Herbal prescription, Danggui-Sayuk-Ga-Osuyu-Senggang-Tang, inhibits TNF-α-induced epithelial-mesenchymal transition in HCT116 colorectal cancer cells

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Abstract. Tumor necrosis factor-α-mediated (TNF-α) epithelial-mesenchymal transition (EMT) is associated with distant metastasis in patients with colorectal cancer with poor prognosis. Although traditional herbal medicines have long been used to treat colorectal cancer, the incidence and mortality in patients with colorectal cancer has continued to increase. Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang (DSGOST) has long been used for treatment of chills, while few studies have reported its anticancer effect. This study aimed to demonstrate the inhibitory effect of DSGOST on TNF-α-mediated invasion and migration of colorectal cancer HCT116 cell lines. MTT was used to measure cell viability. Wound healing and Transwell invasion assay were used to detect migration and invasion of cells, respectively. The intracellular localization of proteins of interest was assessed by immunocytochemistry. Western blotting was performed to determine the expression level of various proteins. A non-toxic dose of DSGOST (50 μg/ml) on HCT116 cells was determined by MTT assay. Furthermore, DSGOST prevented the TNF-α-induced invasive phenotype in HCT116 cells. DSGOST inhibition of the invasive phenotype was also associated with increased expression of EMT markers. Furthermore, DSGOST treatment blocked TNF-α-induced migration and invasion of HCT116 cells. In addition, DSGOST treatment inhibited TNF-α-mediated nuclear translocation of Snail. DSGOST treatment also downregulated TNF-α-induced phosphorylation of AKT and glycogen synthase kinase-3β. Therefore, the findings of the current study suggest that DSGOST exhibits anti-migration and anti-invasion effects in TNF-α-treated HCT116 human colorectal cells.

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Introduction

Colorectal cancer is one of leading causes of cancer-associated mortality worldwide (1,2). Despite advances in detection and therapy in previous years, the incidence and mortality in patients with colorectal cancer is still increasing throughout the world (3-6). The major cause of mortality patients with colorectal cancer is distant metastasis to other organs, such as the liver (7), lung (8), brain (9) and bone (10). Chronic inflammation has emerged as a key contributor for development and metastasis of cancer, including colorectal carcinoma (11,12). Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukins (ILs), including IL-8, IL-6 and IL-1β, promote cancer progression (13). Among them, TNF-α contributes to proliferation and metastasis of colorectal cancer cells and its increased expression is often correlated with poor prognosis of patients (14). Although TNF-α expression has been considered to be a double-edged sword in cancer, it has been suggested that targeting TNF-α may be a successful strategy for cancer treatment (14,15). Activation of the TNF-α pathway promotes nuclear translocation of Snail transcription factor proteins (16). This translocation promotes epithelial-mesenchymal transition (EMT), a process that is considered to be a prerequisite for metastasis in various types of cancer, including colorectal cancer (17-21). Furthermore, Snail overexpression in patients with colorectal cancer has also been correlated with aggressiveness and poor prognosis (22,23). Therefore, inhibition of TNF-α-induced EMT is likely to be effective for treatment of colorectal cancer.

Protein kinase B (AKT), one of the mitogen-activated protein kinases (MAPKs), regulates proliferation, differentiation, development, transformation and apoptosis (24). Disturbance of phosphoinositide 3-kinase/AKT signaling is associated with cancer progression and poor prognosis (25).

Deregulated AKT phosphorylates glycogen synthase kinase-3β (GSK-3β), followed by inactivation of GSK-3β (26). Inactivated GSK-3β fails to tag Snail protein for ubiquitination and subsequent degradation (26). As Snail stabilization is tightly regulated by AKT/GSK-3β pathways, targeting this pathway is a promising strategy for cancer treatment. It has been reported that various anticancer agents inhibit tumor growth via suppressing AKT/GSK-3β/Snail signaling (27-31).

Traditional herbal medicines have previously been researched for colorectal cancer treatment (32-37). Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang (DSGOST; Danggui-Sini-Jia-Wuzhuyu-Shengjian-Tang in Chinese, Tokishigyakukagoshuyushokyoto in Japanese) has long been used to cure patients suffered from Raynaud's syndrome (38,39), dysmenorrhea (40), atopic dermatitis (41), but not cancer. Choi *et al* (42) initially suggested a new use for DSGOST in the treatment of cancer as DSGOST was shown to inhibit pancreatic tumor growth and metastasis by suppressing vascular endothelial growth factor (VEGF)/VEGF receptor 2-dependent tumor angiogenesis. Furthermore, Nagata *et al* (43) demonstrated the inhibitory effect of DSGOST on growth of gastric cancer cells. Taken together, we hypothesize that DSGOST may be beneficial for cancer treatment.

