

Transcriptional Analysis of VEGF-D and TGFβ Genes in MCF7 Cells Exposed to Saponin Isolated from *Holothuria leucospilota* (Sea Cucumber)

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Abstract

Background: Marine natural products contain a wide range of bioactive compounds with therapeutic properties that have revealed crucial properties in the treatment of some diseases. Some of these compounds have recently received considerable attention for drug discovery. In this study we examined the anti-angiogenic effect of saponin isolated from *Holothuria leucospilota* (sea cucumber) through evaluation of vascular endothelial growth factor D (VEGF-D) and transforming growth factor-β (TGFβ) expression in a breast cancer cell line.

Methods: To investigate the effect of SCS on VEGF-D and TGFβ expression in breast cancer cells, the cells were treated with various concentrations of sample. After 48 h the viability of the cells was evaluated by trypan blue staining, and VEGF-D and TGFβ mRNA expression was evaluated by real time-PCR.

Results: Our results revealed that SCS can suppress cell viability and VEGF-D and TGFβ mRNA expression in breast cancer cells. Sea cucumber saponin at a concentration of 12 μg/ml inhibited VEGF-D and TGFβ expression more than 90% compared with controls.

Conclusion: Findings suggest that SCS could inhibit tumor growth via inhibition of angiogenesis.

Keywords: Angiogenesis, Anticancer, Saponin, Sea cucumber

Introduction

Breast cancer is common in women and the mortality rate is high. The high mortality rate is indicative of ineffective therapies. Limitations in facilities and treatment for breast cancer have created the need to investigate new aspects of treatment (1). Bioactive compounds derived from natural products often have less toxic side effect than conventional treatments. For this reason, they are currently being considered by many scientists. Recent studies have demonstrated that some marine organisms contain compounds and novel molecules with therapeutic properties (2). Sea cucumbers are marine invertebrates that have been utilized to treat certain diseases (3). Therapeutic features of sea cucumber can be attributed to the

existence of bioactive compounds such as glycosides, polysaccharides, chondroitin sulfates, and cerberosides (4). Saponins, the main secondary metabolites in the bodies of sea cucumbers, demonstrate exclusive properties based on their structural characteristics. Numerous investigations have shown various medicinal properties of saponins such as anti-microbial, anti-proliferative, antioxidant, and anti-cancer activities (5). One of the most important features of saponins is its cytotoxic effect against cancer cells. The effects of saponins in cancer therapy have been revealed in several studies (6). Saponins can inhibit the growth of some tumors through various mechanisms, such as cell cycle arrest (7), induction of apoptosis (8), and inhibition of

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angiogenesis (9). The formation of new blood vessels from existing blood vessels is called angiogenesis, and this process is controlled by a wide range of activators and inhibitors (10). VEGF and TGF- β are the most important pro-angiogenic factors and interaction of these factors plays a crucial role in angiogenesis (11). Vascular endothelial growth factor D (VEGF-D), is a potent angiogenic factor that exerts its effects via binding to special receptors, and thus can control the function of endothelial cells, and it also protects cells from apoptosis (12). Thus, inhibition of VEGF-D can lead to the inhibition of angiogenesis and induction of apoptosis, which are important goals in the treatment of tumors (13). Transforming growth factor- β (TGF- β) is known as a multifunctional gene in angiogenesis and some investigations indicate that this gene is involved in apoptosis and inflammation. TGF- β has critical roles in tumor pathogenesis and can induce angiogenesis through effects on tumor microenvironment (14). Given the role of VEGF-D and TGF genes in angiogenesis, identification of compounds that can inhibit these factors' activities is important in the prevention of cancer progression. The current investigation was conducted to evaluate the anti-angiogenic effects of sea cucumber saponins (SCS). Our findings indicate that SCS can inhibit tumor growth by inhibiting expression of VEGF-D and TGF β as pro-angiogenic factors.

Materials and Methods

Raw Material

Specimens of the sea cucumber (*Holothuria leucospilota*) were collected from Persian Gulf water. After washing, their body walls were isolated and maintained at -80 °C until use for extraction of saponins.

Cells and Chemicals

The breast cancer cell line MCF7 was acquired from the National Cell Bank of Iran. All materials for cell culture, including the culture media and supplements,

were purchased from Gibco- Invitrogen (USA). The RNA extraction kit was obtained from Roche Company (Germany). The c-DNA synthesis kit was from Thermo Scientific Company (USA). SYBR Green real-time PCR master mix was purchased from Pars tous Iran.

