pathophysiological relationship between the expression of ganglioside GM3 and apoptosis in pre-implanted mouse embryos. After pre-implanted mouse embryos had been incubated with the apoptosis inducers (actinomycin D, camptothecin and cycloheximide), at concentrations of 5 ng/mL for 15 h, the embryos at the 4-cell, morula and blastocyst stages were stained with anti-ganglioside GM3 monoclonal antibody, TUNEL and Hoechst 33342. Prominent expression of ganglioside GM3 in normal embryos was observed at all developmental stages tested, and there was no significant difference in the immunoreactivity against anti-ganglioside GM3 at each stage during the embryonic development. All three apoptosis inducers caused a significant increase in the expression of ganglioside GM3 compared to the developmental stage-matched normal embryos. The highest expression of ganglioside GM3 was observed in the embryos treated with actinomycin D (Fig. 2a), whereas those treated with camptothecin (Fig. 2b) and cycloheximide (Fig. 2c) showed relatively low immunoreactivities against ganglioside GM3. In addition, apoptosis inducer-treated embryos also showed a dramatic decrease in the number of blastomeres, with a significant percentage of apoptotic nuclei demonstrated using the TUNEL assay.

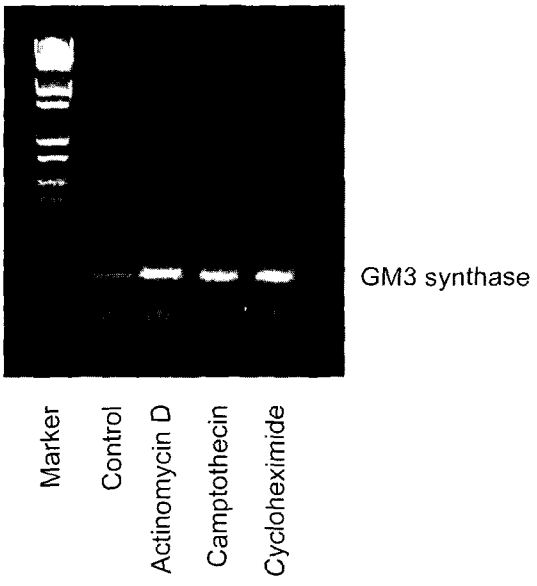

Fig. 1. Semiquantitative RT-PCR analysis of the expression of ganglioside GM3 synthase mRNA in pre-implanted embryos. Total RNAs from the blastocyst stage of mouse embryos were used as templates for the RT-PCR.

DISCUSSION

Ganglioside GM3 is pathologically implicated in the ovarian maturation and morphogenesis of fertilized embryos (Kwak *et al.*, 2003), and actinomycin D, camptothecin and cycloheximide are well known, standardized apoptosis inducers (Yuan *et al.*, 2002; Calabrese, 2001; Naora *et al.*, 1996). With the use of these drugs, the pathophysiological implication of ganglioside GM3 was investigated in early mouse embryonic development, *via* an apoptotic pathway. All three apoptosis inducers tested in this study caused embryonic cell apoptosis and decreased the numbers of blastomeres, in concentration-dependent manners, and these changes were positively paralleled with the changes in the expression of ganglioside GM3. These observations lead us to consider that apoptosis may be an important mechanism in embryonic cell injury and death, and ganglioside GM3 may participate in the regulation of embryonic cell survival, via modulation of the apoptotic pathway in early mouse embryonic development.

