INTRODUCTION

Colon cancer is one of the leading causes of cancer death globally. The worldwide incidence rates of colon cancer have been increasing, which is followed by the increase of need to study on the progression and development of colon cancer (Jemal et al., 2008). There is also an urgent need for novel chemopreventive and/or chemotherapeutic agents with minimal or no side effects and toxicities. In recent years, bioactive compounds derived from natural sources have thus become the focus of a substantial amount of attention from researchers seeking to develop chemopreventive and/or chemotherapeutic agents.

More than 80% of colon cancer cases harbor aberrant Wnt/β-catenin signaling that regulate colon cancer progression through intracellular mechanism or interaction with tumor microenvironment (Song et al., 2015). In colon cancer, Wnt/β-catenin signaling is activated via genetic or epigenetic changes of its components. In the absence of the Wnt ligand, glycogen synthase kinase-3β (GSK-3β) constitutively phosphorylates β-catenin which leads to ubiquitination and degradation of β-catenin. In the presence of Wnt ligand, β-catenin is accumulated in cytoplasm and translocates into the nucleus and acts as a trans-factor on cell survival. The impairment of the regulation of the Wnt/β-catenin pathway (particularly, mutation in APC) plays an important role in the initiation of colorectal cancer (Akiyama, 2000). TGF-β signaling pathway is involved in various cellular processes such as apoptosis, cell migration and cell differentiation (Nicklas and Saiz, 2013). TGF-β receptors consist of TGF-β receptor I (TGFβRI) and TGF-β receptor II (TGFβRII). Binding of TGF-β to the TGFβRII allows phosphorylation of...
the TGFβRI. The TGFβRI then phosphorylates Smad-2/3 (R-Smad). Phosphorylated R-Smad then typically bind to the Smad-4 (co-Smad). These complexes translocate into the nucleus, where they interact with various transcription factors to induce expression of target genes (Nicklas and Saiz, 2013). The TGF-β signaling pathway has dual roles, including being a promoter of tumor metastasis and suppressor of tumor in many human cancers (Sheen et al., 2013). The tumor metastasis includes the epithelial-mesenchymal transition (EMT), which is improved by overexpressed TGF-β at the invasion of cancer cells (Portella et al., 1998; Thuault et al., 2006). Paradoxically, the activation of TGF-β signaling pathway has been known to induce cancer suppression (Sun et al., 1994). However, many colorectal cancers escape the tumor-suppressor effects of TGF-β and become resistant to TGFβ-induced growth inhibition (Hoosein et al., 1989).

Carcinoembryonic antigen (CEA) is glycosylphosphatidylinositol (GPI)-anchored glycoprotein. Typically, CEA is found in colon and gastrointestinal tissues of a developing fetus, whereas the synthesis of CEA remarkably decreases before birth. A low level of CEA is thus maintained in the blood of healthy adult. If the CEA is elevated in the blood of adult, this means that the possibility of cancer increases and in cancer patients, poor prognosis and metastasis are shown (Camacho-Leal and Stanners, 2008). In particular, overexpressed CEA has been reported to inhibit induction of apoptosis by TGF-β signaling pathway via CEA directly binding to TGF-βRI (Li et al., 2010).

(1S,2S,3E,7E,11E)-3,7,11,15-Cembratetraen-17,2-olide (LS-1), a marine cembrenolide diterpene, from Lobophyllum sp. (Fig. 1A) has been reported to have anticancer effects via reactive oxygen species (ROS) generation in HT-29 human colorectal cancer cells (Nguyen et al., 2010; Hong et al., 2012). In addition, LS-1 also showed anticancer effect against SNU-C5/5-FU, fluorouracil-resistant human colon cancer cells, via the apoptosis-induction resulting from the activation of TGF-β pathway with the down-regulation of CEA (Kim et al., 2015). On the other hand, although SNU-C5/5-FU features the high-expression of CEA with low activation of TGF-β signaling compared with SNU-C5, LS-1 showed similar cytotoxicity against both of SNU-C5/5-FU and SNU-C5. In the study, we thus examined the action mechanism of LS-1 on the modulation of the TGF-β and Wnt/β-catenin pathways in the SNU-C5 cells.

