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Spermidine-Induced Apoptosis via Reactive Oxygen Species Generation and Caspase 3 Activation in Mouse P19 Embryonal Carcinoma Cells

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Abstract: The present study was to investigate the effects of spermidine-induced apoptosis in mouse P19 embryonal carcinoma cells. The cell viability using MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay showed that spermidine decreased cell viability after 24-h incubation in a concentration-dependent manner with an IC_{50} value of approximately 20 μM. Using Hoechst33342 staining, chromatin condensation and apoptotic bodies in P19 cells treated with spermidine were observed. Moreover, flow cytometric analysis stained with propidium iodide increased the accumulation of DNA fragmentation in the pre G_0/G_1 phase, indicating apoptotic cell death, which was accompanied by the generation of reactive oxygen species (ROS) as detected with the fluorescence probe 2',7'-dichlorofluorescin diacetate (DCFH-DA). Western blot analysis revealed that spermidine-induced apoptosis was associated with an increase of Bax and caspase 3 expression, meanwhile the expression of Bcl-2 was decreased. The results suggest that spermidine-induced P19 apoptotic cell death is involving ROS, Bcl-2 family as well as caspase 3.

Key words: Spermidine, mouse P19 embryonal carcinoma cells, apoptosis, reactive oxygen species, Bcl-2 family

INTRODUCTION

Polyamines, putrescine, spermidine and spermine, are ubiquitous regulators of cell growth and proliferation, maintenance the function of all normal cell types (Thomas and Thomas, 2001; Wang *et al*., 2004). In mammals, serum levels of spermidine and spermine under physiological condition are ranging from 0.6 to 0.9 μM while under pathological condition such as age-associated memory impairment increased in spermidine levels in the CA1 and CA2/3 (Liu *et al*., 2008), are from 1.2 to 5.7 μM (Hospattankar *et al*., 1980; Facchiano *et al*., 2001). Most of polyamines in cells exist as a polyamine-RNA complex: total spermidine 1.33 mM and spermine 1.58 mM, meanwhile the amount of free polyamine is small: spermidine 0.2 mM and spermine 0.08 mM (Igarashi and Kashiwagi, 2010). Polyamines are also found abundant in foods such as meat, oranges, tea leaves, mushroom, soybeans, cheese and especially fermented soy namely natto a traditional Japanese food which contained 0.08 μmol g^{-1} of spermidine and spermine, considered as an important source of polyamines (Okamoto *et al*., 1997; Soda *et al*., 2009a). The contents of spermidine from high polyamine test diet and human milk breast were 1,540 and 711 nmol g^{-1}, respectively, which were higher than spermine and putrescine (Romain *et al*., 1992; Soda *et al*., 2009b).

Interestingly, administration of spermidine markedly extended the lifespan of both natural ageing such as flies, worms, yeast as well as human immune cells (Eisenberg *et al*., 2009) and age-associated pathology in kidney (Soda *et al*., 2009b). Meanwhile, the administration of spermidine (83 mg kg^{-1} b.wt.) in Wistar rats was not observed of adverse effect, intravenous infusion (600 mg kg^{-1} b.wt.) caused acute toxicity namely a dose-related decrease in blood pressure as well as in plasma calcium and inorganic phosphate (Til *et al*., 1997). Supra-physiological level of spermidine generated products of hydrogen peroxide and aldehydes leading to apoptotic cell death (Agostinelli *et al*., 2004; El-Enshasy *et al*., 2010; Madeo *et al*., 2010). It has been shown that spermidine was cytotoxic to baby-hamster kidney cells which may be through the oxidation by an intracellular copper-containing amine oxidase (Brunton *et al*., 1991). Moreover, spermidine at the concentration of 10 μM was able to induce apoptosis in extravillous trophoblasts

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through the generation of hydrogen peroxide and mitochondrial pathway (Dash *et al*., 2003). Up to now, mechanism of spermidine generated reactive oxygen species (ROS) leading to apoptotic cell death has not yet been elucidated in P19 embryonal carcinoma cells, which exhibited similar characteristics to undifferentiated embryonic and fetal cell types (Jones-Villeneuve *et al*, 1982; McBurney *et al*., 1982). P19 cells have been used to evaluate teratogenicity of a numerous toxic substances (Kultima *et al*., 2004; Kling *et al*., 2005).

