Research Article

Saussurea lappa Clarke-Derived Costunolide Prevents TNFα-Induced Breast Cancer Cell Migration and Invasion by Inhibiting NF-κB Activity

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Saussurea lappa Clarke (SLC) has been used as a traditional medicine in Korea, China, and Japan for the treatment of abdominal pain and tenesmus. Costunolide, a sesquiterpene lactone isolated from SLC, has diverse medicinal effects. However, the anticancer effects of costunolide are still unclear in breast cancer. In this study, we demonstrate that costunolide suppresses tumor growth and metastases of MDA-MB-231 highly metastatic human breast cancer cells via inhibiting TNFα-induced NF-κB activation.

Costunolide inhibited MDA-MB-231 tumor growth and metastases without affecting body weights in the in vivo mouse orthotopic tumor growth assays. In addition, costunolide inhibited in vitro TNFα-induced invasion and migration of MDA-MB-231 cells. Costunolide further suppressed TNFα-induced NF-κB signaling activation, resulting in a reduced expression of MMP-9, a well-known NF-κB-dependent gene to mediate breast cancer cell growth and metastases. Therefore, we conclude that SLC and its derivative costunolide suppress breast cancer growth and metastases by inhibiting TNFα-induced NF-κB activation, suggesting that costunolide as well as SLC may be promising anticancer drugs, especially for metastatic breast cancer.

1. Introduction

Most breast cancer is an epithelial tumor that develops from mammary gland tissue and the inner lining of milk ducts [1]. Metastatic breast cancer is not well cured by surgery, radiotherapy, and chemotherapy [2–4]. Cancer metastasis is the spread of tumor cells from an original site to distant parts of the body. This event consists of multistep processes, which includes tumor cell dissemination, extracellular matrix (ECM) degradation, tumor cell invasion into the ECM, angiogenesis, and secondary metastatic tumor growth [5–7]. Interestingly, primary tumors metastasize to specific organs; for example, aggressive breast cancers selectively metastasize to lung, bone, and brain tissue. This organ tropism seems to be related to different gene expression patterns [8–10].

TNFα is frequently detected in many human cancer tissues including breast, ovarian, and renal cancers [11, 12]. In addition, tumor cells producing TNFα are correlated with poor prognoses [11]. TNFα signaling activation through TNF receptor leads to promoting a recruitment of adaptor proteins and to activating signal cascades including NF-κB pathway [13, 14]. NF-κB regulates diverse physiological and pathological processes including development, metabolism, inflammation, and tissue homeostasis by regulating expression of various genes. In particular, genes regulated by NF-κB play roles such as development, proliferation, survival, and metastasis in cancer [15–17]. NF-κB protein bound to IκBα in the cytoplasm is maintained as an inactive state [18]. In response to NF-κB activation signals, IKKa/β complex is activated, resulting in phosphorylation of IκBα on serine residues 32 and 35. Phosphorylated IκBα is then ubiquitinated, and polyubiquitinated IκBα is degraded through proteosomal pathway. As a result, free NF-κB translocates from the cytoplasm to the nucleus and binds to specific DNA
sequences to regulate expression of target genes, which are related to tumor development and metastases [19–21]. The dried root of *Saussurea lappa* Clarke (SLC) has traditionally been used as an ingredient in Korea, China and Japan for the treatment of either abdominal pain or tenesmus. Several earlier studies indicated that the root of SLC has anti-cancer effect in gastric cancer cells [22, 23]. Costunolide (C_{15}H_{20}O_{3}), a sesquiterpene lactone that is a major component of the root of SLC [24] has been reported to have diverse effects such as anti-inflammatory [25], anti-viral [26], and -fungal [27] effects. Furthermore, costunolide affected various cancers including melanoma [28], intestinal [29], leukemia [30], prostate [31], and breast cancers [32]. While anti-cancer effects of either SLC or costunolide have been reported as mentioned before, antimetastatic effects of either SLC or costunolide on metastatic breast cancer are still poorly understood. In this study, we found that SLC and costunolide inhibit TNFα-mediated breast cancer cell migration and invasion by inhibiting NF-κB activation, thereby suggesting the antimetastatic property of costunolide using highly metastatic MDA-MB-231 breast cancer cells.

