SH003-induced G1 phase cell cycle arrest induces apoptosis in HeLa cervical cancer cells

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Abstract. Cervical cancer is a prevalent disease that may lead to mortality in women. In spite of the development of common therapeutic agents to treat cancer, there are several limitations of their use owing to side effects and drug resistance, which may induce cancer recurrence. The anticancer effects of the new herbal mixture SH003 (comprising Astragalus membranaceus, Angelica gigas and Trichosanthes kirilowii Maximowicz) have been examined in various types of cancer. Thus, the present study hypothesized that SH003 may be an effective treatment for cervical cancer. SH003 treatment inhibited the growth of HeLa cells, whereas it did not affect the growth of rat intestinal epithelial cells. In addition, SH003 treatment increased the expression of apoptosis-related proteins and promoted apoptotic cell death in HeLa cells. SH003 treatment also led to G1 phase arrest in HeLa cells. Furthermore, SH003 treatment induced the production of reactive oxygen species (ROS); however, ROS production did not appear to be related to SH003-mediated apoptosis. Results from the present study indicated that the SH003-induced inhibition of HeLa cell growth may be mediated through G1 phase arrest and extrinsic apoptosis, suggested that SH003 may be a potential treatment for cervical cancer.

Introduction

Cervical cancer is a disease with poor prognosis among women worldwide (1-3). Surgery assessment of local disease state, including tumor size assessment and vaginal and parametrical involvement, is good during early stages of the disease; however, the evaluation of tumor size and vaginal and parametrical involvement are difficult in advanced- and late-stages, such as in those patients who do not qualify for up-front surgery (4,5). Owing to such limitations, alternative approaches and more efficient remedies are necessary to treat cervical cancer other than surgical operation, chemotherapy and vaccination.

Previous studies have reported on the use of traditional herbal medicines as alternative cancer therapies (6-8), including a number of studies on HeLa cervical cancer cells that used herbal medicines as therapeutic agents and compounds for potentially treating cervical cancer (9-11). Our previous study developed a new herbal medicine, called SH003, which is composed of Astragalus membranaceus (Am), Angelica gigas (Ag) and Trichosanthes kirilowii Maximowicz (Tk), based on the principles of traditional Chinese medicine (12,13). Ag and Am have been reported to be effective treatments for a number of diseases, including hematologic diseases, endocrine disorders and cancers (14-19). Therapeutic effects of Tk have also been indicated for various cancer types, such as leukemia, hepatocellular carcinoma, colon cancer, non-small cell lung cancer and gastric cancer (18,20-25). Several previous studies have demonstrated that a mixture of those components named SH003 was better therapeutic effect than each component in other types of cancer (12,13,17,26-30). Thus, SH003 is likely to be used for cancer treatment as novel herbal mixture.

Apoptosis, or programmed cell death, is characterized by cell shrinkage, membrane blebbing, maintenance of organelle integrity, and DNA condensation and fragmentation (31). There are two subtypes of the apoptotic pathway: The extrinsic, or receptor-mediated, pathway and the intrinsic, or mitochondria-mediated, pathway (32). The extrinsic apoptotic pathway involves the so-called ‘death receptors’, such as Fas receptor, tumor necrosis factor receptor and death receptor, and their associated extracellular ligands (33-35). These apoptotic factors lead to the activation of caspase (casp)-3 and casp8 (35,36). Conversely, the intrinsic apoptotic pathway is activated by a number of different factors, such as genomic and metabolic stresses, and the presence of unfolded proteins,
which may cause permeabilization of the outer mitochondrial membrane and the release apoptotic proteins into the cytosol (34). Concurrently, cell cycle checkpoints are triggered and apoptosis is induced in cells exhibiting abnormal cell cycle. In particular, the G1 phase is regulated by cyclin D proteins (including cyclin D1, D2, and D3), cyclin E proteins which associate with cyclin-dependent kinase (CDK) 2 to regulate the progression from G1 into S phase, CDK2, CDK4 and CDK6 (37). The CDKs are a family of serine/threonine protein kinases that are activated at the G1 checkpoint; Cyclin D proteins interact with CDK4/CDK6, and these complexes directly regulate the G1 phase of cell cycle.

The present study investigated the anticancer effects of SH003 in the HeLa cervical cancer cell line. The results demonstrated that SH003 treatment reduced the viability of HeLa cells without affecting that of normal cells. Rat intestinal epithelial cells were used as the control group in the present study, as previously described (38). SH003 treatment led to extrinsic apoptosis and induced the cleavage of casp3, casp8 and poly(ADP-ribose) polymerase 1. SH003 treatment also increased the production of reactive oxygen species (ROS) and induced G1 cell cycle arrest in HeLa cells. Results from the present study suggested that SH003 may be a potential treatment for cervical cancer.