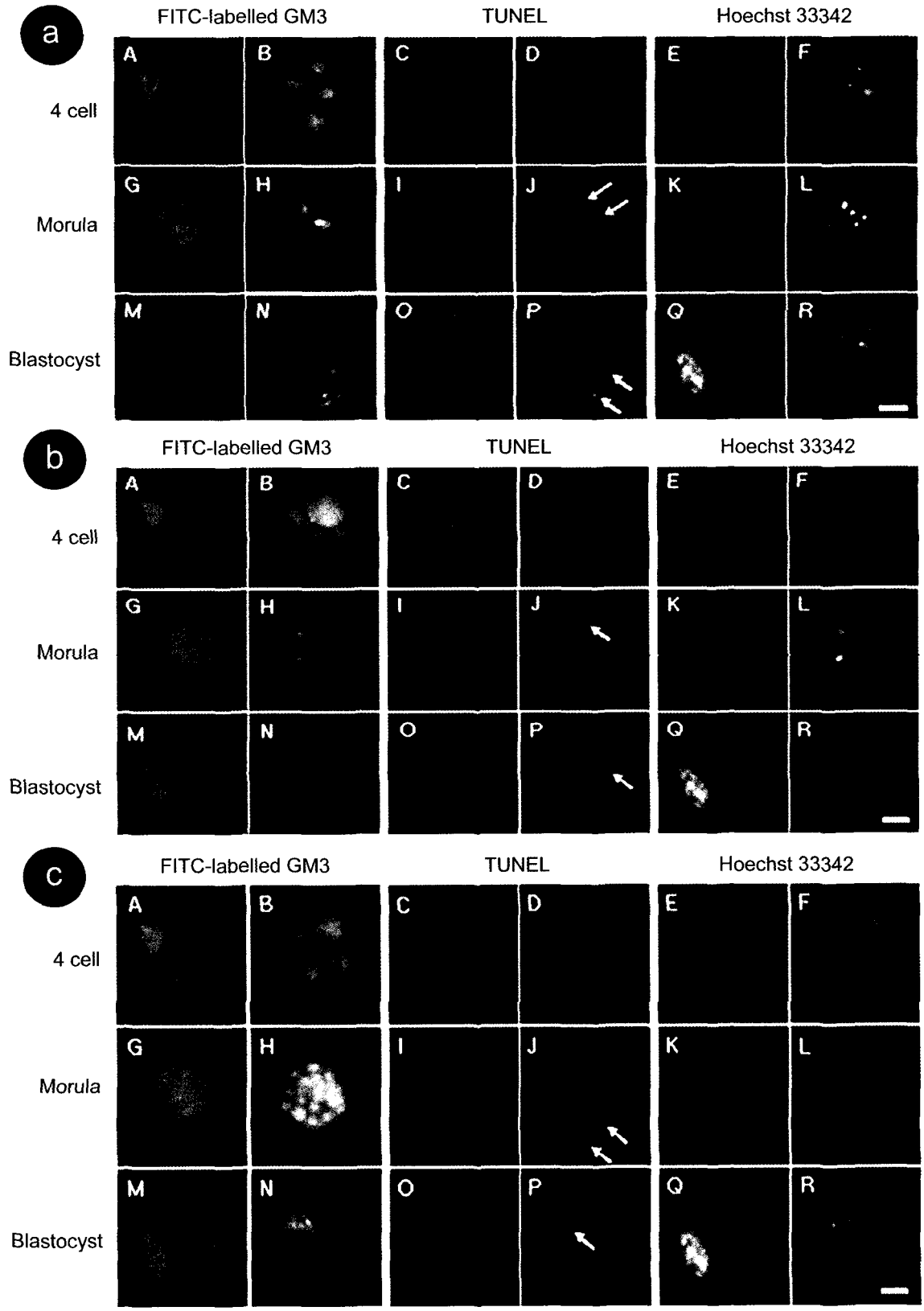

Fig. 2. Immunofluorescence staining of early developmental embryos. The pre-implanted mouse embryos were treated with actinomycin D (a), camptothecin (b) or cycloheximide (c) for 15 h, and were stained with anti-ganglioside GM3 monoclonal antibody, TUNEL and Hoechst 33342 at each stage. Arrows indicate apoptotic fragments. The scale bars represent 50 μm. Control embryos; A, C, E, G, I, K, M, O, and Q, Treated embryos; B, D, F, H, J, L, N, P, and R.