2. Materials and Methods

2.1. Reagents and Cell Lines. Costunolide (molecular weight of 232.32, purity > 99%, see Figure 2(a)) was purchased from Wako (Wako Pure Chemical Industries, Osaka, Japan). RPMI 1640, fetal bovine serum (FBS), antibiotic-antimycotic, and phosphate-buffered saline (PBS) were purchased from Gibco-BRL (Rockville, MD, USA). EZ-western detection kit was obtained from Daellab (Daellab service, Co., Seoul, Korea). TNFα was purchased from R&D systems (Minneapolis, MN, USA).

2.2. Preparation of *Saussurea lappa* Clarke (SLC) Extract *Saussurea lappa* Clarke was purchased from Omniherb (Gyeong Buk, Korea). The 100 g of root of SLC was dipped in 1L of 80% ethanol and sonicated by using an ultrasonicator (Branson, MO, USA) for 30 min at room temperature. The sonicated extract was filtered through a 0.22 mm filter and concentrated. The ethanol extracts were dried in a 42°C bath under reduced pressure using a vacuum pump evaporator (Eyela, Tokyo, Japan). The obtained concentrate was dissolved in DMSO to make a 28.5 g of concentrated extract was dissolved in DMSO to make a 28.5 g/ml solution. The stock solution was stored at −80°C until use.

2.3. Cell Migration and Invasion. Cell migration was measured by wound healing assays. Cells were seeded in 6-well plates and scratched with a 200 μl pipette tip. 24 hours after treatments with *Saussurea lappa* Clarke and costunolide, migrated cell numbers were counted. For invasion assay, cells were seeded in the upper chambers precoated with Matrigel and treated with SLC and costunolide. Low chambers were filled with 10% FBS or TNFα-contained medium, and invasive cells were stained with hematoxylin and eosin to visualize and count. All experiments were performed in triplicate and student's t-test was performed to determine statistics. P values below 0.05 and 0.001 were considered statistically significant. All data was represented as the mean ± standard deviation.

2.4. Immunofluorescence Assays. Immunofluorescence assays were used for p-NFκB nuclear translocation in MDA-MB-231 cell. After treatment with SLC and costunolide for 6 hours, cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.5% Triton X-100 for 10 min. The cells were washed with PBS, blocked with 5% FBS in PBS for 30 min, and then incubated with anti-p-NFκB antibody overnight at 4°C and with anti-Alexa Fluor-488 secondary antibody (Invitrogen, Eugene, Oregon, USA) for 1 hour. Phalloidin (Sigma) and TO-PRO-3 (Invitrogen) were used to contain F-actin and the nucleus, respectively. Images were obtained with Olympus FV10i Self-Contained Confocal Laser System. The object was 20x, and scale bars on the image indicate 50 μm.

2.5. Luciferase Assays. Cells were seeded in 24-well plates and NF-κB-luc plasmid (Stratagene, La Jolla, CA, USA) transfected in MDA-MB-231 cells by using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were treated with SLC and costunolide for 6 hours, and then the luciferase assays were done by using dual-luciferase reporter assay (Promega, Madison, WI, USA). All transfections included the RLT-K-Luc (kindly provided by Sang Hoon Kim) for transfection efficiency. All experiments were performed in triplicate and student's t-test was performed to determine statistics. P values below 0.05 and 0.001 were considered statistically significant. All data was represented as the mean ± standard deviation.