In present study, the anticancer effect of DSOGST on TNF-α-mediated metastatic phenotypes was examined in HCT116 colorectal cancer cells. A non-cytotoxic dose of DSGOST inhibited TNF-α-induced migration and invasion of HCT116 cells. Furthermore, TNF-α induced morphological changes with either downregulation of E-cadherin or upregulation of N-cadherin whereas DSGOST reversed TNF-α-mediated changes. Furthermore, DSGOST suppressed TNF-α-induced nuclear translocation of Snail via inhibition of AKT/GSK-3β pathways. Therefore, these results demonstrated for the first time, the inhibitory effect of DSGOST on TNF-α-induced migration and invasion in HCT116 cells.

Materials and methods

Preparation of DSGOST. DSGOST powder was prepared as described previously (44). DSGOST consists of following herbal drugs; 1 g Angelica radix, 1 g Cinnamomi cortex, 1 g Paeoniae root, 1 g Akebia root, 0.67 g of Asarum, 0.67 g Glycyrrhiza, 1.67 g *Zizyphus jujuba*, 0.67 g Evodia fruit and 1.33 g ginger root. The dried mixture was dissolved in distilled water and stored at -80˚C until use.

Cell culture and cell viability assay. HCT116 human colorectal cancer cell lines were purchased from Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 medium (Welgene Inc., Daegu, Korea)supplemented with 10% fetal bovine serum (FBS; JR Scientific, Woodland, CA, USA) and 1% penicillin/streptomycin solution. For cell viability assay, HCT116 cells were seeded at the density of 4,000 cells/well in 96-wells plate and then treated with DSGOST (0, 10, 50 and 100 μg/ml). After 72 h incubation, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay with an absorbance 570 nm.

Migration and invasion assay. For a cell migration assay, cells were cultured in 6-wells plates until 100% confluence and then the monolayer was scratched the monolayer with a 200 μl pipette tip. Cells were then pretreated with DSGOST for 2 h before incubation with TNF-α (R&D Systems, Minneapolis, MN, USA) for 48 h. The migrated area was analyzed by ImageJ software (64-bit Java 1.6.0_24; National Institutes of Health, Bethesda, MD, USA) and the average was calculated from independent experiments. For the invasion assay, Matrigel was mixed with coating buffer [0.01 M Tris (pH 8.0), 0.7% NaCl] at 1:1 ratio and then added in upper side of BD Transwell chamber in 24-well plates for overnight incubation. HCT116 ($2x10^5$ cells) were pre-incubated with 50 μg/ml DSGOST for 2 h, followed by 20 ng/ml of TNF-α for 48 h in the upper chamber. Culture medium with 10% FBS as chemoattractant was added into the lower chamber. After 48 h incubation, the cells were fixed with 4% paraformaldehyde for 5 min and then permeabilized with 100% methanol for 20 min. Non-invading cells were removed by cotton swab and the cells located at the underside of chamber were stained with 0.05% crystal violet for 20 min at room temperature. The four areas were randomly selected and cells were counted under a phase-contrast microscope.

Cellular fractionation. Cells were pretreated with DSGOST for 2 h, followed by incubation with TNF-α for 6 h. Cell lysates were suspended in 500 μl hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) and incubated on ice for 15 min, followed by centrifugation at 1,000 x g at 4˚C. The supernatant (cytoplasmic fraction) was stored at -80˚C until use. The obtained pellets were suspended in 50 μl cell extraction buffer containing 100 mM Tris (pH 7.4), 2 mM Na_3VO_4, 100 mM NaCl, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 mM EGTA, 0.1% sodium dodecyl sulfate (SDS), 1 mM NaF, 0.5% deoxycholate and 20 mM $Na_4P_2O_7$. After incubation on ice for 15 min, the mixture was centrifuged at 14,000 x g at 4˚C. The supernatant (nuclear fraction) was stored at -80˚C until use.

Western blotting. Cells were collected by scraping and then lysed with RIPA buffer containing 50 mM Tris-HCl, (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS and 1% sodium deoxycholate. Equal amount of proteins [20 μg per well; protein concentration was determined using a Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA, USA)] were separated by 10-12% SDS-PAGE, followed by transfer to nitrocellulose membranes. After blocking with 2% skim milk in PBS-Tween at room temperature for 1 h, membranes were incubated with the appropriate antibodies at 4˚C overnight. Anti-E-cadherin (cat. no. 3195, 1:1,000), tight junction protein-1 (ZO-1; cat. no. 8193; 1:500), N-cadherin (cat. no. 4061; 1:1,000), Snail (cat. no. 3879, 1:1,000), claudin-1 (cat. no. 13255; 1:500), p-AKT (cat. no. 9271; 1:500) and p-GSK-3β (cat. no. 9336; 1:500) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-lamin B (cat. no. sc6216; 1:500) and β-actin (cat. no. sc47778; 1:2,000) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase-conjugated secondary antibodies for mouse (cat. no. 7076; 1:1,000-3,000) and rabbit (cat. no. 7074; 1:1,000-2,000) were purchased from Cell Signaling Technology, Inc. The membranes were incubated with secondary antibodies for 1 h at room temperature. An enhanced chemiluminescence kit (DoGen, Seoul, Korea) was

