SPATA4 Counteracts Etoposide-Induced Apoptosis via Modulating Bcl-2 Family Proteins in HeLa Cells

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Spermatogenesis associated 4 (SPATA4) is a testis-specific gene first cloned by our laboratory, and plays an important role in maintaining the physiological function of germ cells. Accumulated evidence suggests that SPATA4 might be associated with apoptosis. Here we established HeLa cells that stably expressed SPATA4 to investigate the function of SPATA4 in apoptosis. SPATA4 protected HeLa cells from etoposide-induced apoptosis through the mitochondrial apoptotic pathway, in the way that SPATA4 suppressed decrease of the mitochondrial membrane potential, the release of cytochrome c, and subsequent activation of caspase-9 and -3. We further demonstrated that SPATA4 upregulated anti-apoptotic members of Bcl-2 family proteins, Bcl-2, and downregulated the pro-apoptotic member of Bcl-2 family proteins, Bax. Knockdown of SPATA4 in HeLa/SPATA4 cells could partially rescue expression levels of bcl-2 and bax. In conclusion, SPATA4 protects HeLa cells against etoposide-induced apoptosis through the mitochondrial apoptotic pathway. Our findings provide further evidence that SPATA4 plays a role in regulating apoptosis.

Key words spermatogenesis associated 4 (SPATA4); apoptosis; Bcl-2 family; etoposide

Apoptosis, or programmed cell death, is critically important in a variety of biological processes, such as tissue development, homeostasis, and the removal of damaged or infected cells.1–3) Mitochondria play a crucial role in regulating apoptotic cell death. The mitochondrial apoptotic pathway acts in response to various types of intracellular stress, including DNA damage.4,5) The Bcl-2 family proteins have either pro- or anti-apoptotic activity through mitochondrial apoptotic pathway, Bcl-2 family proteins alter the permeability of mitochondrial outer membrane6) and hence regulate the release of apoptotic factors. Those factors trigger the caspase proteolytic cascade, chromatin condensation and DNA fragmentation.7,8) Thus, the Bcl-2 family proteins are critical regulators in mitochondrial apoptotic pathway.

Spermatogenesis related gene 2 (SRG2; GenBank accession no. AF395083) was first cloned from a cryptorchidism mouse model,9) and the human homolog of SRG2, spermatogenesis associated 4 (SPATA4; originally named as TSARG2, GenBank accession no. AY040204) was cloned from a human cDNA library using nested polymerase chain reaction (PCR) and draft human genome searching.9,10) SPATA4 shows 78% similarity to SRG2 in their protein sequences, while it demonstrates no evident homology with other known proteins.11) Using molecular beacon probe to examine the mRNA expression level of SPATA4 gene in cryptorchid testis of various stages, it was found that the gene was up-regulated distinctly,9) while the pathogenesis of cryptorchidism is associated with the spermatogenic-cell apoptosis.12)

Apoptosis of spermatogenic cells is a multi-gene coordinated process. Bcl-2 family proteins were reported to be involved in the apoptosis of spermatogenic cells in mammals.13,14) However, research on spermatogenic-cell apoptosis is still at the initial stage. Recently, several genes homologous to SPATA4 have been studied. A novel mouse gene, mTSARG7 can accelerate growth of GC-1spg cells, causing cell cycle to traverse the S-phase and enter the G2-phase.15) SPATA4 can accelerate the proliferation of MCF7 cells in a similar mechanism.16) Cell cycle progression is closely related to both cell proliferation and apoptosis. Taking together, we speculated that SPATA4 could function in apoptosis.

In the present study, HeLa cells stably expressing SPATA4 were established as a model. Etoposide, a DNA topoisomerase II inhibitor that induces cell apoptosis predominantly through the mitochondrial apoptotic pathway,16–18) was used to trigger cell apoptosis. Our results showed that SPATA4 significantly protected HeLa cells from etoposide-induced mitochondrial apoptosis by regulating Bcl-2 family proteins.

