Mechanism of Apoptosis Induced by Spermine in MCF-7 Breast Cancer Cells

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Received June 17, 2008 / Accepted September 16, 2008

In the present work, we show that spermine (spm)-induced cytotoxicity is due to the mitochondrial-dependent pathway triggered by the intracellular Ca^{2+} increase in MCF-7 human breast cancer cells. Spm induced the intracellular Ca^{2+} increase in a dose-dependent manner in the medium containing 1.5 mM Ca^{2+} . Even in the Ca^{2+} -free medium, spm could induce a minor Ca^{2+} increase in a dose-dependent fashion, suggesting a probable leak from the internal storage. The cytotoxic effect of Ca^{2+} could be further proved by using either BAPTA or ionophore. Spm-induced Ca^{2+} increase led to the release of cytochrome *c* from mitochondria into the cytosol and the change of mitochondrial membrane potential. In MCF-7 cells, caspase-7 plays a key role in the downstream of apoptosis because caspase-3 is absent. In the cells treated with spm, the cleavage of caspase-7 and -12 was increased almost two-fold. The level of anti-apoptotic Bcl-2 protein decreased to 35% of the control; however, the cells showed increased expression of pro-apoptotic Bax protein about two-fold in response to spm. These results imply that the apoptotic signaling pathway activated by spm is likely to be mediated via the mitochondrial-dependent pathway.

Key words : Apoptosis, calcium, cytochrome *c*, MCF-7, spermine

Introduction

Polyamines are ubiquitous cellular components with multiple functions and are essential for life. The three basic polyamines (putrescine, spermidine and spermine) are small, flexible molecules, which specifically bind to nucleic acids and proteins and thus affect their conformation and biological activity [23]. Polyamines are important multifunctional cellular components and are considered mediators of cell growth and division. Recently, polyamines have been also known to be implicated in cell death. It appears that polyamines are bivalent regulators of cellular functions, promoting proliferation or cell death depending on the cell type and on environmental signals [7,16,18,20,26]. Among polyamines, spermine (spm) was most effective in inhibiting cancer cell growth. The cytotoxicity of spm has been suggested by the production of H_2O_2 and aldehyde(s) from spm in the presence of amine oxidase [1,2]. Polyamines are also important in death signaling pathways related to apoptosis. Many evidences indicate that polyamines directly affect the mitochondrial and postmitochondrial phases of apoptosis, by modulating cytochrome *c* release from mitochondria and activation of caspases. Recently, poly-

amines have been also implicated in the regulation of the signal transduction pathways [16,26]

Apoptosis is physiological cell death regulated by genetic mechanisms and is principally characterized by morphological and biochemical changes in their nuclei, including chromatin condensation and internucleosomal DNA fragmentation. Proteolytic cascades involving a family of proteases, called caspases, play a role in the signaling and execution stage of apoptosis. In particular, caspase-3 is the most active effector caspase in the execution stage of apoptosis by cleaving specific cellular substrates in both extrinsic (death receptor-dependent) and intrinsic (mitochondrial-dependent) pathways [4,9]. The activation of caspase-3 during apoptosis is regulated by multiple distinct pathways. Death receptors such as Fas/CD95 activate caspase-8, an initiator caspase, and the activated caspase-8 in turn activates caspase-3. Another pathway to activate caspase-3 is mediated by the mitochondrial alteration. Apoptosis-inducing stimuli lead to the release of cytochrome *c* from mitochondria and other apoptosis-inducing factors, which induce activation of pro-caspases and caspases. The release of cytochrome *c* can be related to the full opening of the mitochondrial permeability transition pore. The activation of caspase-3 during spm-induced apoptosis has already been reported in several cell systems [2,8,21]; however, caspase-3 is known to be absent in ER-positive MCF-7 breast cancer cell due to a 47-base pair deletion in exon 3 of caspase-3 gene. Instead of cas-

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pase-3, caspase-7 is known to act as a major downstream caspase in both intrinsic and extrinsic pathways in MCF-7 cells [24].