The precise functions of polyamines including spermidine in cells depend on cell types and environmental signals (Pignatti *et al*., 2004). It has been suggested that excessively polyamines induced apoptosis of alveolar macrophage through the generation of ROS and caspase-dependence (Lasbury *et al*., 2007). Polyamines analogues, N^1, N^{11}-diethylnorspermine and bisnaphthalimidopropyl spermidine, also played a role in apoptosis on carcinoma cell lines through cytochrome c release, caspase activation (Chen *et al*., 2001; Ralton *et al*., 2009), cleavage of poly (ADP-ribose) polymerase (PARP) which accompanied by increasing the ratio of Bax/Bcl-2 (Chen *et al*., 2010). The aim of this study was to determine the mechanism of spermidine-induced apoptosis on the P19 cells.

MATERIALS AND METHODS

Materials: Spermidine (N^1-[3-aminopropyl]-1,4-butanediamine), propidium iodide (PI), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and bovine serum albumin (BSA) were provided by Sigma (St. Louis, MO, USA). Minimum essential medium alpha (αMEM) and newborn calf serum (NCS) were purchased from Invitrogen (New Zealand). Fetal bovine serum (FBS) (HyClone®) was from Perbio Science bvba (Belgium). Antibodies specific for caspase 3, Bax, Bcl-2 and actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and secondary anti-rabbit Ig G (H and L, horseradish peroxidase-linked) were from Cell Signaling Technology (MA, USA). The enhanced chemiluminescence Western blotting detection reagent was purchased from Amersham Biosciences, USA. All other chemicals used were commercially available reagents or analytical reagent quality.

Cell culture: The mouse P19 embryonal carcinoma cell line was obtained from American Type Culture Collection (CRL-1825, Rockville, MD, USA) and maintained in tissue culture flask supplemented with 90% αMEM, 7.5% heat-inactivated FBS and 2.5% NCS at 37°C under a humidified atmospheric condition of 5% CO_2 and 95% air at Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University (2007-2008) as well as at Department of Physiology, Faculty of Medicine, Srinakharinwirot University (2009-2010). Cell culture medium was routinely changed every 2 to 3 days. Spermidine was dissolved in deionized distilled water to give a stock solution of 100 mM. The stock solution was sterile-filtered through a 0.22 μm membrane and stored at -20°C.

MTT assay: The MTT colorimetric assay, widely used as an index of cell viability/toxicity as well as mitochondrial activity, was performed following standard procedures (Hansen *et al*., 1989). Cell viability was determined by a colorimetric method, which is based on the activity of mitochondrial dehydrogenase enzymes to reduce a yellow water-soluble dye of tetrazolium ring to a dark blue insoluble formazan product. After 24-h incubation with various concentrations of spermidine (1-50 μM), cells were observed under a phase contrast microscope, then MTT solution was added to give a final concentration of 1 mg mL^{-1} and returned to the incubator for 4 h. The reaction was stopped by adding lysis buffer containing 50% (v/v) N,N-dimethyl formamide and 20% sodium dodecyl sulfate (SDS), pH 4.7. The reaction mixture was maintained overnight at 37°C and the optical density at an absorbance of 595 nm was monitored using a microplate reader (Sunrise Classic, Tecan GmbH, Austria). The percent of control was calculated as absorbance units in the presence of spermidine as the percentage of the in control (vehicle alone).