2.6. Western Blot. Total protein (30 μg) was separated by SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked, incubated overnight at 4°C with primary antibodies, washed with PBS-T (PBS with 0.1% Tween-20), and incubated with appropriate HRP-conjugated secondary antibodies at room temperature for 1 hour. Immunoreactive protein was developed using an EZ-western detection kit (Daellab service, Co., Seoul, Korea). Anti-MMP-9, -p-IKK, -IKK, -p-IκB, -IκB, -p-NF-κB, and -NF-κB were purchased from Cell Signaling (Danvers, MA, USA). Anti-Tubulin was purchased from Sigma (Louis, MO, USA).

2.7. RNA Extraction and RT-PCR. Cellular total RNA was extracted with TRIzol reagent (Invitrogen). The RNA concentration and purity were measured using a spectrophotometer. cDNA was synthesized from total RNA (1 μg) by reverse transcription. The primer sequences and product size were as follows: MMP-9 (262 bp) forward: 5'-CACCTGTCCACCCCTCAGAGC-3', reverse: 5'-GGCACCTGTCCGGCGATAAGG-3', GAPDH (300 bp) forward: 5'-CGTCTTACACCCCAAGG-3', reverse: 5'-CGGCCATCACGCCACAGTTT-3'. The products were checked by agarose electrophoresis and analyzed using the Bio-Rad gel documentation system (BioRad, Hercules, CA, USA).

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Figure 1: SLC inhibits TNFα-induced MDA-MB-231 cell migration and invasion by inhibiting NF-κB activation. (a) Cell migration was measured by wound healing assay. MDA-MB-231 cells were seeded and scratched, pretreated with SLC for 1 hour, and then exposed to TNFα for 24 hours. Cell migration was determined by counting cell numbers migrated from the wound healing region. * P < 0.05. (b) MDA-MB-231 cells were seeded on the upper chambers and pretreated with SLC for 1 hour and then exposed to TNFα for 24 hours. Invading cells were stained with hematoxylin and eosin, and the cell numbers were measured. ** P < 0.001. (c) MDA-MB-231 cells were pretreated with SLC for 1 hour, then exposed to TNFα for 6 hours, and stained with p-NF-κB antibody. Phalloidin and TO-PRO-3 were for staining F-actin and the nucleus, respectively. The object was 20x, and scale bars on the image indicate 50 μm. (d) MDA-MB-231 cells were transfected with the NF-κB-dependent luciferase reporter, pretreated with SLC for 1 hour, and then exposed to TNFα for 6 hours. Luciferase assays were done by using dual-luciferase reporter assay. All transfections included the RLTK-Luc for transfection efficiency. * P < 0.05. (e) MDA-MB-231 cells were pretreated with SLC for 1 hour and then exposed to TNFα for 24 hours. MMP-9 protein was measured by western blotting. Tubulin was used for the loading control.
2.8. Gelatin Zymography Assay. Conditioned medium was harvested, concentrated, mixed with nonreducing sample buffer, and separated by SDS-PAGE electrophoresis containing 0.1% gelatin. After electrophoresis, the gel was washed with washing buffer (2.5% Triton X-100 in reaction buffer) and then incubated in reaction buffer (50 mM Tris-HCl, 5 mM CaCl$_2$, 1$\mu$M ZnCl$_2$, and pH 7.4) for 18 h at 37$^\circ$C. To visualize, the gel was stained with Coomassie brilliant blue R-250 and destained in 50% methanol, 40% distilled water, and 10% acetic acid.