During the course of mitochondrial function disruption, Ca^{2+} was also found to increase in spm-treated cells. Ca^{2+} was the first identified endogenous substance to function as both a first and second messenger via the stimulation of an extracellular Ca^{2+} sensing receptor [25]. The concentration of intracellular free Ca^{2+} is maintained at about 100 nM, a very low level relative to the extracellular fluid (1.2 mM). The concentration of Ca^{2+} in the nuclear matrix or in the mitochondria matrix is similar to that of the intracellular Ca^{2+} ; however, other intracellular organelles maintain a large Ca^{2+} concentration gradient versus the cytoplasm. The most important Ca^{2+} storage compartment is the endoplasmic reticulum (ER) in most cells.

In recent years, great attention has been placed on the possible correlation between the effects of members of the Bcl-2 family of proteins on Ca^{2+} homeostasis and their role in the control of apoptosis [5]. Bcl-2 was the first family member proposed to have the ability to alter intracellular Ca^{2+} homeostasis [3]. Anti-apoptotic members in the Bcl-2 family, such as Bcl-2 and Bcl-XL, are preferentially localized in the mitochondrial membrane and protect mitochondria from perturbation, thereby preventing cell death [10]. The specific localization of Bcl-2 in the membranes of mitochondria and ER, and the demonstration that Bcl-2 acts as an ion channel when inserted into lipid bilayers suggest that Ca^{2+} signaling could be a target of the action of this anti-apoptotic oncoprotein [19]. Under normal conditions, mitochondrial Ca^{2+} uptake appears to serve as an activating signal to increase metabolism. However, it has been shown that mitochondrial Ca^{2+} uptake promotes cytochrome *c* release in cell exposed to the proapoptotic agent, staurosporine [15].

Mitochondria are considered as one of the main Ca^{2+} storage compartment in cells rather than as regulators of cytoplasmic Ca^{2+} concentration [16,18]. Since polyamines are aliphatic molecules, they assume an important role in maintaining Ca^{2+} homeostasis [13]. Polyamines can directly interact with the mitochondrial inner membrane at the level of specific binding sites. Therefore, in the present study, we investigated the signaling mechanism of apoptosis induced by spm in the following aspects: i) involvement of intracellular calcium modulation, ii) caspase activation, iii) cytochrome *c* release from mitochondria, and iv) disruption of mitochondrial membrane potential.

Materials and Methods

Chemicals

Spermine (N,N'-bis[3-aminopropyl]-1,4-butanediamine), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT), ionomycin calcium salt, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-1H-imidazole (Nifedipine), 2-[[3-(trifluoromethyl)phenyl]amino] benzoic acid (Fluofenamic acid), 1,2-Bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid tetrapotassium salt (BAPTA), Fluo-3/AM, Pluronic F-127, propidium iodide (PI), Hoechst 33342, ribonuclease A, dimethylsulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM, with L-glutamine and 1,000 mg/l Glucose) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO (New York, NY, USA). All other chemicals were purchased from standard commercial sources.

Cell culture

The MCF-7 breast carcinoma cell line was obtained from the Korean Cell Line Bank, and maintained in DMEM containing phenol red with 10,000 units/ml of penicillin G, 10 mg/ml streptomycin, and 7% heat-inactivation FBS in a humidified atmosphere of 95% air; 5% CO_2 at 37°C. The culture media were changed every 2 or 3 days. Cells were harvested using trypsin-EDTA and then subcultured at weekly intervals.

Measurement of cell viability

Cell viability was determined by MTT assay. MCF-7 cells were plated at 5×10^4 cells/ml in 48 multi-wells in DMEM with 10% fetal bovine serum and were allowed to adhere overnight. Cells were then treated with each different drug or with the same volume of vehicle (0.1% ethanol). For each assay, medium was aspirated from each well and replaced with 400 μ l of 0.5 mg/ml MTT in phenol red-free DMEM medium. The treated cells were incubated in a 5% CO_2 incubator at 37°C for 4 hr. After incubation, the medium was removed and the converted dye was solubilized in a 1:1 mixture of DMSO and EtOH. The color intensity was measured by the ELISA Reader (Tecan, Germany) with 570 nm filter.

