Viscothionin Suppresses Human Non-small Cell Lung Cancer via Inhibiting the STAT3 Signaling Pathway

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ABSTRACT

Lung cancer is a crucial cause of mortality worldwide. Signal transducer and activator of transcription 3 (STAT3) are important signaling factors in malignant diseases and are constantly activated in 22% ~ 65% of non-small cell lung cancer (NSCLC) cells. STAT3 can be activated by interleukin-6 (IL-6) which induces cell growth in various cancer cells. Although viscothionin is studied for various health beneficial effects, the anticancer effect of viscothionin has not been studies so far against lung cancer. Therefore, the purpose of this study was to demonstrate the anticancer effect of a polypeptide, viscothionin in NCI-H460 lung cancer cells, which represents NSCLC. To do this, cultured NCI-H460 cells were treated with viscothionin and/or IL-6, and the cell viability, as well as expression levels of STAT3, Akt, mTOR, Bax, Bcl-2, and Bcl-xL, including...
1. INTRODUCTION

Lung cancer is the major cause of cancer-associated deaths in the United States and across the world and classified based on the type of histology [1]. Lung cancer is divided into two types, one is known as small cell lung cancer (SCLC) and the other is non-small cell lung cancer (NSCLC) [2]. Moreover, there is a about 85-90% population of NSCLC of lung cancer-associated cases under three sub types such as squamous cell (epidermoid) carcinoma, adenocarcinoma, and undifferentiated large cell carcinoma [3]. Signal transducer and activator of transcription proteins belong to the family of transcription factors consisting of seven members, including STAT1-STAT6, STAT5A and STAT5B [4]. STAT3 is known to be associated with tumor formation and tolerance to chemotherapy [5]. The activation of STAT3 is strictly controlled in the growth and differentiation of cells [6]. Recent evidence suggests, both at the initiation of malignant transformation and during cancer progression, STAT3 play an important role in selectively maintaining and inducing procancerogenic inflammatory micro-environments [7,8]. Suppression of STAT3 has been shown to depress cancer cell growth and increase the susceptibility to anticancer drugs in various cancers, including prostate cancer [9], colon cancers [10] and breast carcinoma cells [11]. Cytokines and growth factors, such as interleukin 6 (IL-6), activates STAT3 as a transcription factor [12]. Level of IL-6 is associated with postoperative complication and postoperative recurrence of lung cancer [13]. Previous studies suggest that IL-6 promotes lung cancer cell migration and proliferation through activation of STAT3 [14]. Mistletoes, also known as parasitic plants, are traditionally known for their several pharmacological activities, including anticancer, cardiac depressant, hypertensive, neurological, or relaxation effects [15,16]. Mistletoe extracts have been used against a variety of diseases and used as constituents of herbal remedies [17]. Mistletoes contain viscothionin, alkaloids, lectin, triterpenes, steroids, flavonoids, and polysaccharides [18]. In this study, we used a polypeptide, viscothionin isolated from the Korean mistletoe, Viscum album. Although viscothionin has been studied for its several beneficial effects as bioactivate molecule, there is no any reported study about its anticancer effect. Therefore, in this study, we investigated the anticancer effect of viscothionin in the human lung carcinoma NCI-H460 cells via STAT3 pathway.

2. MATERIALS AND METHODS

2.1 Isolation of Viscothionin from Korean Mistletoe

Viscothionin was isolated from the Korean mistletoe as previously described [19]. Briefly, 50 g of powdered mistletoe was mixed with 750 mL of 2% acetic acid and stirred overnight at 4°C. The homogenate was centrifuged at 6000 rpm for 30 min, and the resulting supernatant was lyophilized. The dried matter was then dialyzed against 25 mM sodium acetate buffer (pH 4.8) at 4°C. The dialysate was loaded on a CM Sepharose Fast Flow cation exchange column (20 × 4.6 cm) and washed with the same acetate buffer. Retained compounds were then eluted with a NaCl gradient (0–0.5 M), and 15 mL fractions were collected using a DC-1200 fraction collector (Eyela, Sunnyvale, CA, USA).

2.2 Chemicals

Human lung carcinoma cells (NCI-H460) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum and RPMI 1640 culture medium were purchased from HyClone, Logan, UT, USA. Antibiotic solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were
obtained from Sigma, St. Louis, MO, USA. Anti-STAT3, anti-phospho-STAT3, anti-AKT, anti-phospho-AKT, anti-mTOR, anti-phospho-mTOR, anti-caspase-3, anti-Bax, anti-Bcl-2 and anti-Bcl-xL antibodies were purchased from Cell Signaling, Danvers, MA, USA. Peroxidase-conjugated goat anti-rabbit IgG antibodies were procured from Millipore, Bedford, MA, USA. Super signal west dura extended duration substrate was from Thermo Scientific, CA, USA. Caspase-Glo3/7 assay kit was procured from Promega, Madison, WI, USA.