2.9. In Vivo Studies. Animal studies were approved by Kyung Hee University Institutional Animal Care and Use Committee (KHU-IACUC). Six-week-old nude (Nu/Nu) mice were
Figure 3: Costunolide inhibits TNF-α-induced NF-κB pathway in MDA-MB-231 cells. (a) MDA-MB-231 cell were treated with costunolide for indicated time periods. Whole lysates were analyzed by western blotting with anti-pIKK, -IKK, -pIκBα, -IκBα, and Tubulin. (b) Cells were fractionated into cytoplasmic and nuclear compartment and western blotting for NF-κB. Tubulin and LaminB were used as loading control. (c) MDA-MB-231 cells were treated with TNF-α and cotreated with TNF-α and costunolide for 15 to 30 min. Whole lysates were analyzed by western blotting with anti-pIKK, -IKK, -IκBα, and Tubulin. (d) MDA-MB-231 cells were pretreated with costunolide for 1 hour, then exposed to TNF-α for 6 hours, and stained with p-NF-κB antibody. Phalloidin and TO-PRO-3 were for staining with F-actin and nucleus, respectively. The object was 20x, and the scale bars on the image indicate 50 μm.

2.10. Immunohistochemistry. Tumors were fixed with 4% formaldehyde for further analyses. Tumor tissues were embedded in paraffin, dissected with 5 μm, and deparaffinized in 100% xylene and ethanol series (100%, 95%, and 70%). Heat-induced antigen retrieval was 10mM sodium citrate buffer for 5min. Endogenous peroxidase was blocked with peroxidase blocking reagent containing 3.5% hydrogen peroxide. Nonspecific antigen was blocked with serum containing PBS followed by incubation with human ki-67 (5 μg/mL) (Abcam, MA, USA) and MMP-9 antibody (1:100) (Cell Signaling, Beverly, MA, USA) overnight at 4°C. It was incubated with biotin-labeled rabbit antibody for 1 hour at room temperature and incubated with ABC and DAB buffer substrate. Sections were visualized with DAB and hematoxylin, mounted, and analyzed using a bright field microscope. The object was 20x, and the scale bars on the image indicate 10 μm.

2.11. Statistics. Data were shown as the means and standard deviations. P values less than 0.05 in the two-tailed Student’s t-test or one-way ANOVA were considered statistically significant.

3. Results
3.1. Saussurea lappa Clarke Suppresses TNFα-Induced Breast Cancer Cell Migration and Invasion via an Inhibition of NF-κB
Activation. Because TNFα expression is abundant in tumor microenvironment, and its expression is correlated with poor prognoses [14, 15], we investigate effects of *Saussurea lappa* Clarke (SLC) on highly metastatic MDA-MB-231 cells. In normal culture condition, SLC treatment (50 μg/mL) inhibited MDA-MB-231 cell migration (data not shown). Next, TNFα increased the MDA-MB-231 cells migration compared to nontreated cells, and 50 μg/mL of SLC suppressed TNFα-induced MDA-MB-231 cells migration by approximately 63% (Figure 1(a)). In addition, 50 μg/mL of SLC significantly inhibited TNFα-induced cell invasion (Figure 1(b)). Next, to clarify the mechanism of SLC to inhibit cell migration and invasion, we performed the immunofluorescence assays to examine NF-κB pathway. As shown in Figure 1(c), TNFα induced nuclear translocation of phosphorylated NF-κB, which was blocked by SLC. In the luciferase assay, SLC inhibited TNFα-induced NF-κB-dependent transcriptional activity (Figure 1(d)). Accordingly, 50 μg/mL of SLC suppressed TNFα-induced mRNA and protein expression of MMP-9 that is well known as NF-κB-dependent gene (Figure 1(e)). Thus, our data indicate that SLC inhibits TNFα-induced highly metastatic MDA-MB-231 human breast cancer cell migration, invasion, and NF-κB activation.

3.2. *Saussurea lappa* Clarke-Derived Costunolide Suppresses Cell Migration and Invasion in Breast Cancer Cells. Since Costunolide (C_{13}H_{20}O_{2}) is a major component of SLC [22], we examined whether SLC-derived costunolide inhibits metastatic properties of breast cancer cells. Costunolide (20 μM) blocked cells migration in normal serum conditions by approximately 45% (Figure 2(a)). Next, to examine costunolide effect on cells invasion, MDA-MB-231 cells were seeded in the upper chambers precoated with matrigel and treated with costunolide in 1% serum contained media, and the low chambers were filled with 10% serum contained media. As shown in Figure 2(b), a treatment of breast cancer cells with 20 μM of costunolide for 24 hours reduced cells invasion.