used for detection of HRP signal. ImageJ software (64-bit Java 1.6.0_24; National Institutes of Health) was used for densitometry.

Immunocytochemistry. The cells (80% confluency) were cultured on coverslip in 6-well plates with serum-free medium for 12 h and then pretreated with or without DSGOST. After 2 h incubation with DSGOST, cells were treated with TNF-α in medium with 1% FBS. Cells were washed with cold-PBS, fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 15 min and blocked with 2% bovine serum albumin (BSA) for 60 min at room temperature. Cells were washed with 0.5% BSA and were incubated with antibodies against E-cadherin (cat. no. 3195; 1:50), N-cadherin (cat. no. 4061; 1:50) and Snail (cat. no. 3879; 1:50) (Cell Signaling Technology, Inc.) at 4˚C overnight. Following incubation, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (cat. no. A-11008; 1:1,000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) secondary antibodies for 1 h at room temperature. Nuclei were stained with TO-PRO-3 iodide (Thermo Fisher Scientific, Inc.). Confocal images were acquired with Zeiss LSM5 PASCAL confocal laser scanning microscope system (Carl Zeiss AG, Oberkochen, Germany). Fluorescence intensities were measured under the same condition for all experiments.

Statistical analysis. Data are presented as the mean ± standard error or standard deviation from at least three experiments with three replicates per each experiment. The statistical differences of means between the groups were analyzed by one-way analysis of variance with post-hoc Tukey test using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

DSGOST inhibits TNF-α-induced EMT in HCT116 cells. To avoid interference resulting from the anti-proliferative effect of DSGOST on HCT116 cells on HCT116 cells, the effect of DSGOST on proliferation of cells was examined by an MTT assay. DSGOST (50 μg/ml) did not affect on the proliferation of cells (Fig. 1A). Thus, the effect of DSGOST (50 μg/ml) on TNF-α-mediated changes in morphology of cells was examined. While the cells in the TNF-α treatment group exhibited an invasive phenotype with a mesenchymal morphology, DSGOST treatment abrogated this change (Fig. 1B).