Preparation of Saponin

Saponin from *H. leucospilota* was purified using various solvent systems. The body walls of sea cucumbers were cut, dried, and ground into powder. The powder was incubated in 70% ethanol for 48h and then refluxed three times with ethanol. The solution was filtered and evaporated and the resulting extract was dissolved in dichloromethane/water. The aqueous phase was then extracted with n-butanol. At the last step, the concentrated n-butanol extract was dissolved in water and loaded on a Diaion HP-20 resin column. The resin was washed with water to remove inorganic salts followed by 80% and 100 % of ethanol for separation of saponins. The obtained extracts were concentrated and lyophilized (15, 16).

Culture Medium

The MCF7 cells were grown in RPMI 1640 complemented with 10% v/v fetal bovine serum and 1% antibiotic (penicillin/streptomycin), and kept at 37 °C under 5% CO₂.

Cell Viability Assay

The effect of the SCS on viability of the MCF7 cells was assessed by trypan blue staining. First, the cells were seeded at 1×10^5 cells/ml and incubated for 24 h. After completion of the incubation period, the SCS was applied at concentrations of 0, 2, 4, 6, 8, and 10 μ g/ml. After 48 h, the cells were washed with PBS and detached from the culture plates with trypsin-EDTA. The cell suspension was stained with 0.2% trypan blue (Sigma, USA), transferred to a hemocytometer, and cells were counted using an inverted microscope (17). Cell viability was determined using the following equation:

$$\% \text{ viability} = (\text{live cell count} / \text{total cell count}) \times 100.$$

Table 1. Primer sequences

Gene	Forward 5'→3'	Reverse 5'→3'
VEGF-D	5'GTATGGACTCTCGCTCAGCAT3'	5'AGGCTCTCTTCATTGCAACAG3'
TGF β	5'GGGACTATCCACCTGCAAGA3'	5'CCTCCTTGGCGTAGTAGTCG3'
GAPDH	5' CAAGGTCATCCATGACAACCTT3'	5' GTCCACCACCTGTTGCTGTAG3'

Determination of the expression level of angiogenesis-related genes

The transcriptional expression of VEGF-D and TGF β was examined by quantitative real-time PCR using gene specific primers. For this purpose, total RNA was purified from the control and experimental subjects using a commercial RNeasy kit (High Pure RNA Isolation Kit, Roche, Germany). cDNA was synthesized with random primers using a RevertAid First Strand cDNA Synthesis Kit as described in the manufacturer's handbook. Real-time PCR was performed on a Bio-Rad CFX96 system using SYBR Green master mix. Alterations in expression were estimated relative to the levels expressed by GAPDH gene as a reference gene. Primer sequences are shown in Table 1.

Statistical analysis

Data was analyzed using SPSS version 16. Analysis

of variance (ANOVA) was performed on viability rate and gene expression. To compare differences between mean values of multiple groups the least significant difference (LSD) test ($p < 0.001$) was applied. P value < 0.05 was accepted as significant.

Results

The effect of SCS on viability of MCF7 Cells

Viability of the MCF7 cells 48 h after treatment with SCS was evaluated using a dye exclusion assay. Based on the results, SCS showed significant cytotoxic effect on the MCF7 at 2-10 $\mu\text{g/mL}$ doses (** $p < 0.01$, *** $p < 0.001$) compared with untreated cells. As shown in the Fig. 1, significantly increases with increasing concentration of SCS and this indicates that SCS significantly inhibited viability of MCF7 cells in a dose-dependent manner.

