ORIGINAL RESEARCH

Cytotoxicity of natural extract from *Tegillarca granosa* on ovarian cancer cells is mediated by multiple molecules

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Abstract

Purpose: To determine the cellular and molecular mechanism of cytotoxicity induced by Haishengsu (HSS), nature extract from *Tegillarca granosa*, toward human ovarian cancer cell lines SKOV-3 and OVCAR-3.

Methods: The cytotoxic effects of HSS on two ovarian cancer cell lines were tested by XTT assay. Cell apoptosis and cell cycle arrest induced by HSS were demonstrated by DNA ladder assay and flow cytometric analysis, respectively. RT-PCR or flow cytometric analysis was used to investigate the expression of bcl-2, caspase-3, p53, β -catenin, E-cadherin, CD24, and CD44.

Results: Continuous exposure to HSS for 48 h produced cytotoxic effects on both cell lines in a concentration dependent manner, which was accompanied by apoptosis and cell cycle arrest. Apoptosis associated gene bcl-2 and caspase-3, tumor metastasis associated gene β -catenin, but not E-cadherin, and CD24, but not CD44, were involved in the effect of growth inhibition induced by HSS. Although p53 mediated apoptosis induced by HSS in OVCAR-3 cells, it was not required in SKOV-3 cells.

Conclusion: HSS has a potential cytotoxic effect on human ovarian cancer cells, which was mediated by multiple signal molecules including bcl-2, caspase-3, β -catenin, and CD24. These findings will provide a theoretical basis for HSS's potential clinical application as a novel marine anti-cancer agent.

Ovarian cancer accounts for about 3% of all cancers among women and ranks second among gynecologic cancers. It is the leading cause of cancer death in the female reproductive system, and an estimated 15,520 deaths are expected in the US in 2008.¹ Although most of the patients with ovarian cancer have a high initial response to the drug treatment, this high response is always followed by chemotherapeutic resistance.² To date, no meaningful increase in survival in ovarian cancer has occurred despite all the efforts and advances in surgery, chemotherapy and radiation ther-

apy. Hence, it is essential to accelerate the pace of drug discovery for the more resistant cancer.

The marine environment provides a rich source of natural products with potential therapeutic application. The rate of pharmaceuticals agent discovery from marine natural products has dramatically increased in the past few years. Until now, it has been estimated that more than 14,000 different pharmacologically active compounds have been isolated from marine organisms, and several these compounds have been used in the clinic.³ Many marine natural products have successfully advanced to the late stages of clinical trials.⁴ *Tegillarca granosa*, widely distributed in China, is the main breed of aquatic seashell and has been used as traditional Chinese seafood for hundreds of years. Purified proteins from *Tegillarca granosa* have several pharmacological actions such as anti-coagulation⁵ and anti-proliferation of tumor cells but not of normal cells.⁶ Haishengsu (HSS) is an extract isolated from *Tegillarca granosa* and major chemical constituents are albumen with a molecular weight ranged from 15 KDa to 23 KDa. Previous studies have showed that HSS has a potent suppressive effect on several types of solid tumor cells in vivo and in vitro.^{7, 8} The purpose of this work was to develop an understanding of HSS's effects on ovarian cancer cells, to determine its therapeutic potential in treating this disease and investigate the mechanism of action.

Materials and Methods

Cell lines and culture

Human ovarian cancer cell lines SKOV-3 and OVCAR-3 were obtained from KCLB (Korean Cell Line Bank, Seoul, South Korea) and cultured in RPMI medium 1640 (GIBCO.BRL Grand Island, NY, USA), containing 10%FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin (GIBCO.BRL).