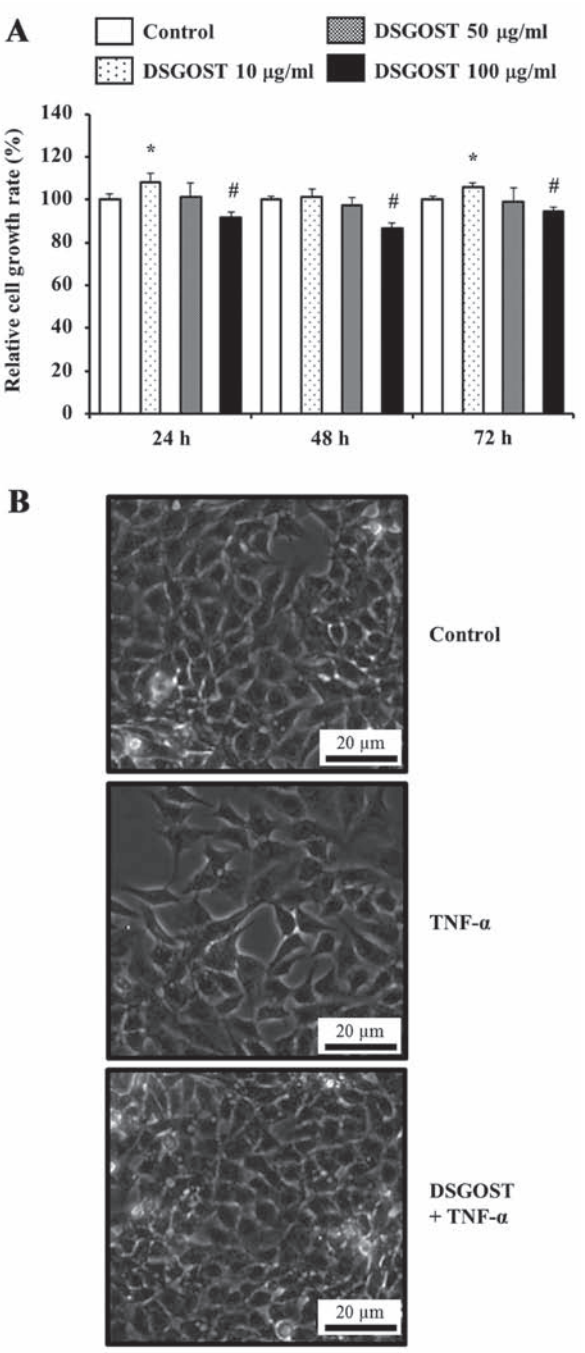

Figure 1. DSGOST inhibits TNF-α-treated invasive phenotype of HCT116 cells. (A) Cell viability assay; 4,000 cells/well of 96-well plates were seeded and treated with indicated doses of DSGOST. Cell viability was measured by MTT assay (sample size of n=3/group). The experiment was performed on three times. Data are presented as the mean ± standard error (*P<0.05, control vs. DSGOST 10 μg/ml; #P<0.05, control vs. DSGOST 100 μg/ml). (B) Cell morphology. The cells were cultured on coverslips in 6-well plates and pretreated with DSGOST (50 μg/ml) for 2 h, followed by incubation with TNF-α (20 ng/ml) for 48 h. Phase contrast images were obtained by confocal microscopy. DSGOST, Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang; TNF-α, tumor necrosis factor-α.

DSGOST reverses TNF-α-mediated EMT in HCT116 cells. TNF-α-induced invasive phenotype is associated with induction of EMT in colorectal cancer cells (16,45). Whether DSGOST prevents TNF-α-mediated changes in EMT markers was examined. Immunocytochemistry data demonstrated that TNF-α treatment induced mesenchymal morphology with decreased E-cadherin expression and increased N-cadherin expression, whereas pretreatment of DSGOST inhibited TNF-α-mediated responses (Fig. 2A). During EMT, epithelial cells acquire a mesenchymal-like phenotype, including the loss of E-cadherin-mediated cell-cell adhesion and the upregulation of N-cadherin. In TNF-α-treated HCT116 cells, epithelial cell markers E-cadherin and ZO-1 were decreased while mesenchymal markers Snail, N-cadherin and claudin-1 were increased. Additionally, DSGOST treatment reversed TNF-α-mediated protein expression (Fig. 2B and C). Thus, DSGOST is may inhibit TNF-α-mediated EMT in HCT116 cells.

DSGOST suppresses TNF-α-promoted migration and invasion of HCT116 cells. EMT process has been considered to

