Effect of Cellular Zinc on the Regulation of C2-ceramide Induced Apoptosis in Mammary Epithelial and Macrophage Cell Lines*

S. E. Han[a], H. G. Lee[a], C. H. Yun[1], Z. S. Hong, S. H. Kim, S. K. Kang, S. H. Kim[2]
J. S. Cho, S. H. Ha and Y. J. Choi**
Department of Animal Science and Biotechnology, School of Agricultural Biotechnology
Seoul National University, Seoul 151-742, Korea

ABSTRACT : Zinc is a trace element that is associated with a stimulation of immune function and regulation of ion balance for livestock production. In this study, the effect of zinc as inhibitor to apoptosis-induced cells was examined *in vitro* using mammary epithelial cell line, HC11 and macrophage cell line, NCTC3749. Cell viability, measured by MTT assay, indicated that 10 g/ml of zinc had a negative impact on cellular activity and 50 ng/ml was chosen for further testing. Apoptosis was induced in cells treated with C2-ceramide in serum-free media. DNA fragmentation and gene expression of acidic sphingomyelinase (a gene responsible for the progress of apoptosis) were distinctively low in zinc treated cells compared with those in non-treated controls. In conclusion, zinc is involved in the regulation of cell proliferation and apoptosis in mammary epithelial cells and macrophages. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 12 : 1741-1745)***

Key Words : Zinc, Cell Viability, Apoptosis, Immunity System

INTRODUCTION

Zinc is a trace element that is associated with a prevention of disease for livestock production (Wang et al., 2004). It is a central component of many metalloenzymes, including alkaline phosphatase, carboxypeptidases, thymidine kinase, and DNA and RNA polymerases. It plays an important role as component of the cell membrane structure and involves intracellular mechanism, such as antioxidant activity and protection against lipid peroxidation (Filipe et al., 1995). Zinc is also involved in the transcription and synthesis of protein, where zinc fingers are essential regulators for the gene expression, particularly in cells with high rate turnover such as gastro-intestinal epithelial cells and leukocytes. Zinc deficiency has been associated with changes or malfunction of immune function such as impairment of antibody and cytokine production, decreased delayed cutaneous hypersensitivity reactions and phagocytosis (Shanker and Prasad, 1998).

Another fundamental cellular process known to be regulated by zinc is the cell suicide process of apoptosis (Wyllie, 1997). Apoptosis is defined as a programmed physiological process of cell death characterized by a distinct set of morphological and biochemical changes, including cytoplasmic membrane blebbing, apoptotic body formation, nuclear condensation, and chromosomal DNA fragmentation. Because changes of the cellular zinc levels are sufficient to alter the susceptibility to apoptosis, zinc may serve as a coordinating regulator of mitosis and apoptosis via molecular targets such as endonucleases or caspases proteases. Zinc prevents the activation of caspase-3 and the downstream caspases through the activation of caspase inhibition pathways (Chai et al., 1999; Wolf and Eastman, 1999). Chemical properties of zinc have numerous advantages and role in cytoprotection. For instance, it protects macromolecules (e.g., microtubules) and subcellular organelles (e.g., membranes) (Kerr et al., 1987). Its affinity for sulfydryl groups coupled with the lack of redox activity enables it to reversibly suppress cysteine-dependent enzymes (including perhaps one or more of the caspases) without causing irreversible damage or inactivation.

In this study, the essential role of cellular zinc on C2-ceramide induced apoptosis was evaluated *in vitro* using mouse mammary epithelial cell line, HC-11 and mouse macrophage cell line, NCTC-3749.