MATERIALS AND METHODS

Cell Lines and Cell Culture HeLa cells were purchased from ATCC and cultured in RPMI-1640 (Gibco, U.S.A.) supplemented with 10% fetal bovine serum (HyClone, U.S.A.) and 0.1% antibiotics (penicillin and streptomycin) at 37°C in a humidified atmosphere containing 5% CO2. To generate stable cell lines, the full-length cDNA of human SPATA4 was cloned into the pcDNA3.1 (−) vector. The primers used for cloning of the human SPATA4 gene were as follows: Fw, 5′-CCG AAT TCA TGG CTG CCG CCG G-3'; Rv, 5′-TAA GCT TGG CAT CACT TCT TCT CAA G-3'. HeLa cells were transfected with the pcDNA3.1 (−) vector or the pcDNA3.1 (−) / SPATA4 plasmid using Lipofectamine™ 2000 (Invitrogen, U.S.A.) and selected with 800 µg/mL of G418. The selected clones were maintained in RPMI-1640 containing 200 µg/mL of G418.

Drug Treatments Etoposide (Sigma, U.S.A.) was dis-
solved in dimethyl sulfoxide (DMSO) in the concentration of 100 mM. In all experiments, exponentially growing cells were seeded at a proper confluence and 12 h later exposed to etoposide for the time as indicated. Concentration of DMSO in culture never exceeded 1%. 

**Determination of Cell Viability** Cell viability was assessed by conventional 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay. Briefly, HeLa cells or HeLa/SPATA4 cells were suspended at a final concentration of 5×10^5 cells/mL and seeded in 96-well plates in a final volume of 0.2 mL. Different concentrations (0–100 μM) of etoposide were added to each well in triplicate. After incubation for the indicated length of time, MTT (0.5 mg/mL) was added and cultures were incubated at 37°C, CO2 free atmosphere for 4 h. After centrifugation, the formazan precipitate was dissolved in 150 μL DMSO and the absorbance at 490 nm was measured using Benchmark microplate reader (Bio-Rad, U.S.A.). Percent cell viability was calculated as the ratio of cell viability of the experimental samples to that of the control samples ×100.

**Morphological Analysis of Apoptotic Cells** 4′-6-Diamidino-2-phenylindole (DAPI) staining was used to detect cells undergoing apoptosis. In brief, cells were fixed with 4% paraformaldehyde at room temperature for 30 min. After washing twice with phosphate buffered saline (PBS), cells were stained with 1 μg/mL DAPI at room temperature for 20 min. Morphologic changes were then observed under phase contrast and fluorescent microscope.

**Annexin V-Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) Apoptosis Analysis** Annexin V-binding assays were performed according to the manufacturer’s instructions (BioVision, U.S.A.). Briefly, approximately 5×10^5 cells/mL in 6-well plates were treated or untreated with 100 μM of etoposide for 24 h. The cells were harvested and used for Annexin V-FITC/PI staining. The stained cells were analyzed by flow cytometry to determine the percentages of Annexin V+/PI− (early apoptosis) and Annexin V+/PI+ (late apoptosis) cells.

**Determination of Mitochondrial Membrane Potential (MMP)** MMP was estimated by flow cytometry using JC-1 staining. Briefly, HeLa/SPATA4 cells were treated or untreated with 100 μM of etoposide at 37°C, CO2 free atmosphere for 4 h. After centrifugation, the formazan precipitate was dissolved in 150 μL DMSO and the absorbance at 527 and 590 nm was measured using a Bio-Rad microplate reader (Bio-Rad, U.S.A.). Percent cell viability was calculated as the ratio of cell viability of the experimental samples to that of the control samples ×100.

**Detection of Cytochrome c Release from Mitochondria** The assay was carried out as previously described. Briefly, cells were harvested and washed twice with PBS, and then resuspended in 200 μL of digitonin lysis buffer (75 mM KCl, 1 mM Na2HPO4, 8 mM NaH2PO4, 250 mM sucrose, 100 μg/mL digitonin). After 5-min incubation on ice, samples were centrifuged at 14000 rpm at 4°C, and cytosol cytochrome c, contained in the supernatant is then subjected to Western blot.

**Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)** Total RNA was extracted from HeLa cells using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was transcribed with RevaTra Ace reverse transcriptase (Toyobo, Osaka, Japan) using oligo(dT)20 as primers. cDNA transcribed from 0.6 μg of total RNA was subsequently used for RT-qPCR analysis with SYBR Green Real-time PCR Mater Mix (Toyobo) in CFX96 Real-Time PCR Detection Systems (Bio-Rad, Hercules, CA, U.S.A.). The primers used in qPCR were shown in Supplementary Table S1. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s.

**Western Blot** Cells were washed twice with cold PBS, and then harvested and lysed with TEN-T buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 5 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mg/mL of aprotinin). Total protein (30 μg) was fractioned by 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride membranes ( Pall, U.S.A.) for blotting. An antibody against SPATA4 (prepared by our laboratory) was used at 1:1000 dilution and Bcl-2 at 1:1000 (Cell Signaling Technology, U.S.A.), Bax at 1:1000 (Cell Signaling Technology), Caspase-3 at 1:500 (Santa Cruz, U.S.A.) and Caspase-9 at 1:500 (Santa Cruz) for blotting overnight at 4°C, respectively. Secondary antibodies were used at 1:10,000 dilution. β-Actin and β-tubulin were used for normalization. Immunoreactivity was visualized using an enhanced chemiluminescent substrate for detection of HRP (Pierce, U.S.A.).

**Statistical Analysis** The values reported in graphs are the mean±standard deviation (S.D.) from three independent experiments. One-way ANOVA followed by Bonferroni multiple comparison was performed to assess the differences. A value of p<0.05 was considered statistically significant.

**RESULTS**

**Stable Expression of SPATA4 in HeLa Cells Enhances Cell Viability under Etoposide Exposure** To study the role of SPATA4 in cell apoptosis, HeLa cells, which do not express SPATA4 normally, were selected as the appropriate cell model to examine the function of SPATA4. After transfection of a vector expressing SPATA4 and selection using G418, HeLa cells stably expressing SPATA4 (HeLa/SPATA4) were established (Figs. 1A, B). Etoposide, a classic compound that induces cell apoptosis predominantly through the mitochondrial apoptotic pathway, was utilized to induce apoptosis of HeLa cells. Control HeLa cells transfected with empty vector and HeLa/SPATA4 cells were treated with various concentrations of etoposide for 24 h, and then cell viability was determined by MTT assays. Etoposide induced control HeLa cell death in a dose-dependent manner, while no significant reduction of cell viability was observed in HeLa/SPATA4 cells after etoposide treatment (Fig. 1C), indicating that SPATA4 increased cellular resistance to cell death. The concentration of 100 μM was chosen for subsequent experiments, at which etoposide exhibited a toxicity of 53.32±2.26% for control cells whereas 27.43±3.65% for HeLa/SPATA4 cells.

**SPATA4 Protects HeLa Cells from Etoposide-Induced...
Apoptosis  Next, we investigated whether SPATA4 affected etoposide-induced apoptosis by examining apoptotic features such as nuclear morphological changes and DNA fragmentation. Control HeLa cells and HeLa/SPATA4 cells were exposed to 100\(\mu\)M of etoposide for 24 h, and subjected to DAPI and Annexin-V/PI staining. Nuclei with typical characteristics of apoptosis were significantly fewer in HeLa/SPATA4 cells than those in control cells (Fig. 2A). Furthermore, DNA ladder was more evident in control HeLa cells than in HeLa/SPATA4 cells after treatment with 100\(\mu\)M of etoposide for 48 h (data not shown). The morphological changes were further confirmed by flow cytometry. Treatment with 100\(\mu\)M of etoposide for 24 h resulted in apoptosis rates of 20.51\(\pm\)2.34% and 8.62\(\pm\)1.36% in HeLa and HeLa/SPATA4 cells, respectively (Figs. 2B, C). Hence, the apoptosis rate of HeLa cell induced by etoposide was dramatically reduced in the presence of exogenously expressed SPATA4.