Ca^{2+} mobilization

Intracellular Ca^{2+} was measured using Fluo-3/AM. The cells grown in DMEM were washed twice and re-

suspended in HBSS; 120 mM NaCl, 6 mM KCl, 1.5 mM CaCl_2 , 2 mM MgCl_2 , 10 mM HEPES and 12 mM glucose. For all Ca^{2+} -free experiments, CaCl_2 -free HBSS with 1mM EGTA was used. For fluo-3/AM loading, 1×10^6 cells were resuspended in HBSS (or Ca^{2+} -free HBSS for Ca^{2+} starvation) containing 3 μM fluo-3/AM, 0.02 μM pluronic F-127. Incubation was performed in the dark at 37°C for 25 min. Cells were then washed with HBSS (or Ca^{2+} -free HBSS) and split into 48 well plates at 2×10^5 cells/wells for each treatment. Fluorescent activity was measured (485 nm excitation/535 nm emission) every 1 or 5 min for 30 min.

Assessment of morphology

Nuclear condensation and/or chromatin fragmentation found during early apoptosis was determined morphologically by the confocal laser scanning microscopy (CLSM) (LSM510, Carl Zeiss, Oberkochen, Germany) after labeling PI and Hoechst dye. Cells with each treatment were harvested onto a clean, fat-free glass slide with cellspin (Hanil Sci. Industrial, Korea). The cells were then fixed for 30 min in 4% paraformaldehyde and then stained in 4 $\mu\text{g}/\text{ml}$ Hoechst 33342 or 10 $\mu\text{g}/\text{ml}$ PI with 50 $\mu\text{g}/\text{ml}$ ribonuclease A for 1 hr at 37°C . The cells were observed and photographed by CLSM.

Measurement of cytochrome *c* release

Cells were attached on a slide glass by cytospin centrifugation for 2 min at 900 rpm using cellspin, and then the cells were fixed with 4% paraformaldehyde (Sigma, MO, USA) at room temperature for 20 min. Fixed cells were washed three times with PBS for 5 min and incubated with 0.2% Triton X-100 for 15 min. After three washes with PBS, the cells were incubated with a cytochrome *c* antibody in 1% bovine serum albumin (BSA) at room temperature for 2 hr. For a secondary reaction, the cells were incubated with a rabbit FITC-conjugated secondary antibody at room temperature for 2 hr. For the counter staining of nuclei, cells were incubated with PI (0.05 mg/ml) at room temperature for 30 min. The sample were washed several times with PBS and mounted and stored at 4°C in the dark until observation. The cells were observed by CLSM.

Measurement of mitochondrial membrane potential change ($\Delta\Psi\text{m}$)

To measure $\Delta\Psi\text{m}$, the fluorescent probe JC-1 (5,5',6,6'-

tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide) was used. Cells of 5×10^5 were incubated in darkness with 100 nM JC-1 at 37°C for 30 min. After washing with PBS, the cells were suspended in the PBS and then mounted with coverslip. The cells were observed by CLSM.

Western blot analysis

Cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C . Proteins were extracted by washing cells twice with ice-cold PBS and incubating them for 60 min on ice in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 2 $\mu\text{g}/\text{ml}$ leupeptin. For Western blotting, an aliquot of 60 μg of protein was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose transfer membrane. Blotting was performed using the primary antibodies of anti-Bcl-2 (1:1,000 dilution) and anti-Bax (1:1,000 dilution), and then with the proper secondary antibody (Sigma, MO, USA). The immunocomplexes were detected by using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech Korea, Inc., Seoul, Korea).

Statistical analysis

All experiments were carried out at least in triplicate. Results were expressed as mean $\pm$ SEM. Statistical significances between the control and the treatment group were determined by one-way analysis of variance and then followed by the Student T test. In all cases, a *p* value of less than 0.05 was considered statistically significant.

Results

Effect of spm on cell viability

The effect of spm on viability of MCF-7 cells was examined using the MTT staining method. When the cells were treated for 4 days with 1-40 μM of spm, cell viability was decreased in a dose- and time-dependent manner as shown in Fig 1.

At the lower concentrations (under 10 μM), spm stimulated the cell growth. The stimulatory effect was quite significant in the day 4 culture treated with 1 μM spm. However, a significant cytotoxicity by spm was noticed at the concentration higher than 20 μM . Cells treated with 40 μM spm showed under 30% viability through the 4 days

Fig. 1. Effect of spm on cell viability in MCF-7 breast cancer cells. Cell viability assay was done by MTT staining method as described in "Materials and Methods". Data are expressed as percent change of the control (con). Data shown are means of three independent experiments, bars, SE.

of incubation, compared to the control.