MATERIALS AND METHODS

Cell line

Mouse mammary epithelial cell line, HC-11 and mouse macrophage cell line NCTC-3749 were obtained from American Type Culture Collection (ATCC, USA). Upon arrival, the cells were thawed at 37°C, washed with

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** Corresponding Author: YunJaie Choi. Tel: +82-2-880-4807, Fax: +82-2-875-7340, E-mail: cyjcow@snu.ac.kr
[1] International Vaccine Institute, Kwanak P. O. Box 14, Seoul 151-600, Korea.
[2] Departments of Biology, Kyung Hee University, Seoul, Korea.
[a] SeungEun Han and HongGu Lee were equally contributed to this research.
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complete media and placed on a 100-mm petridish (Falcon, USA) and incubated in a 5% CO_2 humidified atmosphere. The fresh complete medium was replaced, every other day, thereafter. Confluent cells were dissociated from the plate using cell dissociation buffer for three minutes at 37°C. After the disruption of the monolayer, complete RPMI 1640 medium was added and cells were sedimented by centrifugation. Subsequently, the cells were reconstituted in freezing medium (RPMI 1640 containing 20% FBS and 10% dimethylsulfoxide (DMSO: Sigma, USA), or in fresh medium and seeded at desired number of cells for further experiment.

Cell culture

Cells were cultured in 96-well microtiter plates at 2×10^4 cells per well in RPMI 1640 medium (GIBCO-BRL, USA) containing 2% fetal calf serum (FCS, GIBCO-BRL, USA) for NCTC-3749 and 10% FCS for HC-11, 1% L-glutamin (GIBCO-BRL, USA), and 1% of gentamicin (GIBCO-BRL, USA) in a final volume of 100 μl/well in a humidified atmosphere.

Optimization of zinc concentration for the study

The 20 μM/L was chosen after testing broad range of C2-ceramide in serum free media in order to induce apoptosis. Zinc sulfate at 50 ng/ml was added to study its effect on the inhibition of apoptosis. The plain RPMI1640 did not contain significant amount of zinc.

Cell viability assay

3- [4,5-Dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromids (MTT) assay (Roche, Germany) was employed for the quantification of viable cells, in which the reaction cleaved this substrate to form a pale blue formazan dye only through using metabolic active cells. Cells were seeded in 96-well microtiter plates at a density of 2×10^4 cells/well and treated with apoptosis-inducing reagents with/without zinc. At the designated time points, 10 μl MTT-labeling reagent was added at the level of 10 μl to each well and incubated in a humidified atmosphere at 37°C for 4 h, followed by the addition of 100 μl solubilization solution, and further incubated overnight at 37°C. The results were reported as the optical density (OD) readings, after the subtraction of the OD read-out of the mean plus three standard errors of the mean (SEM) of control wells at 620 nm subtracted from that at 540 nm.

DNA fragmentation

To compare DNA fragmentations of zinc-treated and non-treated cells, the cells were incubated in serum-free media containing C2-ceramide with/without zinc at 50 ng/ml. After incubation, the supernatants were removed, and the cell pellets were treated with 600 μl lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton×100. One microliter of RNase A was further added, and the preparation was incubated at 37°C for 30 minutes. After the addition of 200 μl protein precipitation solution into each tube, the mixtures were centrifuged at 14,000 rpm for 20 minutes. The supernatant was collected, and DNA was purified using the traditional phenol/chlorform extraction method. DNA was dissolved in 20 μl deionized H_2O and loaded on 1% agarose gel. After electrophoresis, the DNA fragments were visualized by ethidium bromide staining.

RT-PCR

All primers used in this experiment were synthesized by Bioneer (Seoul, Korea). The acidic sphingomyelinase were amplified with the following two primers: forward, 5'-TC CTGGACCACGAGACCTACATCTT-3' and reverse 5'-CC TCAGGTAGATAAGCAGCACTCGG-3'. As the internal control, GAPDH was amplified by PCR using primers: forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

Reverse transcription was performed in total RNA extracted from cultured cells using traditional TRIzol™ (Gibco BRL, USA) extraction method according to the manufacturer's instruction. The mixture containing 1 μl of total RNA (1 μg), 1 μl of 3' primer (10 μM), and 12 μl of deionized H_2O was incubated for 5 minutes at 65°C and placed on ice for 3 minutes. One microliter of dNTP mixture (10 mM each), 4 μl of 5×RT buffer, and 1 μl of MMLV reverse transcriptase (200 U/μl: Promega, USA) were further added, and the mixture was incubated at 42°C for 90 minutes. To terminate the reaction, the mixture was incubated for 5 minutes at 94°C and placed on ice for 3 minutes. This single stranded cDNA was stored at -20°C until used in PCR reaction, which was performed by using the GeneAmp PCR system 2400 (Perkin-Elmer, USA).