SPATA4 Inhibits Cytochrome c Release from Mitochondria and Reduces Activation of Caspase-9 and -3  Etoposide induces cell apoptosis predominantly through the mitochondrial apoptotic pathway in which the key steps includes the release of cytochrome c from mitochondria into cytosol.16–18) Mitochondrial membrane potential (MMP) plays an important role in maintaining the physiological function of mitochondria. To investigate whether SPATA4 was involved in regulating etoposide-induced apoptosis of HeLa cells through the mitochondrial apoptotic pathway, we measured the change of MMP using JC-1 staining and flow cytometry. Exposure of control cells to etoposide for increasing time led to a significant reduction in MMP, meanwhile HeLa/SPATA4 cells exhibited little reduction in MMP (Figs. 3A, B). Furthermore, the release of cytochrome c from mitochondria was observed in control cells after etoposide treatment for 12 or 24 h, but not in HeLa/SPATA4 cells as detected by Western blot (Fig. 3C). The activation of caspase-9 and -3 are the downstream events of cytochrome c release. Consistent with the cytochrome c release, activity of both caspase-9 and -3 was significantly decreased in HeLa/SPATA4 cells after treatment with 100\(\mu\)M of etoposide for 24 or 48 h but not in control cells (Fig. 3D).

Bcl-2 and Bax Are Involved in SPATA4-Mediated Protection against Etoposide-Induced Apoptosis  The Bcl-2 family members are pivotal regulators of the mitochondrial pathway of apoptosis. Disruption of the function of Bcl-2 protein leads to permeabilization of the mitochondrial membrane,20) and the ratio of Bax to Bcl-2 has been reported to reflect the apoptotic state of cells.21) Our results showed that etoposide exposure had little effect on SPATA4 expression (Fig. 4A). HeLa/SPATA4 cells displayed a robust increase in both the mRNA and protein levels of Bcl-2 (Figs. 4B, D) but only a minor change of Bax (Figs. 4C, D) compared with control cells. Thus, the Bax/Bcl-2 ratio was markedly decreased in HeLa/SPATA4 cells compared with control cells (Fig. 4E). Etoposide exposure to control cells induced a significant increase in both the mRNA level and protein level of Bax (Figs. 4C, D), and a marked reduction of bcl-2 gene expression but a negligible change in the protein level of Bcl-2 (Figs. 4B, D), thereby increasing the ratio of Bax/Bcl-2 (Fig. 4E). In HeLa/SPATA4 cells, the up-regulation of Bax induced by etoposide treatment was significantly hindered (Figs. 4C, D), while expression Bcl-2 protein displayed little changes (Figs. 4B, D). Moreover, knock down of SPATA4 in HeLa/SPATA4 cells could partially rescue expression level of the bcl-2 and...
Fig. 2. SPATA4 Protects HeLa Cells against Etoposide-Induced Cell Apoptosis

(A) DAPI staining. Control HeLa cells and HeLa/SPATA4 cells were treated with or without 100 µM of etoposide for 24 h, stained with 1 µg/mL DAPI and then examined using fluorescence microscope (magnification ×400). (B) Apoptosis was assessed by Annexin V-FITC/PI assays. Cells in the lower right quadrant represented early apoptosis and those in the upper right quadrant represented late apoptosis. (C) Quantification of apoptotic cells in control HeLa cells and HeLa/SPATA4 cells. Data are expressed as mean±S.D. of results from three independent experiments. ***p<0.001.

Fig. 3. Exogenous SPATA4 Maintains the Mitochondrial Membrane Potential, and Prevents Cytochrome c Release from Mitochondria and the Activation of Caspase-9 and -3

(A) Measurement of the mitochondrial membrane potential. Control HeLa cells and HeLa/SPATA4 cells were exposed to 100 µM of etoposide for 12 or 24 h, stained with the mitochondrial-selective JC-1 fluorescent dye, MMP was estimated by flow cytometry showing decrease in the ratio of red to green fluorescence. (B) Quantification of the change of MMP (Δψm) in control HeLa cells and HeLa/SPATA4 cells. The bars represented mean±S.D. of three independent experiments. *** p<0.001. (C) Detection of cytosolic accumulation of cytochrome c by Western blot. (D) Proteolytic cleavage of caspase-9 and -3, as determined by Western blot.
bax resulted from over-expression of SPATA4 in HeLa cells (Supplementary Figs. S1a, b). After treatment with 100 µM of etoposide for 12 h, nuclei with typical characteristics of apoptosis in HeLa/SPATA4 transfected with small interfering RNA (siRNA)-SPATA4 were obviously increased than those in control cells (Supplementary Fig. S1c). These findings suggested that the protective effects of SPATA4 against etoposide-induced cell death in HeLa/SPATA4 cells might be partially mediated by regulating the expression of Bcl-2 and Bax.