MATERIALS AND METHODS

Reagents

Hoechst 33342, RNase A, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI)
were obtained. We determined the IC_{50} value (compound concentration)-response (% control optical density; Y-axis) curves were determined by measuring the absorbance at 540 nm using a microtiter plate enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instruments Inc., Winooski, VT, USA). Each experiment was repeated at least three times. Concentration (X-axis)-response (% control optical density; Y-axis) curves were obtained. We determined the IC_{50} value (compound concentration resulting in 50% inhibition of growth).

**Morphological analysis of apoptosis by hoechst 33342 staining**

SNU-C5 cells were seeded at 2×10^5 cells/mL on 24-well microplates. After 24 h of incubation, the cells were treated with LS-1 (6.5 μM of IC_{50}) for 24 h. The cells were incubated in a Hoechst 33342, which is a DNA-specific fluorescent (10 μg/mL medium at final) at 37°C for 30 min. The SNU-C5 cells were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera (Olympus, Tokyo, Japan) and photographed (magnification ×20).

**Flow cytometric analysis of apoptosis**

The effect of LS-1 on cell cycle distribution was analyzed by flow cytometry after staining the cells with PI (Fried et al., 1976). SNU-C5 cells (2×10^5 cells/mL) were treated with 6.5 μM of LS-1 for 24 h. The treated cells were detached with trypsin, washed twice with phosphate-buffered saline (PBS) and fixed with 70% ethanol for 30 min at -20°C. The fixed cells were washed twice with cold PBS, incubated with 50 μg/mL RNase A at 37°C for 30 min, and stained with 50 μg/mL PI at 37°C for 30 min in the dark. The stained cells were analyzed using fluorescence activated cell sorter (FACS) caliber flow cytometry (Becton Dickinson, San Jose, CA, USA). Histograms were analyzed with Cell Quest software (Becton Dickinson). The proportion of cells in G_0/G_1, S and G_2/M phases was represented as DNA histograms. Apoptotic cells with hypodiploid DNA were measured by quantifying the sub-G_1 peak in the cell cycle pattern.

**Western blot analysis**

To examine the effect of LS-1 in the SNU-C5 cells, the cells were seeded 2×10^5 cells/mL for 24 h and treated with LS-1 6.5 μM for 12, 24 and 48 h. After treatment, SNU-C5 cells were lysed with lysis buffer for 30 min at 4°C. The lysates were centrifuged at 15,000 rpm, 4°C for 15 min. Protein content was determined according to the method of Bradford assay (Bradford, 1976). The cell lysates were separated by 8-15% SDS-PAGE gels and then transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA) by glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], and 20% MeOH [v/v]) at 200 mA for 2 h. After blocking with 5% skim milk solution, the membrane was incubated with primary antibody against PARP (1:2000), cleaved caspase-3 (1:1000), Bcl-2 (1:1000), Bax (1:1000), p-GSK-3β (1:1000), GSK-3β (1:1000), GSK-3β (1:1000), β-catenin (1:2000), CEA (1:1000), Smad-2/3 (1:1000), p-Smad-3 (1:1000), c-Myc (1:1000) and β-actin antibody (1:1000) at 4°C overnight and incubated with a secondary HRP antibody (1:5000) antibodies at 4°C overnight and incubated with a secondary HRP antibody (1:5000) at room temperature (RT) for 1 h. Protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON Biotechnology, Gyeonggi, Republic of Korea). VECTASHIELD Antifade Mounting Medium with DAPI was purchased from Vector Laboratories (Burlingame, CA, USA).