Spm has been known to induce apoptosis in many cell systems. To examine that in the present experiment with MCF-7 cells, we studied the morphological change of the nuclei in the cells treated with spm by staining with Hoechst and PI dye. MCF-7 cells treated with 40 μ M spm showed severe chromatin condensation and nuclear fragmentation which was a typical early event of apoptotic cell death (Fig. 2).

Effect of spm on intracellular Ca^{2+} level

Ca^{2+} homeostasis is very important for many cell function,

Fig. 2. Induction of apoptosis in breast cancer cells by spm. MCF-7 cells were treated with 40 μ M or 1 mM of spm for 36 hr and stained with Hoeschst 33342 (A) or PI (B). Data shown are from one of three independent experiments.

including growth, differentiation, and death. Intracellular Ca^{2+} level change was examined to find out whether the cytotoxic effect of spm was related to the intracellular Ca^{2+} modulation. Spm at the concentration of 40 μ M caused a significant increase in the intracellular calcium level in the cells grown at medium containing 1.5 mM Ca^{2+} (Fig. 3A). The level of intracellular Ca^{2+} increased continuously until 10 min of incubation time and remained a sustained phase after that up to 40 min. To check if the Ca^{2+} level increase was due to Ca^{2+} influx from the exterior, the same experiment was performed in the EDTA treated Ca^{2+} -free medium. As shown in Fig. 3B, the Ca^{2+} level increase was not prominent, compared to the high level found in the calcium containing medium (notice the fluorescence intensity). The minor increase in the intracellular Ca^{2+} level in the Ca^{2+} -free medium might have resulted from the leak of the stored intracellular Ca^{2+} pool. Thus, the main source of intracellular Ca^{2+} increase induced by spm treatment is most likely due to the Ca^{2+} influx from outside.

Effect of BAPTA and ionomycin on spm-induced apoptosis

In order to determine the co-relationship between Ca^{2+}

Fig. 3. Intracellular Ca^{2+} level change by spm treatment in 1.5 mM Ca^{2+} medium (A) and Ca^{2+} -free medium (B). For each experiment, 1×10^6 cells were seeded in 48 multi-well plate containing HBSS. CaCl_2 was omitted and EDTA was added into the reaction medium for Ca^{2+} -free experiment. Details are mentioned in "Materials and Methods". Data shown are means of three independent experiments, bar, SE.

increase and the apoptotic death, cells were pretreated with 10 μM of BAPTA, an intracellular Ca^{2+} chelate, for 2 hr before being treated with spm. Cell viability and intracellular Ca^{2+} level change was then studied in the cells treated with and without spm. As shown in Fig. 4A, BAPTA pretreatment almost completely blocked spm-induced intracellular Ca^{2+} increase. BAPTA also significantly abolished the cytotoxicity of spm (Fig. 4B), especially in the early stage of spm treatment. When the effect of BAPTA on spm-induced apoptosis was checked by CLSM, BAPTA prevented the nuclear shrinkage caused by the spm treatment (Fig. 5). If the increase of intracellular Ca^{2+} level induced by spm was one of the main causes of cell death, an enforced Ca^{2+} increase by ionophore would give the same results. To check the above hypothesis, cells were treated with ionomycin. At the 10 μM of ionomycin, cells were found to undergo cell death in a time- and dose-dependent manner (Fig. 6). Cell viability dropped to 56% of the control after 12 hr treatment. When PI staining was performed, ionomycin-induced cells underwent apoptosis. These findings further demonstrated that raising intracellular Ca^{2+} induced apoptosis in MCF-7 cells.

Fig. 4. Effect of BAPTA on intracellular Ca^{2+} change (A) and cell viability (B) in the cells treated spm. For each experiment, 2×10^5 cells/well were seeded into the 48 multi-well plate. Ca^{2+} level was measured in the cells exposed to BAPTA 2 hr before treatment with spm. Data shown are the means of three independent experiments, bar, SE.