DISCUSSION

Etoposide could cause accumulation of double-strand breaks within DNA in the nuclei of cells, and it has been widely used to couple DNA damage to apoptosis. Cells are apparently able to recognize such DNA damage and, in turn, eliminate the injured cells through apoptosis. Etoposide-induced apoptosis is accomplished through the mitochondria-dependent pathway, involving mitochondrial transmembrane potential (Δψm) reduction, cytochrome c release, caspase-9 and -3 activation, and eventually cell death. The effect of SPATA4 on resisting etoposide-induced apoptosis was associated with maintenance of mitochondrial membrane potential, the key step in the mitochondrial apoptotic pathway.

Mitochondria participate in apoptotic signaling pathway through the release of mitochondrial proteins into the cytoplasm. Cytochrome c, a crucial protein in electron transport, is released from mitochondria in response to apoptotic signals, and activates Apaf-1, a protease released from mitochondria. Activated Apaf-1 activates caspase-9 and the rest of the caspase pathway. After treatment with etoposide for 12 or 24 h, cytochrome c release from mitochondria could be detected in the cytosolic fraction of control cells, but was effectively inhibited in the HeLa/SPATA4 cells, even was undetectable until 36 h. Consistent with cytochrome c release, caspase-9 and -3 activation were significantly decreased in HeLa/SPATA4 cells after treatment with 100 µM of etoposide for 24 or 48 h.

The Bcl-2 family proteins play critical roles in either promoting or inhibiting cell apoptosis, especially in the mitochondrial apoptotic pathway. Bcl-2 regulates the mitochondrial apoptotic pathway by blocking the release of cytochrome c and subsequent caspase-9 and -3 activation. Such a mechanism confers Bcl-2 a protective effect against etoposide-induced apoptosis. Meanwhile, Bax, another Bcl-2 family protein, acts oppositely by translocating from the nucleus to the mitochondrial membrane and increasing its permeability. That resulted in the release of cytochrome c and other mitochondrial proteins. The balance between the anti-apoptotic and pro-apoptotic members plays a pivotal role in determining cell survival or death. Up-regulation of Bcl-2 and down-regulation of Bax were observed in HeLa/SPATA cells, suggesting that SPATA4 suppressed etoposide-induced apoptosis at least partly via regulating the Bcl-2 family. However, the molecular mechanism through which SPATA4 modulates the expression of Bcl-2 and Bax is presently unknown and will be further studied.

Meanwhile, we also found that SPATA4 enhanced cell viability compared with the control cells after being treated with taxol for 24 h (Supplementary Fig. S2). Taxol is known to inhibit cell growth and trigger significant apoptosis in various cancer cells, and taxol-induced apoptosis was also associated with decreased mitochondrial membrane potential (Δψm) and a significant increase of cytochrome c release from the mitochondria. In our research, toxicity of taxol was found to be dose-dependent for the control cells, while no significant viability reduction was observed in HeLa/SPATA4 cells. These findings suggested that the effect of SPATA4 on mitochondrial apoptosis was possibly universally applicable. Moreover, SPATA4 was reported to be up-regulated distinctly in cryptorchidism compared with the normal testis tissue. As ectopically expression of SPATA4 in HeLa cells markedly protected cells from apoptosis induced by DNA damage reagents, it is possible that the increased expression of SPATA4 in cryptorchid...
testis was triggered by the hot signal, which started up apoptosis, and then SPATA4 hastened spermatogenesis and suppressed the excess apoptosis.

In summary, we demonstrated for the first time that the SPATA4 could repress DNA-damage-induced apoptosis in HeLa cells, possibly through regulating Bcl-2 family proteins and inhibiting cytochrome c release from mitochondria. Our studies suggested a possible role of SPATA4 in cell apoptosis. However, it still remains to be uncovered whether the anti-apoptotic effects of SPATA4 are universally applicable or cell specific.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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