Ganglioside GM3 is ubiquitously distributed on the outer leaflet of vertebrate plasma membranes, and this molecule has the simplest structure known to serve as a precursor for most species of the more complex gangliosides. Therefore, ganglioside GM3 is widely considered a potential key regulator in the combinatorial biosynthetic pathway of gangliosides. Several lines of evidence suggest its important functional roles in regulating a wide range of biological processes, including cell differentiation, cell growth and transmembrane signaling (Paller *et al.*, 1993; Hanada *et al.*, 1992; Bremer *et al.*, 1986; Hakomori, 1981). Regulatory roles have also been suggested for ganglioside GM3 in relation to developmental biology. Eggs of sea urchin are very rich in ganglioside GM3, with euplastic distribution and changes occurring in the eggs after fertilization (Shogomori *et al.*, 1993; Kubo and Hoshi, 1990). Our previous works have also demonstrated that ganglioside GM3 regulates follicular development and ovulation during the estrous cycle (Choo, 1999). Therefore, it is conceivable that ganglioside GM3 may be significantly implicated in ovarian maturation and early embryonic development, with the clarification of the spatiotemporal expression of ganglioside GM3 being potentially meaningful in the understanding of its functional roles in early embryonic development. In immunohistochemical studies using monoclonal antibody, which specifically reacts with ganglioside GM3, the constitutive expression of ganglioside GM3 was observed in the early stages (4-cell, morula and blastocyst) of mouse embryos, and the immunoreactivity against anti-ganglioside GM3 was not significantly different between each stage during early embryonic development. These results suggest that ganglioside GM3 may essentially participate in the early stages of embryonic development, with no spatiotemporal difference of its expression.

In addition, ganglioside GM3 has also been suggested to have pathological regulatory roles. Ganglioside GM3 may be pro-apoptotic in certain types of cells (Morales *et al.*, 2004; Kakugawa *et al.*, 2002), and the malignancy-suppressing effects of CD82 and CD9 are based partially on cell motility inhibition and apoptosis induction promoted by the concurrent synthesis of GM3 and *N*-glycosylation (Ono *et al.*, 1999). These suggestions lead us to consider that ganglioside GM3 may serve as an endogenous substance in the regulation of early embryonic development *via* the mediation of an apoptotic pathway. For clarification of the above-mentioned indication, the relationship between the numbers of embryonic cells and changes in the expression of ganglioside GM3 in the pre-implanted mouse embryos treated with actinomycin D, camptothecin and cycloheximide were examined using a pharmacological strategy to induce apoptosis of embryonic cells. The pharmacological properties and biochemical mechanisms of these drugs on the incidence of apoptosis in many different types of cells, including embryonic cells, have been well documented in previous reports (Alexandre *et al.*, 2000; Hietanen *et al.*, 2000; Moore *et al.*, 1999). Actinomycin D can interfere with DNA topoisomerase I and II, and directly damage DNA. Camptothecin inhibits topoisomerase I within the DNA replication complex, which produces DNA strand breaks. The mechanism by which cycloheximide affects cellular apoptosis is believed to involve caspases, which once activated, start a cascade of proteolytic processes leading to the unfolding of the apoptotic process. When pre-implanted mouse embryos at the 2-cell stage were incubated with these apoptosis inducers, the numbers of embryonic cells that developed to the blastocyst stage were markedly reduced compared to those in normal embryonic cells, and these cells showed prominent apoptotic fragments in their nuclei. These results agree well with the changes in the expression of ganglioside GM3 observed in apoptotic embryonic cells, which may support our suggestion that ganglioside GM3 potentially acts, at least in part, as an endogenous substance in the regulation of early embryonic development *via* the *de novo* mechanisms involved in cellular apoptosis. This suggestion may also be supported by previous reports (Matwee *et al.*, 2000; Weil *et al.*, 1996), which indicated the functional apoptotic pathway might be suppressed during early development. Therefore, the results observed in this study clearly indicate the pathophysiological roles of ganglioside GM3 in early embryonic development.