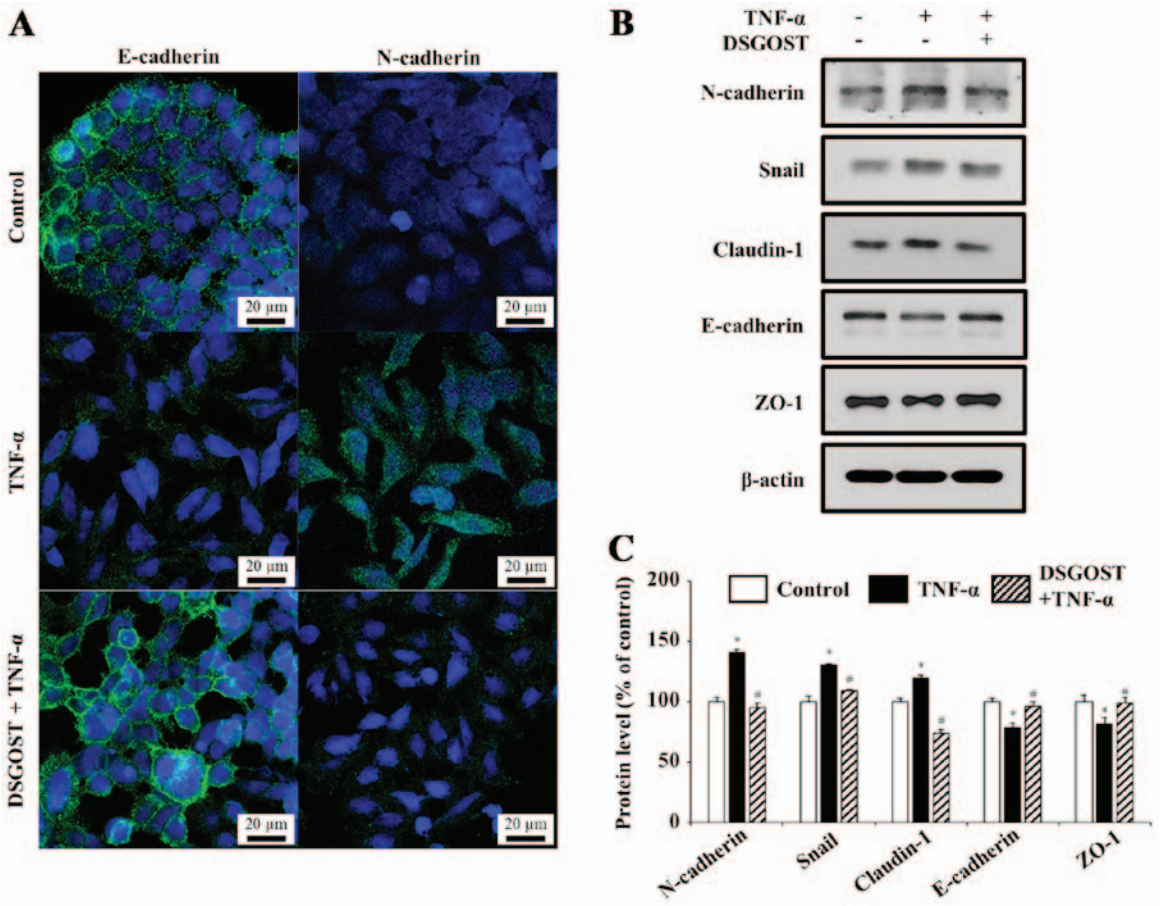

Figure 2. DSGOST reverses TNF-α-mediated EMT in HCT116 cells. (A) Immunocytochemistry. Cells were cultured on coverslip in 6-well plates and pre-treated with DSGOST (50 μg/ml) for 2 h, followed by incubation with TNF-α (20 ng/ml) for 48 h. Cells were immunostained with E-cadherin and N-cadherin specific antibodies. Nuclei was stained with TO-PRO-3 iodide (blue). (B) Representative images and (C) densitometry from western blot analysis. Expression of EMT-associated proteins was analyzed by western blotting. β-actin was used as internal marker. The experiment was performed three times. Values are presented as the mean ± standard deviation (*P<0.05, control vs. TNF-α; $^{\#}$P<0.05, TNF-α vs. DSGOST + TNF-α). EMT, epithelial-mesenchymal transition; DSGOST, Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang; TNF-α, tumor necrosis factor-α; ZO-1, tight junction protein-1.

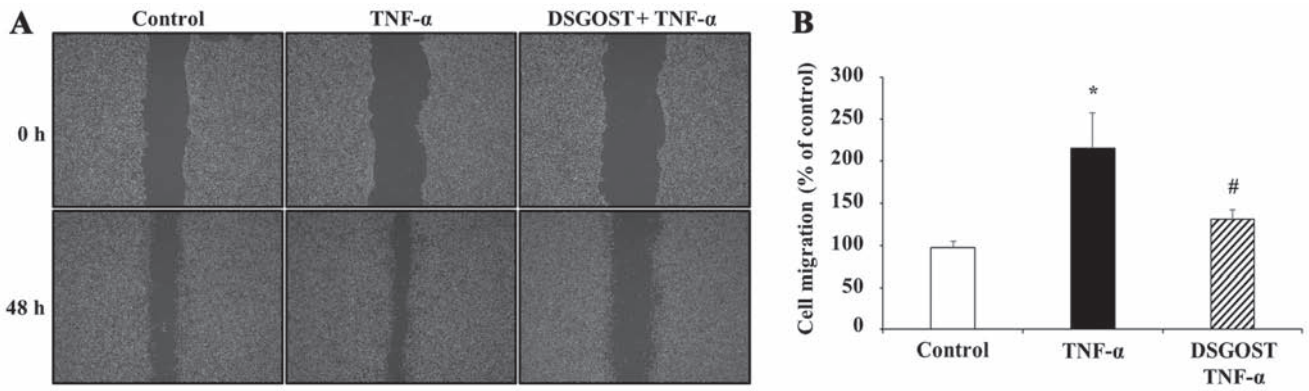

Figure 3. DSGOST inhibits TNF-α-induced migration of HCT116 cells. (A) Representative images from wound healing assay. Cells were cultured on 6-well plates until 100% confluent and then scratched. Cells were pretreated with DSGOST (50 μg/ml) for 2 h, followed by incubation with TNF-α (20 ng/ml) for 48 h (magnification, x40). (B) Migrated area (% of control) was analyzed by ImageJ software. The experiment was performed on three times. Values are presented as the mean ± standard deviation (*P<0.05, control vs. TNF-α; $^{\#}$P<0.05, TNF-α vs. DSGOST + TNF-α). DSGOST, Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang; TNF-α, tumor necrosis factor-α.

be a prerequisite for migration and invasion of various cell types (46). As DSGOST inhibited TNF-α-induced EMT in HCT116 cells, it was also examined whether DSGOST suppresses TNF-α-mediated migration and invasion using wound healing assay and Transwell invasion assays, respectively. TNF-α increased migration and invasion of HCT116 cells, and DSGOST treatment blocked TNF-α-mediated migration and invasion (Figs. 3 and 4). Therefore, DSGOST is may suppress TNF-α-induced migration and invasion of HCT116 cells.