Caspase 3 activity

Caspase 3 activity was measured using Caspase 3 colorimetric assay kit (Promega, USA) according to the manufacturer's procedure. HC-11 cells were adjusted to 10^6 cells/ml, treated with 20 μM C2-ceramide with/without 50 ng/ml zinc sulfate, and incubated for 24 h at 37°C. The cells were dissociated and harvested by centrifugation at 450×g for 10 minutes at 4°C, washed with ice-cold PBS, and resuspended in the cell lysis buffer. The resuspended cells were lysed with freezing and thawing cycle and incubated on ice for 15 minutes. The cell lysates were then centrifuged at 15,000×g for 20 minutes at 4°C, and the supernatant fraction (cell extract) was collected. Protein concentrations of the cell lysates were determined through the bicinchoninic acid (BCA) assay (Pierce Chemical Co., USA). Equal amount of each protein was added to 50 μM

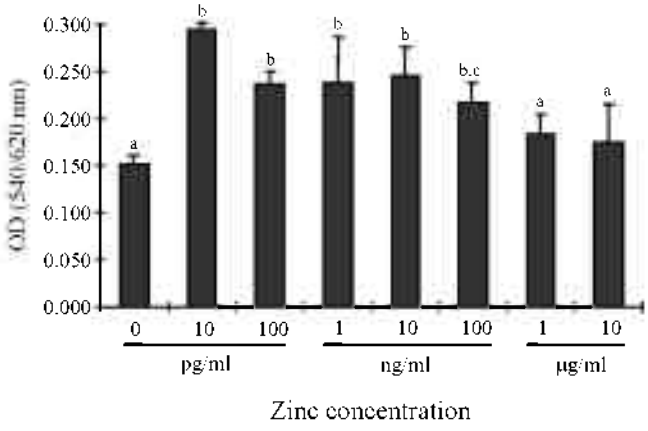

Figure 1. MTT assay on cell viability of NCTC-3749 at 24 h after treatment of zinc sulfate at different levels. * Data with the same letters are not significantly different ($p<0.05$).

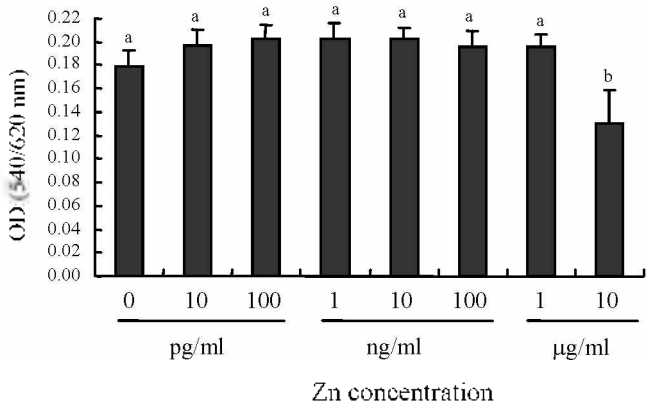

Figure 2. Cell viability of HC-11 as determined by MTT assay at 24 h after treatment of zinc-sulfate at different levels. * Data with the same letters are not significantly different ($p<0.05$).

DEVD-pNA substrate in the assay buffer. Samples were incubated at room temperature and analyzed using a spectrophotometer at optical density value 405 nm.

Statistical analysis

Statistical analysis was carried out by least significant difference (LSD) multiple range test using the General Linear Model (GLM) procedure of SAS (1985).