Fluorescence microscopic assay using Hoechst 33342 staining: Nuclear morphological changes of apoptotic cells were determined using the fluorescent dye Hoechst 33342 as described previously (McKeague *et al*., 2003). P19 cells were seeded onto 24-well plates and treated with spermidine at a concentration of 10 and 20 μM for 24 h. Nuclei were then stained with 2.5 μg mL^{-1} Hoechst 33342 for 15 min. The cells were visualized and photographed under a fluorescent microscope. The healthy cells showed an oval-shaped cell body and its chromatin stained dimly and occupied the majority of the cell body volume. The chromatin of an apoptotic cell was condensed, intensely stained by Hoechst 33342 (blue color) and shifted to the periphery of the cell body.

Flow cytometric analysis for measurement of subG_0/G_1 phase: After P19 cells were treated with spermidine (10-45 μM) for 24 h, flow cytometric analysis was

performed as described previously (Yoshizumi *et al*., 2002). P19 cells were harvested and washed with PBS, fixed in cold 70% ethanol and stored at 4°C for 24 h. After washing with PBS, the cells were resuspended in PBS containing RNase A (200 μg mL^{-1}), incubated at 37°C for 30 min and centrifuged at 1,000×g for 10 min. The cells were stained with PI (100 μg mL^{-1}) and incubated in the dark at 4°C for 30 min. The cells were washed and subjected to flow cytometric analysis of DNA content which was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Excitation was performed at 488 nm and emission was detected at 600 nm. CELLQuest™ software (Becton Dickinson) was used for $subG_0/G_1$ peak detection of the apoptotic cells. Ten thousand cells in each sample were analyzed and expressed as a percentage of total cells.

Measurement of intracellular ROS level: After P19 cells incubated with or without spermidine (10-50 μM) for 24 h, the cells were washed with ice-cold PBS and incubated with 10 μM DCFH-DA (30 min at 37°C) in the dark. DCFH-DA was rapidly de-acetylated by esterases after diffuses through the cell membrane and yields 2',7'-dichlorodihydrofluorescein (DCFH2), which is then oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS (Pae *et al*., 2003). The cells were washed twice with PBS and further incubated in probe free PBS before analysis using a FACScan flow cytometer (Becton Dickinson) for detection of the mean fluorescence intensity (FL2-H) at excitation 488 nm and emission 525 nm for DCF. Ten thousand events (cells) were collected and analyzed by the CellQuest software.

Western blot analysis: P19 cells were treated with various concentrations of spermidine (10-50 μM) for 24 h. Treated cells were washed with ice-cold PBS and solubilized in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1% NP-40, 0.02% NaN_3, 1 mM phenylmethanesulfonyl fluoride, 2 μg mL^{-1} leupeptin and 2 μg mL^{-1} aprotinin for 30 min on ice. Protein concentrations in the samples were determined by the Bradford method. Whole cell extracts were mixed with Laemmli loading buffer (225 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 9% 2-mercaptoethanol and 0.009% bromphenol blue) and boiled for 5 min. Equal amounts of protein were loaded each lane, resolved by 12% SDS-polyacrylamide gel electrophoresis and electro-blotting onto PVDF membrane. The membranes were blocked with TBST (10 mM Tris, 0.1 M NaCl and 0.01% Tween 20) containing 5% non-fat dry milk for 2 h at room temperature. After blocking, blots were probed with specific primary antibodies: Bax, Bcl-2, caspase 3 and actin for overnight at 4°C. After washing with TBST, the membranes were further incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) for 2 h at room temperature. Blots were treated with an enhanced chemiluminescene detection reagent and exposed to CL-X films.

Statistical analysis: All data were expressed as mean±SEM of three or more separate experiments. Statistical differences were evaluated using one-way ANOVA followed by Duncan's multiple comparison procedure. Values of $p<0.05$ were considered statistically significant.

RESULTS

Spermidine exerts cytotoxicity against P19 cells: To evaluate the cytotoxicity of spermidine (Fig. 1A) on P19 cells, cell viability was determined by the MTT assay. P19 cells were exposed to 1 to 50 μM spermidine for 24 h. The viability was reduced in a concentration-dependent manner with the IC_{50} of approximately 20 μM (Fig. 1B). The results demonstrated that spermidine exhibited potent growth inhibitory and cytotoxic activity against P19 cells.