**Cell viability assay**

The effect of LS-1 on the growth of SNU-C5 cells was evaluated using the MTT assay (Scudiero et al., 1988). The cells (2×10^5 cells/mL) were seeded on 96-well microplates for 24 h. The cells were treated with LS-1 (0.1, 1, 10, 20 and 50 μM) for 72 h. After incubation, the cells were treated with 50 μL (5 mg/mL) MTT dye and incubated at 37°C for 4 h. The medium was aspirated, and 150 μL/well of dimethyl sulfoxide were added to dissolve the formazan precipitate. Cell viabilities were determined by measuring the absorbance at 540 nm using a microplate enzyme-linked immunosorbent assay (ELISA) reader (BioLegend, San Diego, CA, USA). Each experiment was repeated at least three times. Concentration (X-axis)-response (% control optical density; Y-axis) curves were obtained. We determined the IC_{50} value (compound concentration resulting in 50% inhibition of growth).

**Statistical analysis**

Results are shown as mean ± standard deviation (SD) from three independent experiments. Student’s t-test was used to determine the data with the following significance levels: *p<0.05; **p<0.01. All assays were performed with at least three independent experiments.

**RESULTS**

**Effect of LS-1 on the apoptosis induction of SNU-C5 cells**

In a previous report, we showed that LS-1 treatment could induce the cell death of SNU-C5 cells (Kim et al., 2015). In the study, we also confirmed that IC_{50} value of LS-1 was 6.5 μM in SNU-C5 cells (Fig. 1B). We examined whether SNU-C5
cell death by LS-1 could result from induction of apoptosis. The cell death by apoptosis has typical characteristics including increase of sub-G_{1}, hypodiploid cells and apoptotic bodies. When SNU-C5 cells were treated with LS-1 of 6.5 μM for 24 h, we could observe apoptotic bodies (Fig. 1C). Also, the sub-G_{1} phase population significantly increased from 1.11% to 15.76% after 24 h treatment (Fig. 1D).

Effect of LS-1 on the expressions of apoptosis-related proteins in SNU-C5 cells

When treated with LS-1, we then examined the expressions of the apoptosis-related proteins. As a result, LS-1 treatment increased the level of Bax, a pro-apoptotic protein, while LS-1 decreased the level of Bcl-2, an anti-apoptotic protein, in a time dependent manner (Fig. 2A, 2B). In addition, treatment of LS-1-induced the cleavage of caspase-3, an executioner caspase (Fig. 2A, 2B) and cleavage of PARP in time dependent manner (Fig. 2A, 2B). These results indicate that LS-1 treatment could inhibit the proliferation of SNU-C5 by the induction of apoptosis.

Effect of LS-1 on the Wnt/β-catenin signaling pathway in SNU-C5 cells

By what mechanism LS-1 might induce the apoptosis of SNU-C5 cells? We thus examined the effect of LS-1 on the apoptosis related pathways. Up-regulated Wnt/β-catenin signaling pathway contributes the progression and development of cancer (Akiyama, 2000). We investigated the action of LS-1 on expressions of Wnt/β-catenin signaling-related proteins including GSK-3β and β-catenin. As a result, LS-1 treatment decreased levels of phospho-GSK-3β, the inactivated form of GSK-3β, and β-catenin (Fig. 3). These data suggest that LS-1 might decrease β-catenin level through GSK-3β activation, and that the LS-1 effect on the apoptosis induction could be associated with the down-regulation of the Wnt/β-catenin signaling pathway.
We thus examined whether LS-1 could also induce the activation of TGF-β signaling. During activation of TGF-β signaling, activated TGF-β receptors induce the phosphorylation of Smad-2 or Smad-3. Interestingly, treatment LS-1 increased the phospho-Smad-3 level, whereas the expression of c-Myc, the target protein of TGF-β signaling, decreased in a time-dependent manner (Fig. 4A, 4B). The phosphorylated-Smad-3 combines the Smad-4 and the complex is transferred into the nucleus (Nicklas and Saiz, 2013). The LS-1 increased the level of phospho-Smad-3 in the nucleus (Fig. 4C). These results indicated that LS-1 could activate TGF-β signaling with decrease of CEA, which is followed by the decrease of c-Myc in SNU-C5 cells.