Next we performed experiments to determine whether costunolide inhibits TNFα-induced cells migration and invasion. 20 μM of costunolide suppressed TNFα-induced MDA-MB-231 cell migration by approximately 62% (Figure 2(c)). In addition, whereas TNFα increased an invasiveness of MDA-MD-231 cells by approximately five folds, costunolide significantly inhibited TNFα-induced cell invasion by approximately five folds (Figure 2(d)).

3.3. Costunolide Inhibits NF-κB Pathway in Breast Cancer Cells. Next we examined costunolide effect on NF-κB signaling pathway in MDA-MB-231 cells. As shown in Figure 3(a), costunolide inhibited phosphorylation of IKK and IκBα, resulting in blocking IκBα degradation in a time-dependent manner. Accordingly, a treatment of the MDA-MB-231 cells with costunolide inhibited the nuclear translocation of p65 NF-κB subunit (Figure 3(b)).

Next, in order to examine whether costunolide suppresses TNFα-induced NF-κB pathway, we stimulated cells with TNFα for 15 to 30 min in the presence or absence of costunolide. As shown in Figure 3(c), TNFα-induced IKK phosphorylation was prolonged until 30 min, which was blocked by costunolide. Furthermore, while TNFα induced IκB degradation, costunolide slowly recovered IκBα expression at 15 min.

To confirm costunolide suppression of NF-κB nuclear translocation, we performed immunofluorescence assay using the anti-pNF-κB antibody. As shown in Figure 3(d),...
Figure 5: Costunolide inhibits orthotopically tumor growth and metastasis. (a) \(1 \times 10^6\) MDA-MB-231 cells were orthotopically injected in nude mice (\(n = 5\)/group). Costunolide was injected into the mammary fat pad and repeated every three days for 30 days. Tumor volumes were measured using calipers. Tumor volume (cubic millimeters) = width\(^2\) \times\) length/2. (b) Body weight measured three times a week. (c) Tumor tissues were stained with hematoxylin and eosin. Photo images were taken at 20x magnification. (d) Tumor tissues were stained with anti-ki-67 antibody. (e) Tumor tissues were stained with anti-MMP-9 antibody. The object was 20x, and, scale bars on the image indicate 10\(\mu\)m.
NF-κB was observed in the cytosol of the cells treated with costunolide. Thus, our data indicate that SLC-derived costunolide inhibits NF-κB pathway.

### 3.4. Costunolide Inhibits NF-κB Transcriptional Activity and MMP-9.

To confirm the inhibition of NF-κB pathway by costunolide, we performed the transcriptional activation of NF-κB by using the luciferase assay. As shown in Figure 4(a), costunolide reduced TNFα-induced NF-κB transcriptional activation by 5-fold in MDA-MB-231 cells. We next examined whether costunolide affects upstream of IKK in TNFα-induced NF-κB pathway, MDA-MB-231 cells were cotransfected with NF-κB reporter gene and TNFRI and then cultured in the presence or absence of costunolide. Costunolide reduced TNFRI-induced NF-κB transcriptional activity by approximately 2.5-fold in MDA-MB-231 cells (Figure 4(a)).

It is known that MMP-9 is regulated by NF-κB, and the promoter region of MMP-9 gene contains binding sites for NF-κB. Thus, we examined whether costunolide inhibits MMP-9; we checked MMP-9 by using RT-PCR, western blot, and zymography. As shown in Figure 4(b), Costunolide inhibited TNFα-induced MMP-9 mRNA, protein, and enzyme activity, when cells were treated with costunolide for 24 hours.