Materials and methods

SH003 preparation. The production of SH003 and its components, Am, Ag and Tk, was commissioned to Hanpoong Pharm and Foods Company (Jeonju, Korea), and the extraction procedures were performed as previously reported (12). Briefly, extracts (~333 g each) of Am, Ag and Tk in 10 l 30% ethanol were obtained by boiling for 3 h at temperature ranging between 80 and 100˚C. The ethanol extract was filtered, concentrated using vacuum evaporation and freeze-dried at <60˚C (average yield=35.5%). Dried extract was then dissolved in 30% ethanol to prepare a stock solution of 100 mg/ml, or one of the individual components (Am, Ag or Tk) for 72 h.

Cell culture. HeLa human cervical cancer cells (Korean Cell Line Bank, Seoul, Korea) and rat intestinal epithelium (RIE; provided by Dr Joon Woo Lee; Seoul National University, Line Bank, Seoul, Korea) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, Gyeongsang, Korea) containing 10% fetal bovine serum (Welgene) and 1% penicillin. Cells were maintained at 37˚C in a 5% CO2 atmosphere.

Reagents. Methyl thiazolyl tetrazolium (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), N-acetyl L-cysteine (NAC) and 7-aminoactinomycin D (7-AAD) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Acrylamide/bis-acrylamide solution (30%) was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Annexin V-fluorescein isothiocyanate (FITC) was purchased from BD Biosciences (San Jose, CA, USA), and 5,5',6,6'-tetrafluoro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine (JC-1) was purchased from Biotium Inc. (Freemont, CA, USA).

Western blotting. Radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Dithiothreitol 3 mg/ml, Phenylmethylsulfonyl fluoride 3 µg/ml, Na2VO3 5 µg/ml, NaF 5 µg/ml and protease inhibitor 4 µg/ml) was used for cell lysis for 20 min at 4˚C, and supernatants were collected by centrifugation at 16,600 x g for 20 min at 4˚C. Protein concentration was measured using a Bradford assay kit (Bio-Rad Laboratories, Inc.). Proteins (15 µg) were separated by 8-12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked at room temperature for 1 h in 2% skim milk containing 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) and probed with the primary antibodies raised against the following proteins at 4˚C overnight: Cleaved (C)-casp3 (cat no. 9661; 1:2,000), C-casp8 (cat no. 9661; 1:2,000), casp9 (cat no. 9502; 1:50,000), poly(ADP-ribose) polymerase 1 (PARP; cat no. 9542; 1:50,000), phosphorylated (p)-p53 (cat no. 9284; 1:50,000), cyclin-dependent kinase (CDK) 4 (cat no. 12790s; 1:50,000) and CDK6 (cat no. 3136; 1:50,000), all from Cell Signaling Technology, Inc. (Danvers, MA, USA); β-actin (cat no. sc47778; 1:50,000), Bcl-2 (cat no. sc7382; 1:10,000), Bcl-2-like protein (Bax; cat no. sc-7480; 1:100,000), CDK2 (cat no. sc163; 1:100,000) and cyclin E (cat no. sc-247; 1:5,000) all from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); cyclin D1 (cat no. 556470; 1:50,000) and retinoblastoma-associated protein (pRb; cat no. 554140; 1:50,000; from BD Biosciences). Membranes were then incubated in room temperature for 1 h with the following horseradish peroxidase-conjugated secondary antibodies (1:1,000): Anti-rabbit immunoglobulin (Ig) G (cat no. 7074) and anti-mouse IgG (cat no. 7076) from Cell Signaling Technology, Inc. Protein bands were visualized using enhanced chemiluminescence (cat no. DG-W250; DoGEN, Seoul, Korea). Blots were semi-quantified by densitometry using ImageJ software version 1.43.67 (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin.