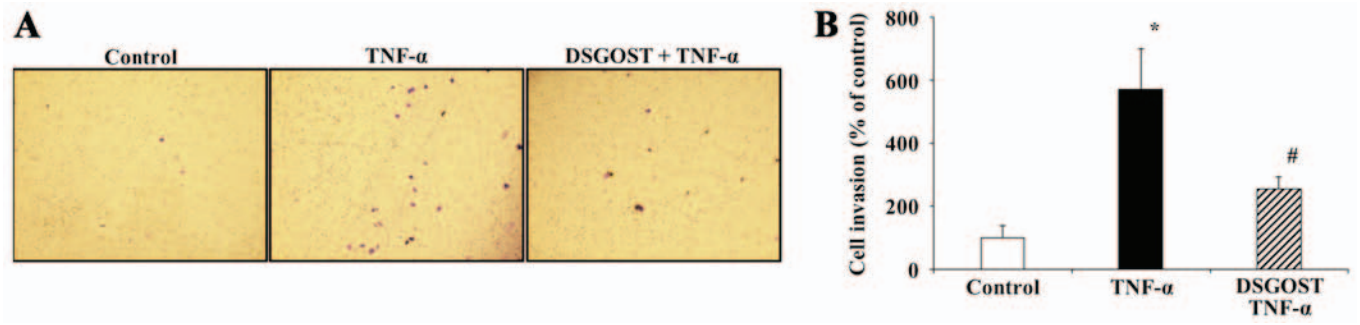

Figure 4. DSGOST inhibits TNF-α-induced Transwell invasion of HCT116 cells. (A) Cells were cultured on Matrigel-coated Transwell chamber. Representative images from Transwell invasion assay. Cells were pretreated with DSGOST (50 μg/ml) for 2 h, followed by incubation with TNF-α (20 ng/ml) for 48 h (magnification, x40). (B) Invading cells were counted under a microscope. The experiment was performed on three times. Graph indicates the mean ± standard deviation (*P<0.05, control vs. TNF-α; $^{\#}$P<0.05, TNF-α vs. DSGOST + TNF-α). DSGOST, Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang; TNF-α, tumor necrosis factor-α.

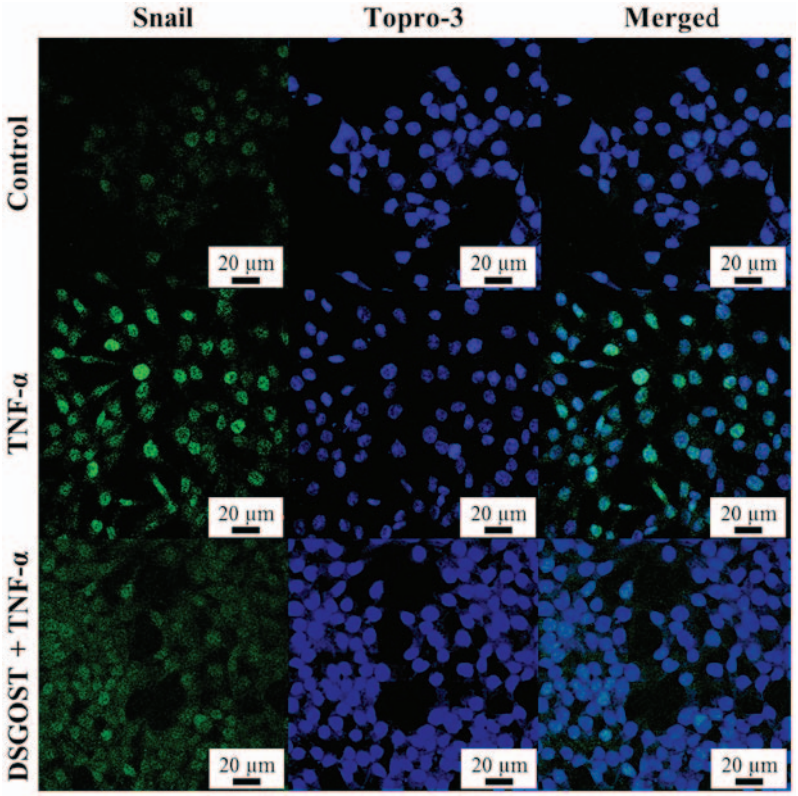

Figure 5. DSGOST blocks TNF-α-induced Snail translocation into nuclei. Immunocytochemistry. Cells were cultured on coverslip in 6-well plates and pretreated with DSGOST (50 μg/ml) for 2 h, followed by incubation with TNF-α (20 ng/ml) for 48 h. Cells were immunostained with Snail antibody (green). Nuclei was stained with TO-PRO-3 iodide (blue). DSGOST, Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang; TNF-α, tumor necrosis factor-α.