Ganglioside GM3 synthase is a key regulatory enzyme for ganglioside biosynthesis, as it catalyzes the first committed step in the biosynthesis of nearly all gangliosides in mammalian cells (Iber *et al.*, 1992; Fishman and Brady, 1976). Recently, Watanabe *et al.* (2002) suggested that the decreased proliferative potential due to ganglioside GM3 overexpression was attributable to the increased number of apoptotic cells in murine urinary bladder cancer. In our previous study, a similar phenomenon was also reported, suggesting that diabetic nephropathy is characterized by proliferation of glomerular mesangial cells and accumulation of extracellular matrix components. The glomerular activity of ganglioside GM3 synthase is significantly reduced by a diabetic condition (Kwak *et al.*, 2003). The above-mentioned information may indicate the pathophysiological implication of ganglioside GM3 synthase in the abnormal differentiation and growth of mammalian cells. In order to clarify the biochemical mechanisms involved in the increased expression of ganglioside GM3 in apoptotic embryos, this study examined the expressional changes in ganglioside GM3 mRNA at the blastocyst stage of apoptotic embryos. The concentration of the apoptosis inducers used in this ex-

periment (actinomycin D, camptothecin and cycloheximide) was chosen to have a significant apoptosis-inducing effect and relatively low detrimental effect on embryonic development. A significant increase in the expression of ganglioside GM3 synthase mRNA was observed in the blastocyst embryonic cells treated with the three apoptosis inducers, which was in close accorded with the elevated level of ganglioside GM3 expression in apoptotic embryos. Although proving that these *in vitro* findings have an important implication for the *in vivo* pathophysiological condition during early embryonic development may be difficult, the results obtained here provide a new rationale, suggesting that increased ganglioside GM3 expression in apoptotic cells during early embryonic development may be mediated, at least in part, by stimulation of its synthetic pathway.

In conclusion, the expression of ganglioside GM3 expression was found to be elevated in the early developmental stage of apoptotic mouse embryos, which was mediated, at least in part, by an increase in the transcriptional expression of ganglioside GM3 synthase mRNA. Therefore, this study suggests a pathophysiological implication of ganglioside GM3 in the regulation of embryonic cell survival via a mediating apoptotic mechanism.

ACKNOWLEDGEMENT

This work was supported by grant No. M10419010001-04N1901-00110 from the Korea Science & Engineering Foundation.

REFERENCES

Alexandre, S., Rast, C., Nguyen-Ba, G., and Vasseur, P., Detection of apoptosis induced by topoisomerase inhibitors and serum deprivation in Syrian hamster embryo cells. *Exp. Cell Res.,* 255, 30–39 (2000).

Betts, D. H. and King, W. A., Genetic regulation of embryo death and senescence. *Theriogenology,* 55, 171-191 (2001).

Bremer, E. G., Schlessinger, J., and Hakomori, S., Ganglioside-mediated modulation of cell growth: specific effects of GM3 on tyrosine phophorylation of the epidermal growth factor receptor. *J. Biol. Chem.,* 261, 2434-2440 (1986).

Byrne, A. T., Southgate, J., Brison, D. R., and Leese, H. J., Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. *J. Reprod. Fertil.,* 117, 97-105 (1999).

Calabrese, E. J., Apoptosis: biphasic dose responses. *Crit. Rev. Toxicol.,* 31, 607-613 (2001).

Choo, Y. K., Distribution of gangliosides GM3 in the rat ovary after gonadotropin stimulation. *Mol. Cells,* 31, 365-375 (1999).

Choo. Y. K., Chiba, K., Tai, T., Ogiso, M., and Hoshi, M., Differential distribution of gangliosides in adult rat ovary during the oestrous cycle. *Glycobiology,* 5, 299-309 (1995).

Fishman, P. H. and Brady, R. O., Biosynthesis and function of gangliosides. *Science,* 194, 906-915 (1976).

Hakomori, S., Glycosphingolipids in cellular interaction, differentiation and oncogenesis. *Annu. Rev. Biochim.,* 50, 733-764 (1981).