Cell viability assays. HeLa (5x10^3) and RIE (4x10^3) cells were seeded in DMEM in 96-well plates and treated with various concentrations (0, 100, 200 or 400 µg/ml) of either SH003 or one of the individual components (Am, Ag or Tk) for 72 h at 37˚C. Subsequently, the medium was discarded and 10 µl MTT solution (Sigma-Aldrich; Merck KGaA) in 90 µl medium was added to each well. Cells were incubated for 2 h at 37˚C, and then 100 µl dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance of each sample was measured at 570 nm using an ELISA microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Mitochondrial membrane potential assay. HeLa cells (3x10^3) were seeded on 60 mm dishes and treated with 100, 200 or 400 µg/ml SH003 for 24 h. Control cells received no treatment. Briefly, 100X JC-1 solution was diluted in 1X in DMSO. Cells were stained with 4 µg/ml JC-1 solution for 15 min at 37˚C. In healthy cells, JC-1 accumulates in the mitochondria and stains them red, whereas in apoptotic cells, the mitochondrial membrane potential collapses and JC-1 remains in the cytoplasm as a monomer (FL-1), emitting green fluorescence. Cells were analyzed using a FACSCalibur Flow Cytometry System (BD Biosciences). Histograms and dot plots were analyzed with CellQuestPro software version 5.2 (BD Biosciences). The M1 gate was calculated based on the control values (no treatment).
ROS generation assay. ROS generation was detected using the dye DCFH-DA. Briefly, HeLa cells (3x10^5) were plated on 60-mm plates and incubated in DMEM at 37°C overnight. Cells were treated with various concentrations of SH003 (0, 100, 200 and 400 µg/ml), with or without NAC (2 mM). Subsequently, 1 µl/ml DCFH-DA was added and cells were incubated for 1 h at 37°C and samples were analyzed using the FACSCalibur Flow Cytometry System (BD Biosciences). Histograms and dot plots were analyzed using CellQuestPro Software version 5.2 (BD Biosciences). NAC (2 mM) was used for inhibition of ROS generation.

Annexin V-FITC/7-AAD apoptosis assay. Rates of apoptosis were measured by annexin V-FITC/7-AAD assay. HeLa cells (3x10^5) were treated with 400 µg/ml SH003 for 48 h and 2 mM NAC for 2 h at 37°C, and then stained with 1.25 µl annexin V-FITC in 1X binding buffer and 1 µl 7-AAD for 15 min at room temperature in the dark. Apoptotic cell population was analyzed by FACSCalibur Flow Cytometry System, measuring the signal by FL-1 (green, 525 nm) and FL-3 (red, 620-767 nm). Cells were analyzed using a FACSCalibur Flow Cytometry System (BD Biosciences). Histograms and dot plots were analyzed with CellQuestPro software version 5.2 (BD Biosciences). The M1 gate was calculated based on the control values (no treatment).

Cell cycle assay. HeLa cells were plated in DMEM in 60-mm dishes at a density of 7x10^5 cells/dish and treated with various concentrations of SH003 (0, 100, 200 and 400 µg/ml) for 24 h at 37°C. Subsequently, cells were harvested, centrifuged at 221 x g for 10 min at 4°C, fixed with 95% ethanol at 4°C overnight, and washed twice in PBS. Subsequently, cells were stained with 50 µl Muse Cell Cycle Assay kit (Merck KGaA) in the dark at room temperature for 30 min. Cell cycle plots were analyzed using the Muse Cell Analyzer (Merck KGaA).

Statistical analysis. Statistical analysis was performed using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation of 3 independent experiments. The statistical significance of the differences between groups was assessed using one-way analysis of variance followed by a post hoc Tukey test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of SH003 and its components on RIE and HeLa cell viability. An MTT assay was performed to evaluate the cytotoxic effects of Am, Ag, Tk and SH003 on normal RIE cells and HeLa cervical cancer cells. RIE cells were treated with varying concentrations (100, 200 and 400 µg/ml) of each extract or SH003 (Fig. 1A). While SH003 and Am treatment exhibited lower toxicity on RIE cells compared with Ag and Tk, SH003 appeared to be more effective at inducing cell death in HeLa cells compared with Am (Fig. 1A). Conversely, SH003 treatment reduced HeLa cell viability at all concentrations (Fig. 1B), which suggested that SH003 may induce toxicity in cervical cancer cells without affecting normal cell viability.

SH003 induces apoptosis in HeLa cells. SH003-induced apoptosis was examined in HeLa cells. Cells were stained with annexin V-FITC and 7-AAD and analyzed by flow cytometry. SH003 treatments at 200 and 400 µg/ml were revealed to significantly increase the percentage of cells in the early and late apoptosis regions (Fig. 2A). In accordance with these data, SH003 treatment appeared to increase the protein expression levels of C-casp3, C-casp8 and PARP1, but not C-casp9 (Fig. 2B), which indicated that SH003 treatment may induce extrinsic apoptotic cell death in HeLa cells. However, these results were not statistically significant.