DSGOST inhibits TNF-α-induced nuclear translocation of Snail via inhibition of AKT/GSK-3β pathways in HCT116 cells. TNF-α activates either the expression or nuclear translocation of Snail protein, resulting in induction of EMT (16). As presented in Figs. 5 and 6, TNF-α promoted nuclear translocation of Snail protein, whereas pretreatment with DSGOST prevented this translocation. Thus, DSGOST inhibits TNF-α-mediated EMT by suppressing Snail translocation into nucleus. As Snail is tightly regulated by GSK-3β, which is phosphorylated and inactivated by AKT (16,47), it was also further examined whether DSGOST inhibits TNF-α-mediated AKT/GSK-3β signaling. While TNF-α induced the phosphorylation of both AKT and GSK-3β, DSGOST inhibited their phosphorylation (Fig. 6B). Therefore, data indicated that DSGOST inhibition of TNF-α-induced Snail nuclear translocation is mediated by blocking the AKT/GSK-3β pathways.

Discussion

While DSGOST has been researched for the treatment of various disorders, recent studies have suggested DSGOST as a novel candidate for treatment of cancer (42,43). Thus, we hypothesized that DSGOST would be an effective treatment for colorectal cancer. Since TNF-α activates the EMT process in colorectal cancer cells, resulting in tumor metastasis, the inhibitory effect of DSGOST treatment on TNF-α-mediated migration and invasion of HCT116 cells was investigated in the current study. The findings showed that DSGOST suppressed

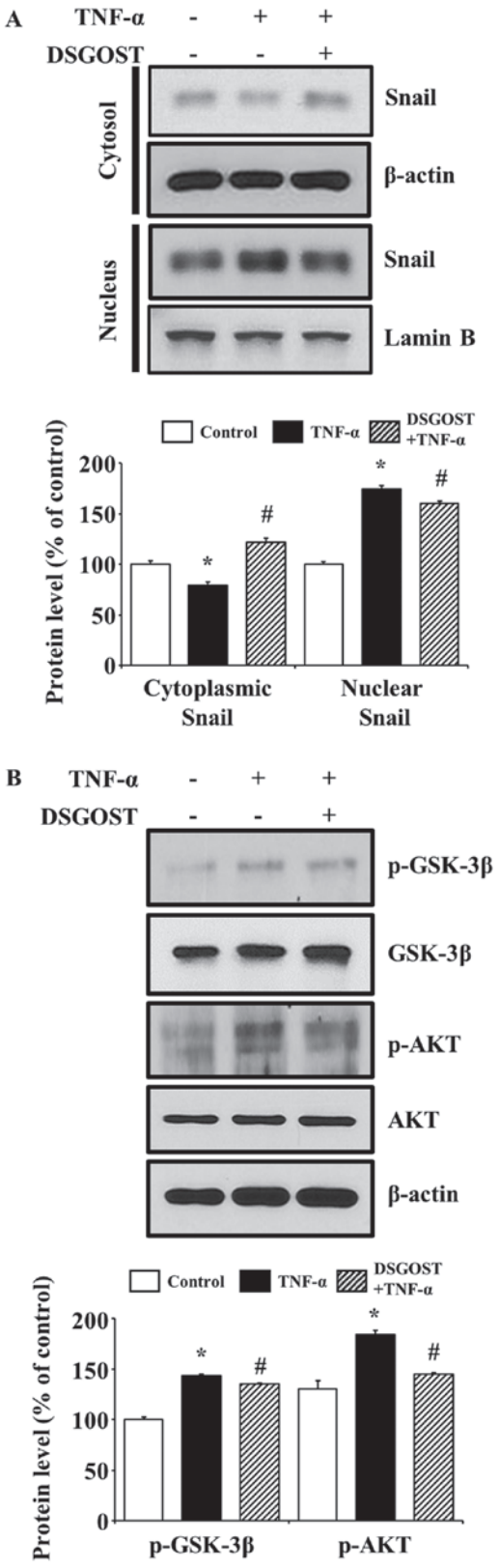

Figure 6. DSGOST blocks TNF-α-induced Snail translocation into nuclei by downregulating AKT/pathways in HCT116 cells. Representative images from western blotting. Cells were pretreated with DSGOST (50 μg/ml) for 2 h, followed by incubation with TNF-α (20 ng/ml) for 6 h. Expression of (A) Snail and (B) p-AKT and p-GSK-3β were analyzed by western blotting. β-actin and lamin B were used as cytosol and nucleus loading controls, respectively. The experiment was performed three times. Values are presented as the mean ± standard deviation (*P<0.05, control vs. TNF-α; #P<0.05, TNF-α vs. DSGOST + TNF-α). DSGOST, Danggui-Sayuk-Ga-Osuyu-Saenggang-Tang; TNF-α, tumor necrosis factor-α; AKT, protein kinase B; GSK-3β, glycogen synthase kinase-3β.