Hakomori, S., Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cell interaction. *J. Biol. Chem.,* 265, 18713-18716 (1990).

Hakomori, S., Yamamura, S., and Hanada, A. K., Signal transduction through glyco (shingo) lipid introduction and resent studies on glyco (sphigo) lipid-enriched microdomains. *Am. N. Y. Acad. Sci.,* 19, 1-10 (1998).

Hanada, K., Nishijima, M., Kiso, M., Hasegawa, A., Fujita, S., Ogawa, T., and Akamatsu, T., Sphingolipids are essential for the growth of Chinese hamster. *J. Biol. Chem.,* 267, 23527-23533 (1992).

Hardy, K., Cell death in the mammalian blastocyst. *Mol. Hum. Reprod.,* 3, 919–925 (1997).

Hietanen, S., Lain, S., Krausz, E., Blattner, C., and Lane, D. P., Activation of p53 in cervical carcinoma cells by small molecules. *Proc. Natl. Acad. Sci. U.S.A.,* 97, 8501–8506 (2000).

Iber, H., Zacharias, C., and Sandhoff, K., The c-series gangliosides GT3, GT2 and GP1c are formed in rat liver Golg. By the same set of glycosyltransferases that catalyse the biosynthesis of asialo-, a- and b-series gangliosides. *Glycobiology,* 2, 137-142 (1992).

Inoki, Y., Hakamata, Y., Hamamoto, T., Kinouchi, T., Yamazaki, S., Kagawa, Y., and Endo, H., Proteoliposomes colocalized with endogenous mitochondria in mouse fertilized egg. *Biochem. Biophys. Res. Commun.,* 278, 183-191 (2000).

Ji, M. Y., Lee, Y. C., Do, S. I., Nam, S. Y., Jung, K. Y., Kim, H. M., Park, J. K., and Choo, Y. K., Developmental patterns of mST3GalV mRNA expression in the mouse: In situ hybridization using DIG-labeled RNA probes. *Arch. Pharm. Res.,* 23, 525-530 (2000).

Kakugawa, Y., Wada, T., Yamaguchi, K., Yamanami, H., Ouchi, K., Sato, I., and Miyagi, T., Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression. *Proc. Natl. Acad. Sci. U.S.A.,* 99, 10718-10723 (2002).

Kawamura, S., Ohyama, C., Watanabe, R., Satoh, S., Hoshi, S., Gasa, S., and Orikasa, S., Glycolipid composition in bladder tumor: a crucial role of GM3 ganglioside in tumor invasion. *Int. J. Cancer,* 94, 343-347 (2001).

Keefe, D. L., Franco, S., Liu, L., Trimarchi, J., Cao, B., Weitzen, S., Agarwal, S., and Blasco, M. A., Telomere length predicts embryo fragmentation after *in vitro* fertilization in women-Toward a telomere theory of reproductive aging in women. *Am. J. Obstet. Gynecol.*, 192, 1256-1260 (2005).

Kim, B. H., Kim, C. H., Jung, K. Y., Jeon, B. H., Ju, E. J., and

Choo, Y. K., Involvement of nitric oxide during *in vitro* fertilization and early embryonic development in mice. *Arch. Pharm. Res.*, 27, 86-93 (2004).

Kotani, M., Ozawa, H., Kawashima, I., and Tai, T., Generation of one set of monoclonal antibodies specific for a-pathway ganglio-series gangliosides. *Biochim. Biophys. Acta,* 117, 97-103 (1992).

Kubo, H. and Hoshi, M., Immunocytochemical study of the distribution of ganglioside in sea urchin eggs. *J. Biochem.*, 108. 193-199 (1990).

Kwak, D. H., Jung, K. Y., Lee, Y. C., and Choo, Y. K., Expressional changes of ganglioside GM3 during ovarian maturation and early embryonic development in *db/db* mice. *Develop. Growth Differ.*, 45, 95-102 (2003).