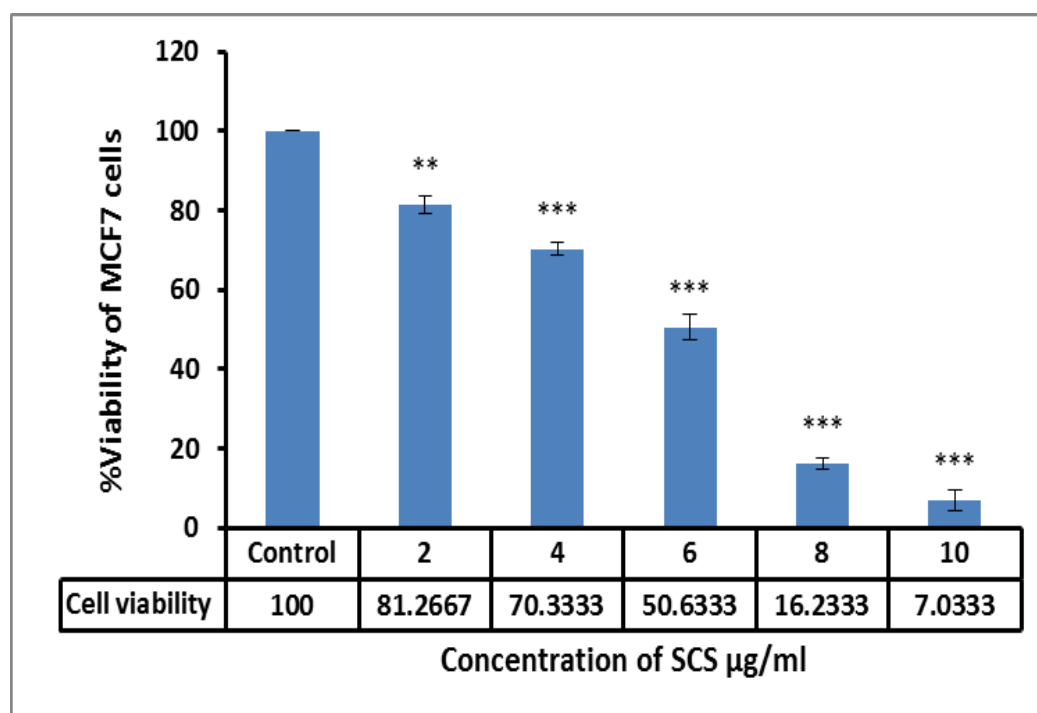


Fig. 1. Viability of MCF7 cells 48 h after treatment with 0, 2, 4, 6, 8, and 10 $\mu\text{g/ml}$ of SCS. Results are presented as the mean \pm SD. ** $P < 0.01$ and *** $P < 0.001$ were considered significant.

The effect of SCS on VEGF-D expression level in MCF7 cells

VEGF-D mRNA expression was not significantly different between the groups treated with different concentrations of SCS, but it was significantly less than in the control group (*** $P < 0.001$). As shown in

Fig. 2, addition VEGF-D expression was significantly less in the cells that received SCS, even at 3 $\mu\text{g/ml}$, than in the control group. This is indicative of a strong inhibitory effect of SCS on VEGF-D as a pro-angiogenic gene.

The effect of SCS on TGFβ expression level in MCF7 cells

TGFβ mRNA expression was also significantly less in the cells that received SCS than in the control group (** $p < 0.01$, *** $p < 0.001$). As shown in Fig. 3, at the concentration of 6 μg/ml of SCS observed an increase in the expression of TGFβ which probably is

represents a transient change in the expression of this gene. Data analysis showed SCS at 3, 6, and 12 μg/ml inhibited TGFβ expression by 93, 70 and 99.5%, respectively, compared with the control group. Therefore TGFβ can be considered as one of the genes involved in angiogenesis that inhibited by SCS.