Fig. 5. Influence of BAPTA on spm-induced apoptosis. A; control, B; 40 μM spm, C; 10 μM BAPTA, D; 40 μM spm + 10 μM BAPTA. The experimental conditions are the same as in Fig. 5. The cells were stained with PI for 1 hr at 37°C , then observed under CLSM. Data shown are from one of three independent experiments.

Fig. 6. Induction of apoptosis by ionomycin. The experimental conditions are the same as in Fig. 5. A; untreated control, B; 5 μM ionomycin, C; 10 μM ionomycin. Data shown are from one of three independent experiments. D; cell viability change in ionomycin treated cells. Data shown are the means of three independent experiments, bars, SE.

Cytochrome *c* release from mitochondria and mitochondrial membrane potential change ($\Delta\Psi\text{m}$)

Cytochrome *c* released from mitochondria is known to have a pivotal role in the progression of apoptosis. Therefore, to check if the apoptotic cell death induced by spm was preceded by release of cytochrome *c*, immunocytochemistry was performed using specific antibody for cytochrome *c*. In the immunofluorescent study, cytochrome *c* in the control cell was found in a punctuate pat-

tern, in keeping with its normal mitochondrial location (Fig. 7). However, a diffuse distribution of cytochrome *c* was found in the spm-induced cells, which supported the hypothesis that spm led to the release of cytochrome *c* from mitochondria into the cytosol. The change of $\Delta\Psi_m$ in response to the spm was further investigated by using JC-1 dye under CLSM. Spm treatment induced a shift from the JC-1 aggregate (red) to the JC-1 monomeric (green) form of the dye, indicating mitochondrial depolarization because of the loss of membrane potential (Fig. 8).

Effect of spm on the caspase activation and Bcl expression

Caspase activation is regulated by the release of mitochondrial polypeptide activators including cytochrome *c* during apoptosis. MCF-7 breast cancer cells do not express caspase-3. Caspase-7, however, plays a key role in the downstream of apoptosis. To prove the involvement of caspase-7 in spm-induced apoptosis, cells were treated with 40 μ M spm for 36 hr, and then the level of cleaved/activated caspase-7 protein was analyzed using Western blotting. As shown in Fig. 9, caspase-7 expression was increased more than two-fold by spm treatment. Caspase-12, which is specifically localized to the ER, has

Fig. 7. Immunofluorescence analysis of spm-induced cytochrome *c* release in MCF-7 cell. Cytochrome *c* was localized on mitochondria in the untreated cells (punctuate staining) but was found to be distributed throughout the cytosol (diffuse fluorescence in the cells) when treated with the spm for 36 hr. The experimental conditions are mentioned in "Materials and Methods". A; control, B; 40 μ M spm, C; 1 mM spm.

Fig. 8. Analysis of mitochondrial membrane potential change in spm-induced apoptosis. JC-1 dye-stained MCF-7 cells were analyzed with CLSM in the absence (A), presence of 40 μ M and 1 mM spm (B and C). The untreated cells showed a significant population of cells with the J aggregate (red) form of the JC-1 dye indicating a normal mitochondrial membrane potential. A significant shift of J aggregate (red) to J monomer (green) was observed in the population of cells treated with spm for 36 hr. The experimental details are mentioned in "Materials and Methods". Data shown are from one of three independent experiments.

been reported to be activated during ER stress-induced apoptosis. When the level of caspase-12 expression was checked to investigate whether spm-induced apoptosis is related to ER stress, there was 236% increase in caspase-12 expression. These results indicated that both caspase-7 and -12 are related in spm-induced apoptosis. The detailed involvement of ER-associated caspase-12 in the presence of spm is requires further study.

The Bcl-2 family proteins are regulators that play a major role in apoptosis. They exist as multigene subfamilies with many homologues that are individually expressed in various tissues. The Bcl-2 family includes death promoting (Bax) and death inhibiting (Bcl-2) members, most of which

Fig. 9. Western blot analysis showing the effects of spm on Bcl family proteins and caspase protein levels. The experimental details are mentioned in "Materials and Methods". Data shown are from one of three independent experiments. The numbers are from densitometric scanning.

are resident proteins in the mitochondrial membrane. When we examined the expression level of them in the cells treated with spm, there was significant decrease in the level of Bcl-2 protein. However, the cells showed increased expression of Bax almost two-fold in response to spm, as compared to the control (Fig. 9). This result implies that the apoptotic signaling pathway activated by spm is likely to be mediated via the mitochondrial-dependent pathway.