Spermidine induces apoptosis in P19 cells: To determine whether the observed decrease in cell viability exerted by spermidine was a consequence of an apoptotic process,

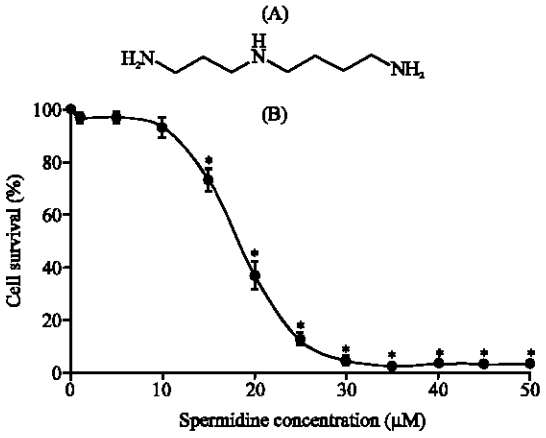

Fig. 1: (A) Structure of spermidine [N-(3-aminopropyl)-1,4-butanediamine or aminopropyl-tetramethylenediamine] (B) spermidine-induced cytotoxicity of P19 cells. The cells were treated with various concentrations of spermidine (1-50 μM). Cell viability was measured after 24-h incubation by MTT reduction assay. The results are presented as mean values and bar represented ±SEM, n = 3. *$p<0.05$ compared to control

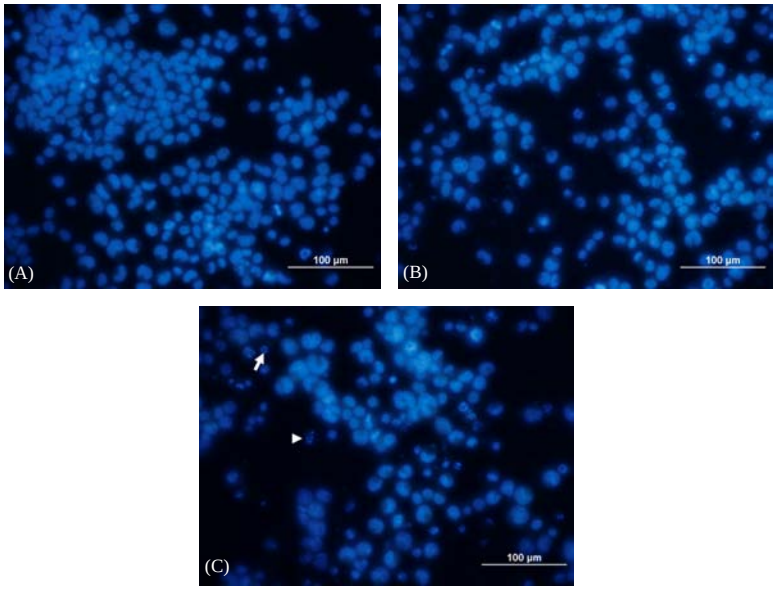

Fig. 2: Morphological changes of P19 cells induced by spermidine staining with Hoechst 33342. Typical staining profiles are cells (A) non-exposed to spermidine (control), (B) exposed to 10 µM and (C) 20 µM spermidine for 24 h. Arrows point fragmented nuclear and chromatin condensation. Arrow heads point apoptotic bodies. Scale bar = 100 µm

cellular and nuclear morphological changes using Hoechst 33342 staining was performed under microscope. The untreated P19 cells exhibited intact chromatin and the normal round-to-oval shape of living cells (Fig. 2A). In contrast, most of spermidine-treated P19 cells (20 µM) for 24 h, displayed the typical morphological changes of an apoptotic process including cell shrinkage, nuclear condensation, fragmented chromatin accumulation to the inside of nucleolus membrane with a shape like crescent moon or ring and the formation of apoptotic bodies (Fig. 2C), which emitting bright fluorescence. However, small amount of apoptotic cell death were found after exposure of spermidine at the concentration of 10 µM (Fig. 2B).