RESULTS

Optimization of zinc level

Zinc ion was added in zinc sulfate form, at various levels, to study its effect on cell proliferation in both mouse mammary epithelial cell line HC-11 and mouse macrophage cell line NCTC-3749. The viability, as measured by MTT assay, increased significantly ($p<0.05$) only in zinc-treated NCTC-3749 incubated for 24 h (Figure 1), whereas no increase was observed in HC-11 cells (Figure 2). Cell

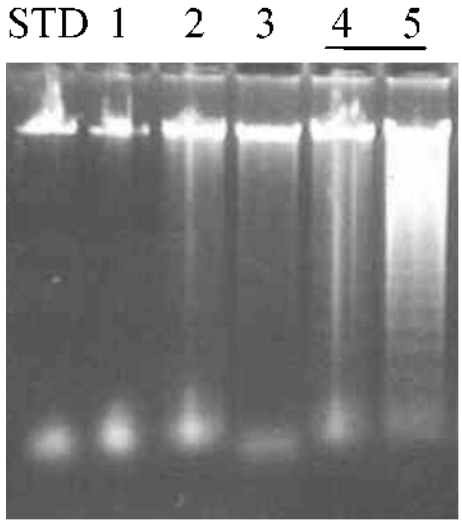

Figure 3. DNA fragmentation in HC-11 cells cultured with or without zinc sulfate. In different lengths of time, cells were incubated in 20 µM C2-ceramide with or without of 50 ng/ml zinc from zinc-sulfate. 1) control (without zinc); 2) 12 h after zinc treatment; 3) 12 h (without zinc); 4) 24 h after zinc treated; 5, 24 h (without zinc).

viability of both cell lines at 10 µg/ml of zinc level, dramatically decreased indicating its toxic effect. With NCTC-3749, 10 pg/ml of zinc ion was already sufficient to induce significant level of proliferation compared to non-treated control. The concentration of 50 ng/ml of zinc-sulfate was chosen for further studies in order to examine its effect on the inhibition of apoptosis since this level was safe and at the same time effective based on the results from cell viability assay.

Zinc inhibits C2-ceramide induced apoptosis

The cells were co-cultured with or without zinc ion in the presence of C2-ceramide as a cell death inducer at 20 µM in HC-11 cell line (Figure 3). DNA fragmentation, a hallmark of apoptosis, was inhibited when both cell lines, HC-11 and NCTC-3749 (figures for NCTC-3749 were not shown) were cultured with zinc for 24 h (Figure 3). MTT assay was performed to determine whether zinc ion could protect the cells from apoptosis. Results of MTT assay showed that zinc could protect cells from C2-ceramide induced apoptosis in serum-free media for up to 12 h and afterward zinc still could slow down the process of apoptosis compared to apoptosis induced and cells not treated with zinc up to 48 h (Figure 4). When HC-11 and NCTC-3749 cells were treated with zinc ion (50 ng/ml) for 20 h, the expression levels of acidic sphingomylinase, an apoptosis-associated gene, decreased significantly due to the zinc treatment in both HC-11 cells (Figure 5). The caspase-3 activity was further examined to determine if zinc could modulate caspase-3 activity in cultured cells. HC-11 cells were treated with C2-ceramide in the serum-free

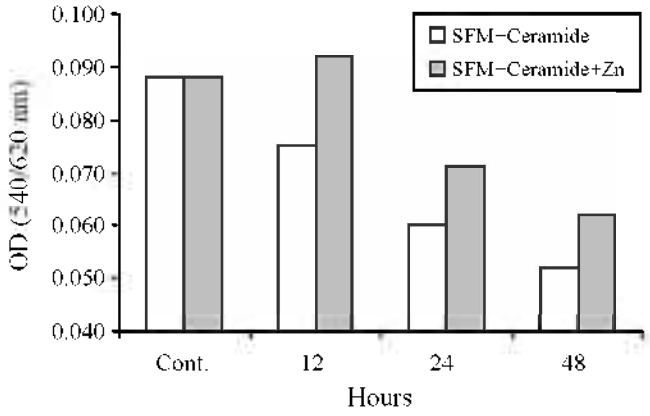

Figure 4. Effect of zinc on cell viability of apoptosis-induced HC-11 (a) and measurement of Caspase-3 activities of C2-ceramide-treated HC-11 (b) with or without zinc by PCR. Cells were co-cultured with serum-free media (SFM) containing C2-ceramide with or without zinc (50 ng/ml) to induce apoptosis. Same results were obtained from three independent experiments.