**DISCUSSION**

In this study, we investigated whether the anticancer effect of LS-1 could result from apoptosis via the modulation of Wnt/β-catenin and the TGF-β pathways. LS-1, a marine cembranolide diterpene, from *Lobophytum sp.* is reported to have an anticancer effect against colon cancer cells such as HT-29, SNU-C5/5-FU and SNU-C5 (Hong et al., 2012; Kim et al., 2015). However, the action mechanism of LS-1 on SNU-C5 human colon cancer cells has not been fully elucidated. To the best of our knowledge, this study is the first to demonstrate that LS-1 could induce the apoptosis via not only activation of TGF-β signaling with down-regulation of CEA but also down-regulation of Wnt/β-catenin signaling pathway.

The components of *Lobophytum sp.* include lobophyteone, lobohedleolide, lobocassolide and pakisterol A besides LS-1. LS-1 and lobophyteone showed cytotoxic effect against A549 lung cancer cells and HT-29 colon cancer cells, while there have been no reports of the biological effects of the other components (Nguyen et al., 2010). We also reported that LS-1 treatment induced the cell death in SNU-C5 cells with the IC50 value of 43.07 μM (Kim et al., 2015). Moreover, LS-1 barely inhibited the proliferation of HEL-299, which is a normal cell line, with an IC50 value of 43.07 μM (Kim et al., 2015). In this study, we found that the treatment of LS-1 showed typical characteristics of apoptosis such as increase of sub-G1 hypodiploid cells and apoptotic bodies in SNU-C5 cells (Fig. 1). Generally, apoptosis is mediated by apoptosis-related proteins, such as pro- and anti-apoptotic molecules. During apoptosis, pro-apoptotic molecules including Bak and Bax are up-regulated and anti-apoptotic molecules such as Bcl-2 and Bcl-XL are down-regulated. Here, we showed that LS-1 induced the increase of Bax, while LS-1 decreased the Bcl-2 level (Fig. 2A, 2B). Up-regulated Bax, a pro-apoptotic molecule, induces release of the cytochrome c from mitochondria, which leads to cleavage of caspase-9. The cleaved-caspase-9 initiates a protease cascade leading to the cleavage of caspase-3, which lead to cleavage of PARP, a nuclear enzyme that is involved in DNA repair in response to various stresses. In response to LS-1 treatment in SNU-C5 cells, caspase-3 was activated (Fig. 2A, 2B), leading to the cleavage of PARP (Fig. 2C, 2D).

Wnt/β-catenin signaling pathway plays a pivotal role in the progression and development of cancer (Akiyama, 2000). Moreover, previous study indicated that activation of the Wnt signaling pathway is a critical event in the development of colon cancer (Schneikert and Behrens, 2007). The activated GSK-3β leads to degradation of the β-catenin. Thus, be-

**Fig. 4.** Effect of LS-1 on the regulation of TGF-β signaling pathway. (A) The SNU-C5 was treated with LS-1 for 12, 24 and 48 h. The levels of CEA, c-Myc, Smad-2/3 and p-Smad-3 were analyzed by western blotting using specific antibodies. (B) Data represent the percentage of CEA, c-Myc, Smad-2/3 and p-Smad-3 expression in SNU-C5. (C) Modulation of the p-Smad-3 expression by LS-1 was examined by immunofluorescent stain of p-Smad-3. The data are presented as the mean value ± SD from three independent trials. *p<0.05 and **p<0.01 compared with the control.

**Effect of LS-1 on the TGF-β signaling in SNU-C5 cells**

We examined expression of CEA, whose down-regulation is associated with induction of apoptosis (Kim et al., 2015). Treatment of LS-1 decreased the CEA level in a time-dependent manner in SNU-C5 cells (Fig. 4A, 4B). Recent studies reported that CEA could interact directly with TGF-β receptor I, which leads inhibition of TGF-β signaling (Li et al., 2010).
cause of inactived-GSK-3β did not degrade the β-catenin, the β-catenin acts as a transcription factor in nucleus (Akiyama, 2000). Interestingly, we confirmed that the LS-1 induced the activation of GSK-3β and down-regulation of β-catenin in SNU-C5 cells (Fig. 3). Recently, flavonoids, a class of natural compounds, act as anticancer drugs through inhibition of Wnt/β-catenin signaling pathway in colon cancer cells (Amado et al., 2014). The vitamin D could also induce apoptosis of colon cancer cells via inhibition of the Wnt/β-catenin signaling pathway (Pendas-Francisco et al., 2008).