To confirm whether spermidine-induced apoptosis in P19 cells, the percentage of apoptotic cells was quantitatively assessed by flow cytometric analysis, after staining with PI which stains DNA by intercalating between the bases with a stoichiometry of one dye per 4-5 base pairs of DNA. The viable cells have normal amounts of DNA while cells that are undergoing late apoptosis have less DNA. The subG_0/G_1 peak in flow cytometric detection considered as an indicator of cell apoptosis. As the results, spermidine produced a concentration-dependent increase in the apoptotic cell population at the concentration ranging from 10 to 45 µM. The percentage of apoptotic cells (sub G_0/G_1 peak) increased significantly

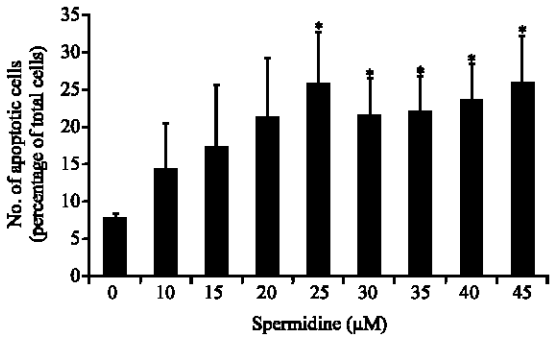

Fig. 3: Flow cytometric analysis showing effects of spermidine-induced apoptosis in P19 cells. The cells were treated with vehicle and spermidine (10-45 µM) for 24 h and analyzed by flow cytometry after propidium iodide staining. Numeric data indicated the mean percentage of apoptotic cells. The results are represented as mean±SEM, n = 3. *$p<0.05$, compared with control group

($p<0.05$) from 7.83±0.57% in control to 25.73±7.10% in the cells exposed to 25 µM spermidine for 24 h (Fig. 3). Present result was coincident with the previous study that the concentration of vitamin K_3 leading to apoptosis in human breast cancer cell line MCF-7 was higher than the IC_{50} (Akiyoshi *et al*., 2009). The results suggested that

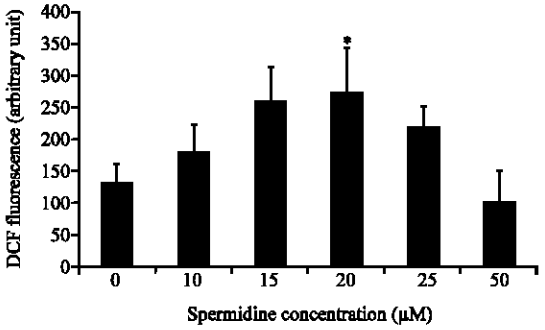

Fig. 4: Spermidine induced the generation of intracellular ROS in P19 cells measured by flow cytometry, staining with DCF-DA

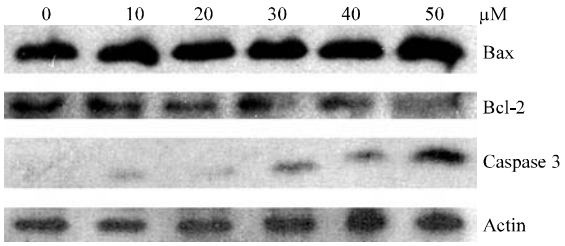

Fig. 5: Spermidine induced the expression of Bax and caspase 3 and reduced the expression of Bcl-2 in P19 cells after the exposure of spermidine (10-50 μM) for 24 h. Equal amount of cell lysates were separated by 12% SDS-PAGE. Immunoblotting was performed using specific antibodies

spermidine induced P19 cell death after 24-h incubation through the process of apoptosis.

Spermidine induces the generation of intracellular ROS: To determine whether spermidine induced apoptosis via generation of ROS, intracellular ROS production was measured with a flow cytometer using DCFH-DA staining. Exposure of P19 cells to various concentrations of spermidine (10-50 μM) increased levels of intracellular ROS in a concentration-dependent manner ranging from 10 to 20 μM and significantly increased at 20 μM (Fig. 4). Our results suggested that spermidine-induced apoptosis was accompanied by the generation of ROS.