Kwak, D. H., Rho, Y. I., Kwon, O. D., Ahn, S. H., Song, J. H., Choo, Y. K., Kim, S. J., Choi, B. K., and Jung, K. Y., Decrease of ganglioside GM3 in streptozotocin-induced diabetic glomeruli of rats. *Life Sci.*, 72, 1997-2006 (2003).

Matwee, C., Betts, D. H., and King, W. A., Apoptosis in the early bovine embryo. *Zygote,* 8, 57–68 (2000).

Moore, R. M., Lundgren, D. W., and Moore, J. J., Cyclooxygenase inhibitors decrease apoptosis initiated by actinomycin D, cycloheximide, and staurosporine in amnion-derived WISH cells. *J. Soc. Gynecol. Investig.*, 6, 245–251 (1999).

Morales, A., Colell, A., Mari, M., Garcia-Ruiz, C., and Fernandez-Checa, J. C., Glycosphingolipids and mitochondria: Role in apoptosis and disease. *Glycoconj. J.*, 20, 579-588 (2004).

Naora, H., Nishida, T., Shindo, Y., Adachi, M., and Naora, H., Constitutively enhanced *nbl* expression is associated with the induction of internucleosomal DNA cleavage by actinomycin D. *Biochem. Biophys. Res. Commun.*, 224, 258-264 (1996).

Nojiri, H., Takaku, F., Terui, Y., Miura, Y., and Saito, M., Ganglioside GM3; Acidic membrane component that increase during macrophage-like cell differentiation can induce monocytic differentiation of human myeloid and monocytoid leukemic cell lines H2-60 and U937. *Proc. Natl. Acad. Sci. U.S.A.*, 83, 782-786 (1986).

Ono, M., Handa, K., Withers, D. A., and Hakomori, S., Motility inhibition and apoptosis are induced by metastasis-suppressing gene product CD82 and its analogue CD9, with concurrent glycosylation. *Cancer Res.*, 59, 2335-2339 (1999).

Paller, A. S., Arnsmeier, S. L., Alvarez-Franco, M., and Bremer, E. G., Ganglioside GM3 inhibits the proliferation of cultured keratinocytes. *J. Invest. Dermatol.*, 100, 841-845 (1993).

Perez, G. I., Tao, X., and Tilly, J. L., Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. *Mol. Hum. Reprod.*, 5, 414-420 (1999).

Shogomori, H., Chiba, K., Kubo, K., and Hoshi, M., Non-plasmalemmal localization of the major ganglioside in sea urchin eggs. *Zygote,* 1, 215-223 (1993).

Summers, M. C., Mcginnis, L. K., Lawitts, J. A., Raffin, M., and Biggers, J. D., IVF of mouse ova in a simplex optimized medium supplemented with amino acids. *Hum. Reprod.*, 15, 1791-1801 (2000).

Svennerholm, L., Gongliosides and synapic transmission. In *advances in experimental biology and medicine: Structure* and function of gangliosides. *Adv. Exp. Med. Biol.*, 125, 533-544 (1980).

Varki, A., Biological roles of oligosaccharides: all of theories are correct. *Glycobiology,* 3, 97-130 (1993).

Watanabe, R., Ohyama, C., Aoki, H., Takahashi, T., Satoh, M., Saito, S., Hoshi, S., Ishii, A., Saito, M., and Arai, Y., Ganglioside G(M3) overexpression induces apoptosis and reduces malignant potential in murine bladder cancer. *Cancer Res.*, 62, 3850-3854 (2002).

Weil, M., Jacobson, M. D., Coles, H. S. R., Davies, J., Gardner, R. L., Raff, K. D., and Raff, M. C., Constitutive expression of the machinery for programmed cell death. *J. Cell Biol.*, 133, 1053-1059 (1996).

Yuan, Q., Ray, R. M., and Johnson, L. R., Polyamine depletion prevents camptothecin-induced apoptosis by inhibiting the release of cytochrome *c*. *Am. J. Physiol.*, 282, C1290-C1297 (2002).