TNF-α-mediated migration and invasion. Furthermore, DSGOST also blocked TNF-α-mediated EMT via inhibiting AKT/GSK-3β/Snail signaling.

In the late 1970s, TNF-α was considered to be an anticancer agent by suppressing tumor cell proliferation (48,49). On the other hand, several *in vitro* studies have reported the tumor promoting effect of TNF-α (16,50,51). Furthermore, clinical studies have reported that TNF-α expression levels in serum and tissues were elevated in patients with tumors (52-55), suggesting that TNF-α may be a prognostic marker in patients with cancer. While the role of TNF-α in tumor malignancy is still controversial, the effect of recombinant TNF-α on EMT in HCT116 cells was investigated in the current study. The data showed that TNF-α treatment induced EMT phenotype. Furthermore, TNF-α also promoted migration and invasion. Although the cell type, TNF-α concentration and incubation period of TNF-α in the present study were limited, and the precise mechanisms of the effects remain to be demonstrated, the data provide evidence that TNF-α may contribute to tumor metastasis by inducing EMT.

The role of Snail in EMT has been validated in several cancer types (18-20,22). Poor clinical outcomes of patients with colorectal cancer are associated with Snail overexpression (22,23). Wang *et al* (16) reported that TNF-α-induced EMT is regulated by Snail protein in at least two types of colorectal cancer cells, HCT116 and Caco-2. Furthermore, the same study clearly showed that AKT/GSK-3β signaling is responsible for TNF-α-mediated Snail stabilization in those cells, while inhibitors of nuclear factor-κB (NF-κB), MAPK and p38 failed to abrogate TNF-α-induced Snail expression (16). Since Snail stabilization is tightly regulated by AKT/GSK-3β signaling pathways, DSGOST inhibition of AKT/GSK-3β signaling indicates that one of the potential biological mechanisms of DSGOST inhibition of TNF-α-mediated EMT in HCT116 colorectal cancer cells is via blocking of AKT/GSK-3β/Snail signaling.

The potential effectiveness of multi-target approaches in cancer therapy has been emphasized previously (56). Phytochemicals and herbal mixtures, which may act on multiple targets, have anticancer activity (57-66). Genistein, a dietary component with an anticancer effects against breast and prostate cancer, has been shown to regulate apoptosis, cell cycle, and NF-κB and AKT pathways (58). Proanthocyanidins have been reported to exhibit anticancer effects via antioxidative and anti-inflammatory properties (59). Ginsenoside, from ginseng plants, modulates the immune system, apoptosis and metastasis (57). Traditional Chinese medicine theory-based multi-herb prescriptions have benefits in the treatment of diseases including breast (60) and lung (61) cancer. SH003, a herbal prescription composed of *Astragalus membranaceus*, *Angelica gigas* and *Trichosanthes kirilowii* Maximowicz, exerts inhibitory effects against tumor growth by targeting apoptosis, autophagy and angiogenesis in several cancer types (62,64-66). A previous study reported the antitumor and anti-angiogenesis effects of DSGOST (42). The present study also demonstrated that DSGOST inhibits TNF-α-mediated invasion and migration of HCT116 cells. Since both invasive phenotype and tumor angiogenesis contribute to tumor metastasis and poor prognosis of patients with cancer, we hypothesize that the multi-target properties of DSGOST

may be useful for improving the prognosis of patients with cancer by dual inhibition of invasion and tumor angiogenesis. However, further *in vitro* and *in vivo* studies are required to demonstrate the anticancer property of DSGOST with a clear biological mechanism in several cancer types.

In conclusion, the data of the current study demonstrates, that DSGOST inhibits TNF-α-mediated EMT via suppressing AKT/GSK-3β/Snail pathways. Although the precise mechanism of DSGOST remains unclear, the results of the present study suggests that DSGOST is potentially beneficial for treating colorectal cancer. Furthermore, taken together with previous results indicating DSGOST inhibition of tumor angiogenesis, this suggests that DSGOST may be a novel multi-target herbal medicine for inhibition of tumor metastasis.

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