Cell cytotoxicity assay

The sensitivities of the cells to HSS were detected by using the Count™ cell viability assay kit (Welgene, Seoul, South Korea). Briefly, 100 µl of cell suspension (5×10^4 cells/ml) were dispensed in quadruple in 96-well microplates and cultured for 24 h. Then, the cells were treated with various concentrations of HSS diluted in 100 µl of conditioned medium (the final concentrations were 0.01%~0.1%). After incubation for 48 h, 10 µl of the reaction solution of PMS/XTT (1:50 volume) were added to each well containing 80 µl medium and the plates were further incubated for 4 h in an incubator. Cell viability was quantified by measuring the absorbance of the samples with a Versa-Max™ microplate reader at a wavelength of 450 nm, and the non-specific readings was eliminated by using a wavelength of 690 nm.

Assessment of apoptosis

After treatment by HSS, cells were harvested and apoptosis was evaluated by Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. The DNA solution were separated on 2% agarose gel and stained with ethidium bromide.

Cell cycle analysis

Flow cytometry analysis of PI-stained cells was performed to demonstrate the progression of the cell cycle. Briefly, 2×10^6 cells treated and untreated by HSS were harvested, washed and fixed in 70% ethanol overnight at -20°C. Prior to flow cytometry, cells were washed and stained with 500 µl of PI staining solution (50 mg/L PI, 0.1% Triton X-100, 0.1 mmol/L EDTA, and 50mg/L RNase A). DNA content was determined with a FACScan flow cytometer (Becton Dickinson) and the proportion of cells in a particular phase of cell cycle was determined with CellQuest software.

TABLE 1. Primers, product sizes, and PCR conditions

Molecules	F/R*	Sequence (5'~3')	Size (bp)	Annealing Temperature T	PCR conditions
Bcl-2	F	GTGGAGGAGCTCTTCAGGGA	308bp	58.6°C	95°C 15min 94°C 40sec T °C 40sec 72°C 1min 30 cycles 72°C 10min
	R	AGGCACCCAGGGTGATGCAA			
Caspase-3	F	AGTGGAGGCCGACTTCTTGT	173bp	58.6°C	
	R	CTGTTGCCACCTTTCGGTTA			
P53	F	TGTCATGGCGACTGTCCAGC	282bp	61.5°C	
	R	GCTCGACGCTAGGATCTGAC			
E-cadherin	F	GCCAAGCAGCAGTACATTCTACACG	342bp	62.7°C	
	R	GCTGTTCTTCACGTGCTCAAAATC			
β-catenin	F	ACTACCACAGCTCCTTCTCTGAG	447bp	61.5°C	
	R	GATAGCGTGTCTGGAAGCTTCCT			
CD24	F	ACCCAGCATCCTGCTAGAC	287bp	57.5°C	
	R	CTTAAGAGTAGAGATGCAGAA			
CD44	F	AACCTGCCGCTTTGCAGGTGT	342bp	57.5°C	
	R	ATCAAAGGCATTGGGCAG			
GAPDH	F	ACAACCTTTGGTATCGTGGAA	458bp	57.1°C	
	R	AAATTCGTTGTCATACCAGG			

* F: Forward primer; R: Reverse primer

RNA extraction and reverse transcription-PCR

RNA was extracted from cells using an Ultraspec™-IIRNA Kit (Biotecx Laboratories, Inc., Houston, TX, USA) according to the manufactures' protocol. Complementary DNA was synthesized using 0.5-2 µg of total RNA with SuperScript™ III Reverse Transcriptase kit (Invitrogen); and 2 µl cDNA product was amplified by reverse transcription – PCR (RT-PCR) analysis. GAPDH was used as the internal control in all reactions. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. Primers, product size, and PCR conditions are listed in Table 1.

Flow cytometry

Detached cells were washed, resuspended in cold HBSS containing 2% heat-inactivated FBS, and blocked for 10 min with FcR reagent. Then, the antibodies of anti-CD24-fluorescein isothiocyanate (FITC) or anti-CD44-FITC (BD PharMingen San Diego, CA, USA) were added at appropriate dilution and incubated for 20 min on ice in the darkness followed

by washing. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Side scatter and forward scatter profiles were used to eliminate cell doublets. Dead cells were eliminated by using the viability dye 7-AAD. Data were analyzed using CellQuest software.