2.3 Cell Culture

Human lung carcinoma NCI-H460 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% antibiotic solution in a humidified incubator with 5% CO₂ at 37°C. Cells harvested in the exponential growth phase were plated to a 6-well culture plate (Nunc, Tokyo, Japan) at a density of 2×10⁴ cells 24 h before treatment. Cells were treated with viscothionin (5 μg/ml) and IL-6(10 ng/ml).

2.4 Cell Viability Assay

Cell viability was determined using a 3-(4,5-dimethylthiazolidyldimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit according to the instruction of the manufacturer. Briefly, NCI-H460 cells were plated in 48-well plates at a density of 2×10⁴ cells per well and were treated with 0.1, 1, 5 or 10 μg/ml viscothionin for 24 h at 37°C. After the incubation period, 10 μL of the MTT solution was added to each well, followed by 3 h incubation at 37°C in 5% CO₂. The resulting formazan crystals were subsequently dissolved in MTT solubilizing solution. Absorbance was then determined at 540 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.5 Western Blotting

Total proteins were collected from NCI-H460 cells after treatment with 5 μg/ml of viscothionin and IL-6 after 24 h. The proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% and 12% gels and proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% skim milk in PBS and then incubated with primary antibodies against STAT3, phosphor-STAT3, AKT, phosphor-AKT, mTOR, phosphor-mTOR, Bax, Bcl-2, and Bcl-xL diluted at 1:1000 in 1% skim milk in PBS overnight at 4°C. Blots were then incubated with peroxidase-conjugated goat anti-rabbit IgG for 1 h. Immunoreactions were visualized with Super signal west dura extended Duration substrate and analyzed using a Chemi Imager analyzer system (Alpha Innotech, San Leandro, CA, USA).

2.6 Caspase-3 Activity Analysis

Caspase-3 activation activity was assessed using Caspase-Glo3/7 assay kit as an index of apoptosis. The chondrocytes were re-plated at the density of 1.0×10⁴ onto 48-well plates. After 24 h of incubation, the NCI-H460 cells were treated with 5 μg/ml viscothionin. After this treatment, 60 μL supernatant was transferred from each well to a fresh 96-well plate. Equal volumes of Caspase-Glo 3/7 reagents were added to each well, and the plate was incubated for 1 h at room temperature before the luminescence was measured (Bio-Tek Instruments, Winooski, VT, USA).

2.7 Statistical Analysis

All data were obtained from at least three individual experiments. Values were expressed as the mean ± SEM. Statistical analysis between groups was performed by one-way ANOVA. The statistical significance was set at p<0.05.

3. RESULTS AND DISCUSSION

3.1 Natural Products are Still the Main Sources of Health Care and New Drug Development in the Era of Modern Medicine

To examine the effect of viscothionin on the cell viability of the lung cancer NCI-H460 cells, we used MTT reagent to detect the changes in the cell viability. We treated NCI-H460 cells with viscothionin at various concentrations (0.1-10 μg/ml) for 24 and 48 h. As a result, treatment of viscothionin decreased cell viability significantly in NCI-H460 cells (Fig. 1). In this study, we demonstrated that viscothionin was cytotoxic towards human lung cancer NCI-H460 cells in a dose-dependent and time-dependent manner (Fig. 1). These results indicate that viscothionin possesses strong cytotoxicity and cell proliferation inhibitory effect on NCI-H460 cancer cells.

As STAT3 is associated with cancer proliferation, we detected the expression of p-STAT3 in NCI-
H460 cells following viscothionin treatment at various concentrations (0.1-10 µg/ml) for 0-24 h. As expected, viscothionin decreased the p-STAT3 protein expression levels in a dose (Fig. 2A) and time (Fig. 2B) dependent manners. In addition, the levels of p-STAT3 protein in both viscothionin and IL-6 exposed NCI-H460 cells were also measured (Fig. 2C). As a result, only the IL-6 treated group showed the highest increment in p-STAT3 protein level compared to the only viscothionin treated group. Interestingly, one-hour pre-treatment with 10 ng/ml of IL-6 and viscothionin treated group significantly suppressed the protein level of p-STAT3 compared to IL-6 alone treated group. These results indicate that viscothionin significantly decreased p-STAT3 protein level in alone and with IL-6 in NCI-H460 cells. The expression of STAT3 protein contributes to cancer malignancy involved in the migration or invasion of cells [6]. Interestingly, STAT3 suppresses the immune system in the tumor microenvironment [20], by inducing the expression of immunosuppressive agents [21] or promoting pro-inflammatory mediators [20]. Thus, by inhibiting the activation of STAT3, it is possible to suppress the expression of various genes regulated by STAT3 and to inhibit the proliferation and metastasis of cancer cells. In our study, we verified that viscothionin treatment effectively suppressed the proliferation of cancer cells by down regulating the expression of STAT3, leading to reduction of Akt/mTOR pathway in NCI-H460 cells (Figs. 2, 3).