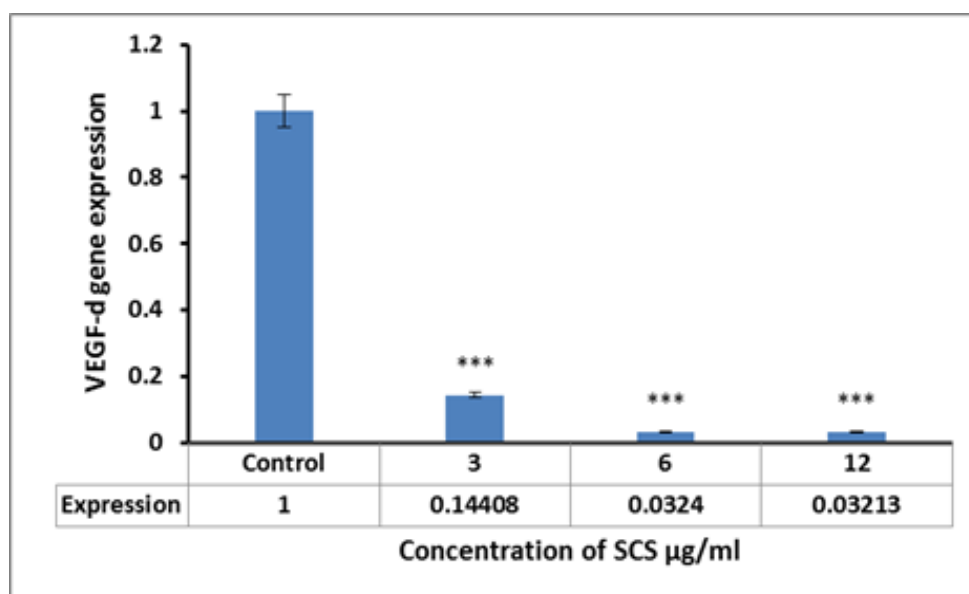


Fig. 2. MCF7 cells were exposed with 3, 6, or 12 μg/ml of SCS for 48h and mRNA expression levels of VEGF-D was assessed by real time pcr examination that demonstrated inhibitory effects of SCS on expression of VEGF-D. Saponin extract showed significant inhibitory effect on the MCF7 at 3-12 μg/mL doses (*** $p < 0.001$) compared with untreated cells.

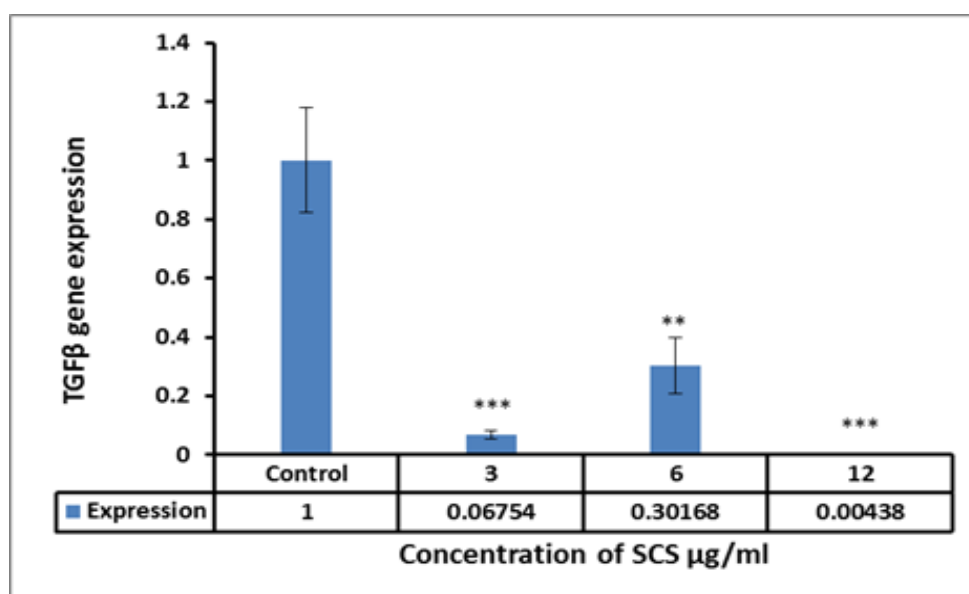


Fig. 3. Effects of SCS on TGFβ mRNA expression in MCF7 cells 48 h after addition of 3, 6, or 12 μg/ml of SCS TGFβ expression levels were assessed by real time pcr and detected a significant decrease in the expression of TGFβ in treated cells compared with control group. All data expressed as mean±SD. ** $P < 0.01$ and *** $P < 0.001$ were considered significant.

Discussion

Because the inhibition of angiogenesis is an important strategy to control of cancer progression, thus identifying compounds with anti-angiogenic properties is of particular importance (18). The use of conventional therapies in breast cancer, like other cancers, can cause damage to healthy cells. For this reason scientists are looking for a new approach to minimize the side effects of current treatments. Natural products and bioactive compounds derived from them may be a solution to this problem. Natural products derived from plants and marine organisms have health benefits that have been used to treat some diseases (19). Saponins are secondary metabolites found in plants and some marine organisms, and anti-cancer effects of these compounds has been demonstrated in some studies (20). To determine whether the saponins isolated from *H. leucospilota* can suppress angiogenesis in breast cancer cells, we evaluated VEGF-D and TGF β expression in MCF7 cells treated with SCS by quantitative real-time PCR assay. Our results revealed that the SCS has a dose-dependent inhibitory effect on MCF7 cell viability (Fig. 1) and VEGF-D (Fig. 2) and TGF β (Fig. 3) expression indicating the anti-angiogenic property of SCS. Studies of the effects of anticancer natural products such as saponin suggest that these compounds suppress cancer cells with various strategies such as inhibition of angiogenesis.