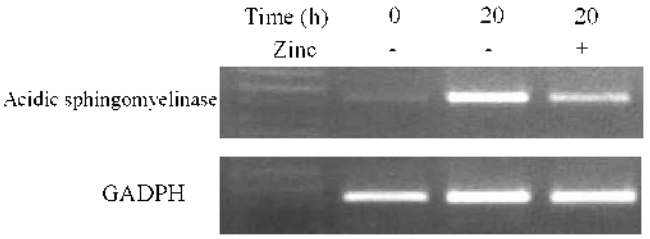

Figure 5. Levels of mRNA expression of acidic sphingomyelinase in HC-11 by RT-PCR. Cells were co-cultured with or without zinc (50 ng/ml) with C2-ceramide and mRNA was isolated at 0 and 20 h post zinc treatment. House-keeping gene GAPDH was used as the loading control.

media in the presence or absence of zinc sulfate at 50 ng/ml. There was a trend of alleviating caspase-3 activity in both HC-11 and NCTC-3749 cells with more obvious activity in HC-11 than in NCTC-3749 (Figure 6(a), (b)).

DISCUSSION

Cells entering apoptosis pathway undergo distinct structural changes that are consistent throughout all cell types (Kerr et al., 1987). These include the separation of dying cell from its neighbors, loss of microvilli, condensation of cytoplasm, and increased cell density to form dense masses underlying the nuclear membrane. In the present study, we demonstrated that the zinc significantly delayed cell (epithelial cells and macrophages) death ($p<0.05$). The effect appeared to be more obvious in NCTC-3749 macrophages. Since macrophages responded well in zinc treatment, this could be an important indicator since these cells serve as a major part of the first line of immune defense system. On the other hand, in HC-11 epithelial cells, zinc did not enhance the proliferation dramatically, probably because zinc has less effect on the cellular

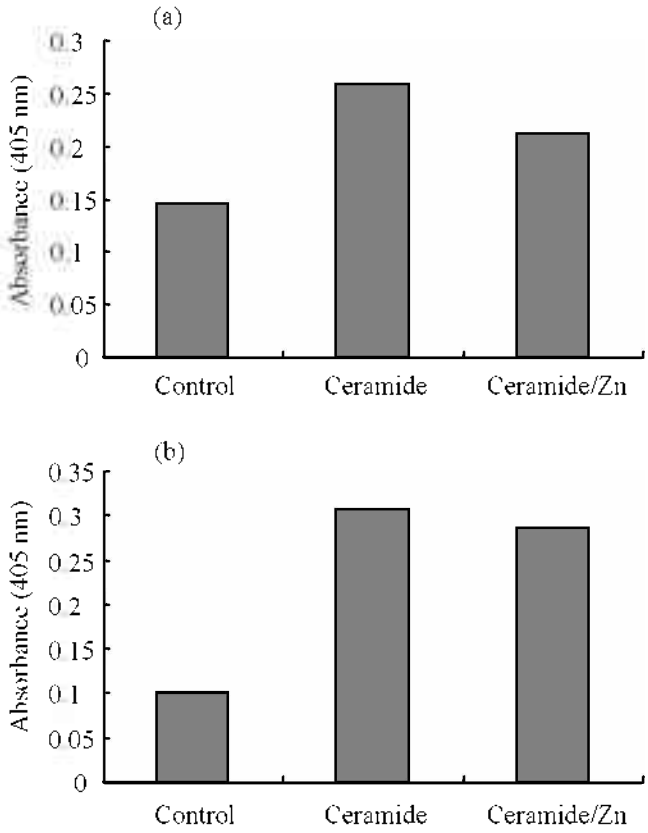

Figure 6. Measurement of Caspase-3 activities of C2-ceramide-treated HC-11 (a) and NCTC-3749 (b) with or without zinc by PCR. HC-11 cells were treated with 20 μM of C2-ceramide, and with or without zinc-sulfate (50 ng/ml) at 37°C for 24 h. Cell extracts were tested for caspase-3 activity according to the procedure provided by the manufacturer (Promega, USA). Same results were obtained from three independent experiments.

homeostasis under the normal condition in mammary epithelial cells.