SH003 does not affect the intrinsic mitochondria-mediated apoptosis pathway in HeLa cells. As SH003 treatment did not appear to affect the expression level of C-casp9 (Fig. 2B), the effects of SH003 on the intrinsic mitochondria-mediated apoptosis pathway were examined by measurement of the Bax/Bcl-2 ratio, which is important in the intrinsic apoptosis pathway. No significant differences in Bax/Bcl-2 ratios were indicted for HeLa cells exposed to SH003, which suggested that SH003 may not induce intrinsic apoptosis (Fig. 3A). In addition, changes in mitochondrial transmembrane potential within the cells were analyzed by staining HeLa cells with Annexin V-FITC/7-AAD apoptosis assay.
Figure 2. SH003 treatment induces apoptosis in HeLa cells. (A) HeLa cells (1x10^5 cells/dish) were seeded on 60 mm dishes and treated with 0, 100, 200 or 400 µg/ml SH003 for 48 h. Cells were harvested and stained with 7-AAD and annexin V-FITC in 1X binding buffer. Apoptotic cell death was analyzed by FACSCalibur flow cytometer FL-1 and FL-3 channels. Experiments were performed in triplicate, and results are presented as the mean ± standard deviation; **P<0.01 and ***P<0.001 vs. control (0 µg/ml). (B) Total cell lysates were prepared and the equal amounts of protein (15 µg) were separated by SDS-PAGE, and the membranes were probed with C-casp3, C-casp8, C-casp9 and PARP1 antibodies. β-Actin was used as a loading control. Arrows indicate the cleaved forms of the proteins. 7-AAD, 7-aminoactinomycin D; C-casp, cleaved caspase; FITC, fluorescein isothiocyanate; PARP1, poly(ADP-ribose) polymerase 1.

Figure 3. SH003 treatment does not affect intrinsic mitochondria-mediated apoptosis. HeLa cells were treated with 100, 200 and 400 µg/ml of SH003 for 24 h prior to testing. (A) Western blotting was performed by using anti-Bax and anti-Bcl-2 antibodies; β-actin was used as a loading control. Bax/Bcl-2 ratio was calculated by ImageJ software, following densitometric analysis. (B) Following SH003 treatment, cells were harvested and stained with JC-1, and mitochondrial membrane potentials were detected by FACSCalibur flow cytometry using FL-1 and FL-2 channels to detect JC-1 monomers and J-aggregates, respectively. Experiments were performed three times, and results are presented as the mean ± standard deviation; *P<0.05, **P<0.01 and ***P<0.001 vs. untreated control. Bcl, B-cell lymphoma; Bax, Bcl-2-like protein; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine; N.S., not significant.
SH003 increases ROS generation in HeLa cells. Elevated ROS levels have previously been implicated in apoptosis (39-41). To investigate whether SH003 treatment influenced ROS generation, HeLa cells were stained with DCFH-DA and analyzed by flow cytometry. The varying concentrations of SH003 treatment significantly increased ROS generation in a dose-dependent manner (Fig. 4A). However, SH003-induced apoptosis was not related with an increase in ROS levels, as co-treatment with SH003 and NAC resulted in increased apoptosis (Fig. 4B) and reduced cell viability compared with SH003 alone (Fig. 4C). These results indicated that apoptosis induced by SH003 is not dependent on the ROS-mediated pathway.

SH003 induces G1 cell cycle arrest in HeLa cells. Chemotherapy commonly inhibits cancer growth by inducing cell cycle arrests (42-44) and the present findings indicated that SH003 may have potential to induce cell cycle arrest in cancer cells. Western blot analysis was conducted to investigate whether SH003 induced cell cycle arrest in HeLa cells. SH003 treatment was demonstrated to decrease the protein expression levels of CDK2, CDK4, CDK6 and cyclin D. In addition, SH003 treatment decreased p-p53 and pRb levels (Fig. 5A). In addition, cell cycle analysis was performed with a Muse Cell Analyzer. The number of cells at the G1 phase increased in HeLa cultures treated with varying concentrations of SH003 (Fig. 5B). These results indicated that SH003 may reduce G1 phase cell cycle associated protein expression and arrests the cell cycle at the G1 phase.

Discussion

As therapeutic agents without toxic side effects, traditional herbal medicines have been the focus of several studies on cancer (45,46). One such traditional herbal medicine, SH003, has been reported previously as a potential novel anticancer agent for breast and prostate cancer (12). In the present study, SH003 treatment was demonstrated to induce extrinsic apoptosis in HeLa cervical cancer cells.