Spermidine elevates the expression of Bax and caspase 3 and reduces Bcl-2 expression: The involvement of Bcl-2 family in apoptosis has been widely established. To clarify the molecular mechanism of spermidine-induced apoptosis, the expression of protein Bax, Bcl-2 and caspase 3 detected by Western blot was examined in P19 cells after exposed to spermidine (10-50 μM) for 24 h. The results showed that the spermidine increased the expression of pro-apoptotic Bax and caspase 3, meanwhile Bcl-2 expression was reduced (Fig. 5). These findings strongly suggested that the spermidine-induced apoptosis in P19 cells was due, at least in part, to elevate the expression of Bax and caspase 3 and reduction of Bcl-2.

DISCUSSION

The present data demonstrated the involvement of Bcl-2 family in spermidine-induced apoptosis in mouse P19 embryonal carcinoma cells through the increment of ROS, Bax and caspase 3, as well as the reduction of Bcl-2.

Spermidine decreased cell viability on P19 cells with the IC_{50} 20 μM, approximately. Our investigation was concomitant with the previous study that spermidine induced toxicity on myocytes was apparent at 20 μM (Tipnis and He, 1998). Effects of intracellular polyamines level in cells are rather depended on cell types. It has been found that after starvation, 24-h exposure of spermidine caused apoptotic cell death on numerous cells including primary rat aorta smooth muscle cells (IC_{50} 3.3 μM), primary cultures of bovine aortic endothelial cells (IC_{50} 10 μM) and a human cutaneous melanoma cell line Mel 120 cells (IC_{50} 12 μM) (Facchiano *et al*., 2001). In addition, spermidine-induced apoptosis in extravillous trophoblast cells was at the concentration starting from 10 μM via the generation of hydrogen peroxide and mitochondrial pathway (Dash *et al*., 2003).

Spermidine entered prokaryotes via ATP binding cassette (ABC) transporters, consisting of a periplasmic substrate binding protein, two transmembrane proteins and a membrane-associated ATPase (Igarashi *et al*., 2001) and mammalian cells at least in part via glypican-1 and phosphorylated caveolin-1 dependent endocytic mechanism (Roy *et al*., 2008; Igarashi and Kashiwagi, 2010). Spermidine induced apoptosis via a marked increase of intracellular transglutaminase activity found in rat aortic smooth muscle cells (Facchiano *et al*., 2001). Over-accumulation of intracellular polyamines in an *Escherichia coli* strain caused by deficient in spermidine acetyltransferase, an enzyme that metabolized spermidine, showed a decrement of cell viability (Higashi *et al*., 2008), which may be due to polyamine catabolism producing the generation of ROS which undergo conversion to secondary highly ROS and reactive nitrogen species leading to apoptosis and transformation (Schipper *et al*., 2000; Seiler, 2005; Babbar *et al*., 2007). In the present study, exogenous spermidine led to be metabolized producing over-accumulation of intracellular ROS which

triggered P19 cells undergoing apoptosis. It has been shown that ROS was the upstream mitochondrial membrane potential depolarization, Bax delocalization, cytochrome c release and caspase activation which can cleave death substrates such as PARP and induce DNA fragmentation and the declined expressions of Bcl-2 (Ansah *et al*., 2008; Kim *et al*., 2010a, b; Liu *et al*., 2010). Moreover, ROS can directly attack the major cellular components such as DNA strand breaks and lipid peroxidation and finally induced apoptotic cell death (Svobodova *et al*., 2007). The present study provides novel evidence that over-accumulation of spermidine was able to inhibit growth and proliferation of P19 cells through the generation of ROS and mitochondrial pathway.

CONCLUSION

The results of this study showed that spermidine induced apoptosis in P19 cells were mediated by the generation of ROS, an increase of Bax and caspase 3 expression and a decrease of Bcl-2 expression. These results also suggest that the ingestion of polyamine-containing diet should be planned and careful monitoring of pregnant women.

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