In a study in 2005, Stanley and colleagues found that *Ganoderma lucidum* inhibited angiogenesis via down regulation of VEGF and TGF- β 1 in PC-3 cells (21). This is similar to the results of our study. Tian et al (2007) showed that philinopside E, through specific interactions with the kinase insert domain-containing receptor (KDR) extracellular domain, block its interaction with VEGF, and the downstream signaling and from this pathway can inhibit angiogenesis (22). Recently, much attention has been focused on the extraction of bioactive compounds from ginseng and evaluation of its therapeutic properties. Ginsenoside Rg3 is a bioactive compound isolated from ginseng that exerts a strong inhibitory effect on growth and angiogenesis in ovarian cancer cells (23). Diosgenin is a saponin purified from *Trigonella foenum graecum*, which like other saponin compounds, has anti-proliferative effects. Diosgenin inhibited PC-3 cell proliferation in a dose-dependent

manner. Diosgenin prevented the progression of angiogenesis by suppressing VEGF expression in PC-3 cells, which resemble to our data in anti-angiogenic activity of SCS (24). Ds-echinoside A (DSEA), a triterpine glycoside extracted from *Pearsonothuria graeffei* sea cucumber, has an inhibitory effect on HepG2 cell viability and is also inhibits tube formation in ECV-304 cells. Western blot results showed that this compound has a strong inhibitory effect on nuclear factor- κ B (NF- κ B) and VEGF (25). Chan et al (2011) reported that polyphyllin D, is a steroidal saponin extracted from *Paris polyphylla* inhibited angiogenesis in HMEC-1 cells at 0.1–0.4 μ M concentrations (26).

Wenjing et al (2012) reported that astragaloside IV (AS) inhibited viability of uveal melanoma cells and suppressed angiogenesis through inhibition of VEGF-a expression. Therefore, astragalos may be considered as a therapeutic agent for the treatment of angiogenesis-dependent diseases, including cancer (27).

Radix astragali, a medicinal herb with pharmacological features, contain many bioactive compounds, including astragalosides (AST). Evaluation of anti-angiogenic properties of AST in in vivo models showed that the AST reduced the levels of p-Akt, p-mTOR, VEGF, VEGFR1, and VEGFR2 proteins. Therefore, it can be regarded as a compound with anti-cancer properties (28).

Because saponins have diverse structures they have diverse biological properties. Liu et al. (2013), reported different results than we found in our study. In the Liu et al. study, total saponins extracted from *Panax notoginseng* enhanced angiogenesis by increasing VEGF expression and related receptor signalling (29).

Pulsatilla saponin D (SB365), an active herbal compound extracted from *Pulsatilla koreana* strong inhibited the viability of several cancer cell lines such as MIA PaCa-2, BXPc-3, PANC-1, AsPC-1, and HPAC. SB365 also inhibits angiogenesis and via down regulation of HIF-1 α and VEGF genes. These features qualify SB365 as a candidate for use in cancer treatment (30). Theasaponin E1 (TSE1) is a saponin present in tea seed. TSE1 has been shown to exert its effect on anti-angiogenic effects through the inhibition of VEGFR and NF- κ B (31). Prior studies revealed the suppression of VEGF and TGF- β 1 via the inhibition

of NF- κ B (21). Therefore, it is predicted that TSE1 may have an inhibitory effect on VEGF and TGF- β genes. Taken together, these reports suggest that natural products could play important roles in cancer treatment. Therefore, identification of bioactive compounds is an important step in the treatment of diseases.

The results of our study indicate that the saponins extracted from the sea cucumber can prevent angiogenesis through inhibition of angiogenesis-

related genes, such as VEGF-D and TGF β . Thus, saponins and other biological compounds could be effective in the treatment of cancer and other angiogenesis-related diseases.

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