TGF-β signaling has important roles in many processes including cell cycle regulation, cell migration and apoptosis (Nicklas and Saiz, 2013). During activation of TGF-β signaling, TGF-β receptor II (TGF-βRII) induces the phosphorylation of TGF-β receptor I (TGF-βRI). The Phospho-TGF-βRII leads to phosphorylation the receptor-regulated Smad (R-Smad), which be composed of Smad-2 and Smad-3. Phosphorylated R-Smad could bind with common mediated Smad (Co-Smad), such as Smad-4. These complexes migrate towards the nucleus, bind transcription promoters and cause the transcription of target gene (Nicklas and Saiz, 2013; Han et al., 2008). Some of the target of the TGF-β signaling is cell cycle check point genes including p21 and p27 (Xu and Pasche, 2007). Thus, these target genes evokes G1 arrest in the cell cycle (Chiarugi et al., 1994; Xu and Pasche, 2007). Many colon cancers escape the tumor-suppressor effect of TGF-β signaling and resistant to TGF-β induced growth inhibition (Xu and Pasche, 2007). α-Tocopherol succinate has been shown to upregulate cell surface expression of TGF-βRII leading to increased cellular responsiveness to TGF-β-induced apoptosis in human breast cancer cells (Constantinou et al., 2008). FK506, the canonical ligand of FKBP, could also lead to apoptosis through activation of the TGF-β signaling in chronic lymphocytic leukemia B cells (Romano et al., 2008). On the other hand, CEA, a tumor marker on colon cancers, also relates with cell survival against chemotherapy agent (Camacho-Leal and Stanners, 2008). Abnormal CEA expression and alteration of TGF-β signaling are common features of colon cancers (Samaras et al., 2007; Han et al., 2008). A recent study reported that CEA contributes the inhibition of apoptosis via activation of TGF-β signaling through direct interaction with TGF-βRII (Li et al., 2010). In previous report, when examined the characteristics of several colon cancer cells such as SNU-C5/5-FU, HT-29, LOVO, HCT-116 and SNU-C5 on the activation of TGF-β signaling and the CEA expression, SNU-C5/5-FU showed the high-expression of CEA with low activation of TGF-β signaling compared with SNU-C5. Nevertheless, LS-1 showed similar cytotoxicity against both of SNU-C5/5-FU and SNU-C5 and induced activation of TGF-β signaling with the decrease CEA level in SNU-C5/5-FU (Kim et al., 2015). In the study, we thus examined the action mechanism of LS-1 on the modulation of the TGF-β pathway in the SNU-C5 cells. LS-1 reduced the CEA expression and increased the phosphorylation of Smad-3 in SNU-C5 cells (Fig. 4). These results indicated that LS-1 could induce activation of TGF-β signaling with the decrease of CEA in SNU-C5 cells besides SNU-C5/5-FU. We need to examine LS-1 effect on the activation of TGF-β signaling in HT-29 colon cancer cells which relatively shows high-activation of TGF-β signaling with low or none CEA expression in further study.

Taken together, the induction of apoptosis by LS-1 was also accompanied by the decrease of β-catenin through activation of GSK-3β, and activation of TGF-β signaling with down-regulation of CEA. The results suggest that LS-1 could induce the apoptosis via modulation of Wnt/β-catenin signaling and TGF-β signaling pathways in SNU-C5 cells, which means that LS-1 might have a potential in colon cancer treatment.

CONFLICT OF INTEREST

No competing financial interests exist for any of the authors of this study.

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