Conventional chemotherapeutic agents for cancer treatment commonly induce apoptotic death (9-11), and traditional herbal medicines have exhibited anticancer effects against cervical cancer cells by promoting apoptosis (9-11). Previous studies have demonstrated that SH003 treatment suppressed the growth of breast and prostate cancer cells by inducing apoptosis (12,47,48). It has been also reported that SH003 co-treatment with doxorubicin may have synergistic effects on the promotion of apoptotic death in MDA-MB-231 human breast cancer cells (49). Consistent with these previous results, the present study demonstrated that SH003-induced growth inhibition of HeLa cells may be mediated by the induction of apoptosis.

SH003 comprises Am, Ag and Tk, which have previously been indicated as effective treatments for a variety of diseases, such as hematologic diseases, endocrine disorders, leukemia, hepatocellular carcinoma, colon cancer, non-small cell lung cancer and gastric cancer (14-25). Although the mechanism of action remains unclear, the mixture of Ag, Am and Tk may complement the individual properties of each component. Previous studies have demonstrated that

with JC-1 and measuring the monomeric form (via channel FL-1) and J-aggregates (via FL-2). SH003 did not induce a low mitochondrial transmembrane potential, suggesting that SH003 does not induce apoptosis through the mitochondria-mediated pathway (Fig. 3B), but through the extrinsic pathway.

Figure 4. SH003 treatment increases ROS generation in HeLa cells. (A) HeLa cells were treated with various concentrations (0, 100, 200 and 400 µg/ml) of SH003 and incubated with DCFH-DA for 1 h. ROS generation of HeLa cells was measured by FACSCalibur flow cytometry. *P<0.01 vs. untreated control. (B) Cells were pretreated with 2 mM NAC for 30 min, followed by treatment with concentrations of SH003 for 48 h. Cells were stained with DCFH-DA for 1 h, then stained with 7-AAD and annexin V-FITC in 1X binding buffer. Apoptotic cell death was analyzed by FACSCalibur flow cytometer. **P<0.01 vs. untreated control; ***P<0.001 vs. SH003 treatment only. (C) HeLa cells were pretreated with 2 mM NAC for 30 min, followed by treatment with SH003 for 48 h. MTT solution was added to the cells and incubated for 2 h, followed by the addition of 100 µl of DMSO to each well. Cell viability was analyzed by ELISA microplate reader. ***P<0.001 vs. untreated control; **P<0.01 vs. SH003 treatment only. All data are presented as the mean ± and standard deviation of 3 independent experiments. DCFH-DA, 2',7'-dichlorofluorescein diacetate; FITC, fluorescein isothiocyanate; MTT, methyl thiazolyl tetrazolium; NAC, N-acetyl L-cysteine; ROS, reactive oxygen species.
SH003 may be more effective to inhibit the growth of breast cancer cells compared with the effects of the separate components (12,48,49). Consistent with these previous results, the present data indicated that SH003 treatment exhibited better effects on the inhibition of HeLa cancer cell growth than its constituents’ effect. In addition, SH003 appeared to be less toxic to normal RIE cells compared with cells treated with Am, Ag or Tk alone, which suggested that SH003 may be a possible treatment for cervical cancer without serious adverse effects. However, further studies, investigating the effects of SH003 and its components on apoptosis, ROS levels and cell cycle arrest on normal cervical cells, are required in order to establish the safety profile of SH003.

Cell cycle entry is critical for homeostatic control in the cell (31); however, when cells suffer from cellular damage, cell cycle arrest is induced by various mechanisms such as the inhibition of cyclin and CDK expression. These regulations are commonly referred to as cell cycle checkpoint (50).
In the present study, SH003 treatment reduced the levels of expression for G1 phase associated CDKs (CDK2, CDK4 and CDK6) and cyclin D. Although the present data indicated that inhibition of cell growth by SH003 is mediated through cell cycle arrest at G1 phase, further study should be performed to determine the mechanisms of CDK inhibition.

Increased ROS production has been associated with promoting apoptosis (40,51,52). In the present study, SH003 treatment increased ROS production in a dose-dependent manner, whereas treatment with NAC did not block ROS generation (data not shown) and apoptosis in SH003-treated HeLa cells. The present data indicated that SH003-mediated apoptosis was not correlated with increased ROS production.

In conclusion, the present study is the first to suggest, to the best of our knowledge, that SH003 induces extrinsic apoptosis in HeLa cells. Although a mechanism of SH003 action on treating cervical cancer cells is required to clarify which of the active compounds of SH003 show antitumor effects against cervical cancer cells, the present study suggests that SH003 may be effective for the treatment of cervical cancer.

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