### 3.5. Costunolide Inhibits Tumor Growth and Metastasis.

To examine costunolide effect on breast cancer growth and metastases in vivo, MDA-MB-231 cells were orthotopically injected into the 4th mammary fat fads. A day after tumor cell injection, costunolide at 20 μM was injected into the mammary fat fad three times a week for 30 days. In addition, tumor volume and body weight of mice were also measured three times a week. As shown in Figure 5(a), costunolide reduced tumor volume (P = 0.007628), and no significant weight loss in mice treated with either costunolide or vehicle was observed (Figure 5(b)). When tumor tissues were stained with hematoxylin and eosiin, we found that tumor cohort treated with costunolide compared to that with control was well differentiated (Figure 1(c)). In addition, tumor and organ (lung and liver) tissues were stained with anti-ki-67. Costunolide, compared to control reduced ki-67 positive cell in tumor, lung, and liver (Figure 1(d)). When tumor tissues were stained with MMP-9 antibody, costunolide inhibited a number of MMP-9 positive cells (Figure 1(e)). Thus, our data indicate that costunolide inhibits tumor growth and metastasis.

### 4. Discussion

TNFα-induced NF-κB pathway is a well-known molecular target for cancer therapy. Tumor cells released NF-κB-dependent MMPs by NF-κB-mediated TNFα production of immune cells in tumor microenvironment [33]. In this study, we found that *Saussurea lappa* Clarke-derived costunolide suppressed TNFα-induced MDA-MB-231 breast cancer cell migration and invasion by inhibiting NF-κB activity (Figure 5(c)). Thus, SLC as well as costunolide appears to be useful for treating highly metastatic breast cancer.

Matrix metalloproteinases (MMPs), a family of zinc-dependent endoproteinase is necessary for extracellular matrix (ECM) degradation among metastasis process. MMPs also affect many biological processes such as normal tissue remodeling, wound healing, angiogenesis, embryogenesis, and many diseases including cancer, atheroma, and arthritis [34]. MMP-9 is frequently overexpressed in many cancers and correlates with poor prognosis and survival in cancer patients [35–37]. In addition, MMP-9 is important for tumor metastasis by cleaving basement membranes, which allows migratory phenotype cells to be more invasive and motile [38–40]. MMP-9 is regulated by stimulators (phorbol 12-myristate 13-acetate, PMA; transforming necrosis factor alpha, TNFα; growth factor, UV; and stress) and transcription factors (nuclear factor kappaB; NF-κB and activator protein-1; AP-1) [39, 40]. In addition, MMP-9 is important for tumor metastasis by cleaving basement membranes, which allows migratory phenotype cells to be more invasive and motile. Thus, TNFα-induced MMP-9 expression via NF-κB is important for cancer growth and metastasis. In our study, TNFα-induced cell migration and invasion were inhibited by either SLC or costunolide. SLC and costunolide suppressed TNFα-induced NF-κB translocation to nucleus and transcriptional activity. In addition, costunolide specifically inhibited IKK phosphorylation and IkBα degradation. Those inhibitions further reduced NF-κB-dependent MMP-9 expression. As a result, costunolide suppressed *in vivo* tumor growth and metastasis.

This study concludes that (a) SLC suppresses TNFα-induced MDA-MB-231 cell migration and invasion by
inhibiting NF-κB-dependent MMP-9 expression, (b) SLC-derived costunolide inhibited serum or TNFα-induced MDA-MB-231 cell migration and invasion, (c) costunolide inhibited TNFα-induced NF-κB translocation resulting from the suppression phosphorylation and IkBα degradation (d) costunolide blocked TNFα-induced NF-κB transcription activity and TNFα-induced MMP-9 expression, and (e) costunolide decreased in vivo tumor growth and metastasis without weight loss (Figure 6). In sum, we provide evidence that the anti-cancer effect of both SLC and its component costunolide on MDA-MB-231 result from the inhibition of TNFα-induced NF-κB activation. Therefore, SLC-derived costunolide could be useful for treating highly metastatic breast cancer growth and metastases.

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