Discussion

Polyamines are known to be cytotoxic in many cell systems *in vivo* and *in vitro*. Among polyamines, spm is known to be more effective than spermidine and putrescine [21,22]. The action of spm is partly through the activation of caspases or partly through the generation of oxidative products in several cancer cell lines including human breast cancer and prostate cancer cells [6,8]; however, the precise mechanism of spm-induced apoptosis is yet unclear. The present work demonstrates that spm induces apoptotic cell death through the intracellular Ca^{2+} modulation in MCF-7 cells.

Ca^{2+} is an essential intracellular regulator of signal transduction [5,6]. Diseases are often associated with the perturbed regulation of intracellular free Ca^{2+} [8]. The pathological consequences of perturbed Ca^{2+} regulation are not surprising considering the role of Ca^{2+} in a variety of cellular processes as diverse as muscle contraction, apoptosis, gene expression, synaptic transmission, and proliferation. Because of the importance of Ca^{2+} in disease, transporters of Ca^{2+} such as voltage-gated plasmalemmal Ca^{2+} channels and Ca^{2+} -ATPase can be potential therapeutic targets for which screening assays are useful. One area of research receiving attention in regard to alterations in Ca^{2+} homeostasis is cancer. Recently, Ca^{2+} influx via a pathway known as capacitative Ca^{2+} entry (CCE) has been observed in MCF-7 breast cancer cells [15], and inhibitors of Ca^{2+} influx can inhibit the proliferation of breast cancer cells [11]. Furthermore, an inhibitor of CCE blocks the growth of prostate cancer cells *in vitro* and a mouse *in vivo* model. Another Ca^{2+} pathway altered in cancer is the plasma membrane Ca^{2+} -ATPase, which is a pump responsible for maintaining low levels of resting intracellular Ca^{2+} . The expression of the plasma membrane Ca^{2+} -ATPase is altered in human breast cancer cell lines [12]. The above findings

indicate that Ca^{2+} homeostasis is essential for the normal cell function.

In the present work, spm increased intracellular Ca^{2+} levels via extracellular Ca^{2+} influx and partly by releasing stored intracellular Ca^{2+} . It has been reported that spm induces intracellular Ca^{2+} increase by regulating the Ca^{2+} -sensitive receptors in the plasma membrane of the cardiac cells [25]. Spm could also induce intracellular Ca^{2+} increase through the IP3 receptors in ER.

Further, in the present study we showed that the increase in intracellular Ca^{2+} level was directly related to apoptosis. This finding was further checked by using BAPTA and ionomycin. The increase in intracellular Ca^{2+} by ionomycin was accompanied by apoptotic cell death. However, BAPTA, and intracellular Ca^{2+} chelate, abolished the cytotoxic effect of spm. In general, an increase in cytosolic Ca^{2+} stimulates the mitochondrial uptake of Ca^{2+} . Mitochondrial Ca^{2+} accumulation, in turn, changes the mitochondrial microenvironment [19]. Mitochondrial Ca^{2+} overload is known to lead to the opening of the permeability transition pore. In the present work, intracellular Ca^{2+} level increases, induced by spm, lead to mitochondrial permeability change, clearly demonstrated by JC-1 dye staining. Spm treatment resulted in the membrane depolarization. The opening of a permeability transition pore by depolarization leads to the release of cytochrome *c* and other apoptosis inducing factors which can activate pro-caspases that eventually lead to cell death. The release of cytochrome *c* from the cells treated with spm was clearly demonstrated by fluorescence microscopy in the present work.