The expression levels of p-Akt and p-mTOR proteins in viscothionin treated NCI-H460 cells were measured (Fig. 3). As expected, the viscothionin (5 µg/ml) alone treated cells showed a significant decrease in p-Akt and p-mTOR protein levels compared with the control. In addition, both viscothionin and IL-6 treated group significantly suppressed the protein level of p-Akt and p-mTOR compared to IL-6 alone treated group. These results indicate that viscothionin significantly suppressed the expression levels of p-Akt and p-mTOR proteins in NCI-H460 cells. IL-6 is an activator of STAT3 [22], and inflammatory cytokine that mediates cell survival and cell proliferation of apoptotic signals [23,24]. In a study conducted by Bloem et al. [25], IL-6 and STAT3 regulate Bcl-2 and Bcl-xL, the anti-apoptotic genes. Another study suggested that specific STAT3 excision in intestinal epithelial cells inhibits tumor formation and tumor growth, IL-6-deficient mice have fewer and smaller adenomas than wild-type mice [7,8]. In this study, we confirmed that exogenous IL-6 treatment stimulated STAT3 activation in NCI-H460 cells, and viscothionin in treatment inhibited IL-6-induced STAT3 activation along with inhibition of Akt/mTOR signaling transduction.

![Graph](image.png)

**Fig. 1. Effect of viscothionin on cell death**

Cell viability was measured in NCI-H460 lung cancer cells treated with viscothionin (0.1-10 µg/ml). Data are expressed as the mean ± SEM of three independent experiments, **p<0.01 vs. 24 h control, ++p<0.01 vs. 48 h control. CON: Control**
Fig. 2. Effect of viscothionin on STAT-3 protein expression in NCI-H460 lung cancer cells
(A) NCI-H460 cells were treated with viscothionin (0.1, 1, 5, 10 ug/ml) for 24 h. (B) NCI-H460 cells were treated with viscothionin 5 ug/ml for 3, 6, 9, 12 and 24 h. (C) NCI-H460 cells were pre-treated with IL-6 (10 ng/ml) for 1 h and then treated with viscothionin 5 ug/ml for 24 h. Data are expressed as the mean ± SEM of three independent experiments, **p<0.01 and ***p<0.001 vs. control, ++p<0.01 vs. Visco alone, ##p<0.01 vs. IL-6 alone. CON: Control; Visco: Viscothionin; and IL-6: Interleukin 6

Fig. 3. Effect of viscothionin on Akt and mTOR protein levels in NCI-H460 lung cancer cells
NCI-H460 cells were treated with viscothionin (5 ug/ml) and IL-6 (10 ng/ml) for 24 h. Data are expressed as the mean ± SEM of three independent experiments, **p<0.01 vs. control, ++p<0.01 vs. Visco alone, ##p<0.01 vs. IL-6 alone. CON: Control; Visco: Viscothionin; and IL-6: Interleukin 6
Fig. 4. Effect of viscothionin on apoptosis protein levels and caspase-3 activity in NCI-H460 lung cancer cells

NCI-H460 cells were treated with viscothionin (5 μg/ml) and IL-6 (10 ng/ml) for 24 h. (A) The protein expression levels of Bcl-2, Bcl-xL, and Bax in NCI-H460 cells were measured by western blot. (B) The bar graphs indicate the average Bcl-2, Bcl-xL, Bax, and caspase-3 activity. Data are expressed as the mean ± SEM of three independent experiments. **p<0.01 vs. control, +p<0.05 and ++p<0.01 vs. Visco alone, ##p<0.01 vs. IL-6 alone. CON: Control; Visco: Viscothionin; and IL-6: Interleukin 6

To validate the above results, we further examined whether viscothionin-mediated growth inhibition of NCI-H460 cells was due to apoptosis. In this regard, determination of protein expression levels of Bax, Bcl-2, and Bcl-xL may determine whether initiation of cell death or survival was signaling pathways mediated. In addition, caspase-3 is the most intensively studied effector caspase. As a result, the expression level of apoptotic protein Bax was increased in viscothionin treated cells when compared with control cells, whereas a significant decrease in the expression levels of anti-apoptotic proteins Bcl-2 and Bcl-xL were observed in viscothionin treated cells (Fig. 4A and 4B). Viscothionin increased the expression level of Bax and decreased the expression level of Bcl-2 and Bcl-xL in NCI-H460 cells, which indicated that viscothionin, can induce apoptosis in the NCI-H460 cells. Interestingly, the caspase-3 activity also increased by the addition of viscothionin (Fig. 4B).

4. CONCLUSION

Herein in this study, we documented the anticancer effects of viscothionin against NCI-H460 lung cancer cells. Our findings highlighted the fact that viscothionin could inhibit lung cancer cell proliferation via suppressing the expression levels of STAT3 regulated p-Akt and p-mTOR proteins, including anti-apoptotic proteins Bcl-2 and Bcl-xL and by increasing the expression levels of apoptotic protein Bax. These findings
reinforce the suggestion that the underlying mechanism of viscothionin is mainly due to the inhibition of the STAT3 signaling pathway. Thus, the present study implies that viscothionin may be a potential novel therapeutic agent for treating the lungs cancer.

CONSENT
It is not applicable.

ETHICAL APPROVAL
It is not applicable.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

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