Statistical analysis

The results are expressed as means ± SD. All statistical analyses were analyzed using ANOVA (SPSS11.5 software). $P < 0.05$ was considered to be statistically significant.

Results

HSS inhibits growth of human ovarian cancer cells accompanied by cell cycle arrest and apoptosis

The cytotoxic effect of HSS on two human ovarian cancer cell lines was determined by XTT assay. Treatment with increasing concentrations of HSS (0-0.1%) led to a concentration-dependent inhibition of cell growth (Figure 1). IC₂₅ and IC₅₀ value were

FIGURE 1. Dose response of HSS in human ovarian cancer cells. SKOV-3 and OVCAR-3 cells were treated with varying concentrations of HSS. Means $\pm$ SD, $P < 0.01$ vs control, $P < 0.01$ or $P < 0.05$ among groups in each cell line.

calculated by SPSS 11.0 soft after 48 h of treatment in SKOV-3 and OVCAR-3. $IC_{25} = 0.06\% \pm 0.01\%$, $IC_{50} = 0.09\% \pm 0.02\%$ in SKOV-3 and $IC_{25} = 0.06\% \pm 0.01\%$, $IC_{50} = 0.10\% \pm 0.02\%$ in OVCAR-3, respectively. In subsequent experiments, IC_{25} and IC_{50} concentration of HSS were used.

DNA ladder assay revealed that there was a dose-dependent increase in DNA fragmentation formation in ovarian cancer cells after treatment of HSS for 48 h at concentration of IC_{25} and IC_{50} (Figure 2). DNA cell cycle analysis showed that HSS treatment resulted in a significant alteration in cell cycle progression in each cell line at IC_{50} concentrations of HSS (Figure 3). In HSS-treated SKOV-3 and OVCAR-3 cells, the increase of S phase cells was accompanied by a decrease of G0/G1 phase cells and no change of G2/M phase cells.

HSS administration regulates mRNA level of apoptosis-related genes

Cells treated with IC_{25} and IC_{50} concentration of HSS showed dose-dependent down-regulation at the transcriptional level of bcl-2 and up-regulation in caspase-3 (Figure 4). Treatment of HSS also induced up-

FIGURE 2. Effects of HSS on DNA fragmentation in SKOV-3 and OVCAR-3 cells. Shown is representative electrophoretic fragmentation pattern of DNA isolated from cells treated by HSS at a concentration of IC_{25} and IC_{50} for 48 h.

regulation in p53 gene expression in a dose-dependent manner in OVCAR-3 cell line. As expected, p53 transcript level could not be detected in each group of SKOV-3 cells because SKOV-3 cells were deficient in p53⁹ (Figure 4).

Wnt signal pathway mediates HSS-induced growth inhibition of ovarian cancer cells

β -catenin and E-cadherin (a central mediator and a negative regulator of Wnt signaling pathway, respectively) have been widely implicated as an oncogene in human cancer.^{10,11} We found that β -catenin mRNA transcript was down-regulated dose-dependently after treatment with HSS for 48h in both cell lines. Although an E-cadherin mRNA transcript was present in all of total RNA extracts prepared from each cell line,

FIGURE 3. Cell cycle profiles of HSS-treated and untreated human ovarian cancer cells. Percentages of cells in G0/G1, S, and G2/M phases are showed in each graph. * $P < 0.01$, # $P < 0.05$ vs Control.

no difference was observed in these two cell line before and after treatment with HSS (Figure 4).

Effects of HSS Administration on mRNA and protein expression of CD24 and CD44 in ovarian cancer cells

CD24 mRNA expression was down-regulated, concentration-dependently after 48h of HSS treatment in both SKOV-3 and OVCAR-3 cells Figure 5A. We

also observed a concentration dependent decrease in CD24 membranous protein expression in these two cell lines (Figure 5B, top panel). However, no difference was found in mRNA (Figure 5A) and protein (Figure 5B, bottom-left panel) expression of CD44 between HSS-treated and untreated SKOV-3 cells. HSS treatment had no effect on CD44 mRNA expression in OVCAR-3 cells (Figure 5A). Moreover,

FIGURE 4. RT-PCR analysis of expression of genes related to apoptosis and cancer metastasis in HSS-treated or untreated SKOV-3 and OVCAR-3 cells.