During the apoptosis, condensation of the chromatin commences around the periphery of the nucleus, and later involves most of the nucleus. This is followed by nuclear fragmentation and budding of the cell to produce membrane-bound apoptotic bodies, which are shed into luminal cavities or phagocytes. Zinc ion has been used as a structural component, such as zinc finger proteins, and therefore it is probable that zinc ion could play an important role against apoptosis and resistance from death. DNA fragmentation, associated with various phenomena of apoptosis such as activation of the caspase family members of proteases and modulation of certain gene expressions, is a final offspring of these chain reactions and a good indication of apoptosis. In this regard, further studies on apoptosis were designed to ascertain the result in delaying DNA fragmentation in zinc treated HC-11 and NCTC-3749 cells. Acidic sphingomyelinase, a gene responsible for the progress of C2-ceramide induced apoptosis, is also known to be zinc-dependent (Lina et al., 1993). Upon apoptosis,

this gene is the first one to appear ubiquitously distributed in the cells. Furthermore, it participates cell regulatory programs in response to extrinsic factors such as TNF-α and irradiation. The activity of zinc-stimulated acidic pH-optimum sphingomyelinase was first detected in fetal bovine serum (Spence et al., 1989), in addition to alkaline-pH optimum sphingomyelinase activity in intestinal cells (Duan et al., 1995). In the early part of the study, we confirmed C2-ceramide induced apoptosis in HC-11 cells and this acidic sphingomyelinase gene expression increased linearly during the apoptosis (Kim et al., 2004). When zinc ion at 50 ng/ml was added to apoptosis-induced cells in order of cells, the expression of the apoptosis associated gene was obviously less than in untreated control cells. This result was also observed in the DNA fragmentation test where zinc treatment induced delayed type response, whereas untreated cells showed typical DNA fragmentation. Both results clearly demonstrated the effect of cellular zinc on reducing apoptosis at early stage and it proves that zinc could exert its ability to delay apoptosis.

Activation of caspases occurs because of interaction with either an extrinsic factor, such as growth factor withdrawal, exposure to radiation, malformation of cell matrix, and inducing chemotherapeutic agents, or an intrinsic factor, such as Fas/Apo-1 receptor-mediated cell death process (Vaux and Strasser, 1996; Liu et al., 1997; Perry et al., 1997; Aiuchi et al., 1998). In this experiment, the caspase-3 activity was examined to determine if zinc could modulate caspase-3 activity in cultured cells. HC-11 or NCTC-3749 cells were co-cultured with C2-ceramide with or without zinc ion at 50 ng/ml. The caspase-3 activity in both HC-11 and NCTC-3749 cells significantly increased in ceramide treated groups. It is interesting to note that zinc treatment, however, did not totally block the apoptosis in ceramide-treated cells. This is probably due to the effect of zinc that was not solely inhibiting apoptosis but rather combination effects of enhancing proliferation and at the same time inhibiting apoptosis. This was confirmed with trypan blue stained and visualized counting of the cells (data not shown).

It is worthwhile to note that addition of zinc ion resulted in higher cell viability in NCTC-3749 (macrophage) than in HC-11 (epithelial cell), whereas inhibition of apoptosis in HC-11 was superior to that in NCTC-3749. This result implies that under normal condition, zinc ion does not play a vital role in cell viability, but under certain situation like activation of immunity system or inducing apoptosis; zinc inhibits unnecessary cell death and perhaps helps proliferation. Furthermore, when immune cells have to be activated, zinc ion may be one of the key factors to aid in the activation of the cells.

In conclusion, zinc is involved in the regulation of cell proliferation and apoptosis, particularly in reducing and delaying the C2-ceramide induction of apoptosis in mammary epithelial cells and macrophages.

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