Bax and Bid are also known to induce a change in mitochondrial membrane polarization. Bax is a pro-apoptotic member of Bcl-2 family. Bcl-2 and Bcl-X_L of this family can promote cell survival, but Bax promotes cell death. The overexpression of Bax accelerated cell death [17]. When we checked the expression of Bcl-2 family proteins in the cell treated with spm, the level of Bcl-2 protein significantly decreased. However, the expression of Bax protein increased almost two-fold, which might lead to the change of mitochondrial membrane permeability. Caspase activation is initiated by the release of mitochondrial proteins, including cytochrome *c*. Caspases lead to the drastic morphological changes of apoptosis by proteolysing and disabling a number of key substrates, including gelsolin, protein-tyrosine kinase (PTK), and focal adhesion kinase (FAK) [9]. In MCF-7 breast cancer cells which show the ab-

sence of caspase-3, caspase-7 is known to be in the downstream of the caspase cascade and plays a pivotal role in the terminal execution stage of apoptosis. Caspase-7 is able to cleave various substrates, which contributes to the typical biochemical feature of apoptosis. When we checked the level of cleaved caspase-7 by Western blotting, it was increased more than two-fold, indicating that spm induced the apoptotic cell death through the activation of caspase-7 in MCF-7 cells.

In the present work, we proved that spm induces apoptosis through the modulation of intracellular Ca^{2+} levels in association with the disruption of mitochondrial function.

ER has been known as a major Ca^{2+} storage site. Recent study has shown that the ER Ca^{2+} store may play an important role in apoptotic induction [14]. Ca^{2+} released from ER induced mitochondria to release cytochrome *c* and Bax, a pro-apoptotic protein. To the contrary, a reduction of Ca^{2+} released from ER prevented mitochondrial damage. It has been reported recently that overexpression of Bax caused a loss of ER content [3]. Caspase-12 is specifically localized on the cytoplasmic side (outer membrane) of ER and is activated during ER stress-induced apoptosis [27]. In our present results, spm treatment in MCF-7 cells induced almost two-fold increase in Bax expression. Thus, we checked if there were any changes in the caspase-12 protein level. As mentioned earlier, caspase-7 expression increased more than two-fold by spm treatment, and the caspase-12 protein level also increased two-fold. These results may suggest the possibility of ER-mitochondrial cross-talk in spm-induced apoptosis. Further studies should be performed to pin down the precise interaction of ER-mitochondrial Ca^{2+} modulation during spm-induced apoptosis.

In summary, we have provided the evidence that spm induces the increase in intracellular Ca^{2+} level in MCF-7 breast cancer cells. The Ca^{2+} level change leads to apoptotic cell death via the mitochondrial-dependent pathway including loss of mitochondrial membrane potential, cytochrome *c* release, and caspase activation.

Acknowledgement

This work was supported for two years by Pusan National University Research Grant.

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초록 : MCF-7 유방암 세포주에 있어서 spermine에 의해 유도된 세포사멸 기작

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폴리아민은 미생물에서부터 동·식물에 이르기 까지 모든 세포들에서 발견되는 극성을 띤 분자이다. 세포의 성장과 분화에 폴리아민이 중요한 역할을 한다는 것은 이미 오래 전부터 알려져 온 사실이나 정확한 작용 기작은 잘 밝혀져 있지 않다. 최근에 와서는 폴리아민이 다양한 방법으로 세포 독성을 유발한다는 결과가 보고되고 있다. 본 연구에서는 spm의 세포독성 효과가 세포 내 칼슘이온 농도 증가에 따른 미토콘드리아-의존 기작에 의하여 일어난다는 것을 보여준다. Spm에 의해 유도된 세포 내 칼슘이온 농도 증가는 주로 외부로부터의 칼슘 유입에 의한 것으로 생각되며, 세포 내 칼슘 증가는 미토콘드리아로부터의 cytochrome c 방출과 미토콘드리아막의 탈분극에 의한 membrane potential 변화를 초래하였다. 세포사멸에 주도적인 역할을 하는 caspase의 확인에 있어서는, MCF-7 세포는 caspase-3이 결핍되어서 caspase-7이 중심적인 역할을 하는 것으로 이미 알려져 있다. 본 연구에서 확인 한 결과 spm 처리 시 caspase-7과 -12가 활성화되었다. 또한 세포사멸 조절 단백질인 Bcl-2 종류 단백질들의 발현을 조사 한 결과 세포사멸 억제 단백질인 Bcl-2의 발현은 크게 억제되었으며, 촉진단백질인 Bax는 spm 처리시 단백질 양이 거의 2배로 증가되었다. 이상의 결과에 의하면, spm에 의해 유도되는 세포사멸과정은 세포질 내 칼슘이온 농도 증가에 의한 미토콘드리아의 변화가 주도적인 역할을 하는 것으로 생각된다.