CD44 protein was not expressed in OVCAR-3 cell line before and after treatment by HSS (Figure 5B, bottom-right panel).

Discussion

In this study, HSS inhibited the growth of human ovarian cancer cells, which was accompanied by induction of apoptosis and cell cycle arrest of G1/S transitions. HSS triggered apoptosis via regulating expression of Bcl-2 and caspase-3 in a p53-independent pathway. HSS also could block Wnt signaling pathway via down-regulation of β-catenin and inhibit CD24 expression.

FIGURE 5. Effects of HSS treatment on expression of CD24 and CD44 in SKOV-3 and OVCAR-3 cells. * $P < 0.01$ vs Group without drug, # $P < 0.01$ vs IC₂₅ group, Δ $P > 0.05$ vs Group without drug.

Studies to date have demonstrated that induction of apoptosis in cancer cells is the target for many therapeutic natural extracts.¹² The mitochondrial pathway is an important mechanism of apoptosis. Bcl-2 and caspase-3 are very important members involved in the pathway. In our study, up-regulation of caspase-3 expression and the reduction of bcl-2 expression in the HSS-treated SKOV-3 and OVCAR-3 cells may be responsible for the HSS-induced apoptotic processes. Previous studies showed that tumor suppressor protein p53 was an important molecule for apoptosis and cell cycle arrest.¹³ Although HSS treatment induced apoptosis and cell cycle arrest in OVCAR-3 cells accompanied by up-regulation of p53 mRNA, apoptosis and cell cycle arrest were also observed in p53-deficient SKOV-3 cells, which indicated that growth suppression induced by HSS in ovarian cell lines is not likely mediated by p53-dependent pathway, as several studies suggested that many natural phytochemicals could induce apoptosis and cell cycle arrest in p53-independent pathway.^{14, 15}

Abnormal expression of β -catenin and E-cadherin plays a critical role in the process of invasion and metastasis of tumors.^{11, 16} We found HSS-induced growth inhibition of ovarian cancer cell was accompanied by down-regulation of β -catenin, suggesting HSS possibly possess potential inhibition effect on tumor invasion and metastasis. However, we did not observe E-Cadherin up-regulation, nor an expected association of β -catenin down-regulation in HSS-treated cells, which was consistent with a previous report of a lack of association between E-cadherin and β -catenin.¹⁷ The inhibitory effect of HSS on tumor invasion and metastasis should to be confirmed through further studies in vivo.

Expression of CD24 and CD44 in epithelial carcinomas of the ovary correlates with poor prognosis.¹⁸⁻²⁰ CD24 and CD44 are now emerging as a possible therapeutic target for ovarian cancer. Additionally, in several solid tumors including ovarian cancer,²¹⁻²³ these two molecules have been used as surface markers to identify cancer stem cells which may be respon-

sible for tumorigenesis and contribute to resistance to cancer therapy and recurrence. Here, we found HSS inhibited the expression of CD24 at both mRNA and protein level, while CD44 was not involved in the process of HSS-induced growth inhibition in SKOV-3 and OVCAR-3 cells, suggesting CD24 maybe play an important role in ovarian cancer cell growth inhibition induced by HSS. However, further studies are required to evaluate what the relation is between CD24 and other molecular such as bcl-2, caspase-3, and β -catenin.

In conclusion, these results indicate that HSS could inhibit growth of human ovarian cancer SKOV-3 and OVCAR-3 cells and showed strong cytotoxicity. Bcl-2, caspase-3, β -catenin, and CD24 were involved in the process of cytotoxic effect induced by HSS. It is tempting to speculate that HSS might be utilized as a potential therapeutic agent against human ovarian cancer. Further investigation is warranted to specify the active components and mechanism of their anti-cancer action.

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