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Salvia miltiorrhiza extract inhibits TPA-induced MMP-9 expression and invasion through the MAPK/AP-1 signaling pathway in human breast cancer MCF-7 cells

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Abstract. Cancer cell invasion is crucial for metastasis. A major factor in the capacity of cancer cell invasion is the activation of matrix metalloproteinase-9 (MMP-9), which degrades the extracellular matrix. *Salvia miltiorrhiza* has been used as a promotion for blood circulation to remove blood stasis. Numerous previous studies have demonstrated that *S. miltiorrhiza* extracts (SME) decrease lipid levels and inhibit inflammation. However, the mechanism behind the effect of SME on breast cancer invasion has not been identified. The inhibitory effects of SME on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 expression were assessed using western blotting, reverse transcription-quantitative polymerase chain reaction and zymography assays. MMP-9 upstream signal proteins, including mitogen-activated protein kinases and activator protein 1 (AP-1) were also investigated. Cell invasion was assessed using a matrigel invasion assay. The present study demonstrated the inhibitory effects of the SME ethanol solution on MMP-9 expression and cell invasion in TPA-treated MCF-7 breast cancer cells. SME suppressed TPA-induced MMP-9 expression and MCF-7 cell invasion by blocking the transcriptional activation of AP-1. SME may possess therapeutic potential for inhibiting breast cancer cell invasiveness.

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Introduction

Salvia miltiorrhiza roots have traditionally been used for the treatment of gynecological disorders in Chinese medicine (1). *S. miltiorrhiza* extracts (SME) have been suggested to decrease lipid levels and inhibit inflammation (2-4), and have demonstrated cytotoxic effects on cells derived from various types of human cancer, including the lung, colon and pancreas (5-7). Previously, Tanshinone II-A, extracted from *S. miltiorrhiza,* inhibited invasion and metastasis of human carcinoma cells (8,9). More notably, certain studies have demonstrated that SME exhibits antitumor effects in breast cancer cells (10,11). However, the inhibitory effect of SME on breast cancer invasion was not known.

Breast cancer is a malignant tumor with the ability to spread beyond the breast tissue. The patients with breast cancer exhibited a high mortality rate (12), primarily caused by metastasis and invasion of cancer cells. Consequently, the primary approach for breast cancer treatment has been the development of effective anti-metastasis and anti-invasion drugs (13,14).

Invasion and metastasis of cancer cells are characterized by the degradation of the extracellular matrix (ECM) by proteases secreted from cancer cells (15,16). A total of ~24 types of human matrix metalloproteinases (MMPs) are synthesized and secreted from cells (17,18). Of those, MMP-9 is well known as a key enzyme that regulates breast cancer cell invasion (19). High concentrations of MMP-9 were identified in breast cancer tissue compared with those in normal breast tissue (20). A variety of stimulators such as tissue plasminogen activator (TPA), tumor necrosis factor-α and epidermal growth factor may increase the expression of MMP-9 in cancer cells (21-24).

Cytokine and TPA-mediated MMP-9 expression is controlled by the transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) in cancer cells (19,25,26). AP-1 is the collective name for referring to dimeric transcription factor proteins composed of Jun proto-oncogene, Fos proto-oncogene and the activated transcription factor protein families (27). Dimerized Jun/Fos bind to specific AP-1 sites of DNA, but their functions depend on cell type and activating agent (28-31). NF-κB forms a complex with its cellular protein inhibitor (inhibitory κBα; IκBα) and thereby remains inactive in the cytoplasm (32). When TPA phosphorylates IκB this complex dissociates, releasing NF-κB, which then translocates to the nucleus where it interacts with DNA binding sites on the MMP-9 promoter (33,34). Previously, Hsieh and Wu (28) suggested that *S. miltiorrhiza* roots have capacity to regulate NF-κB.

In the present study, it has been hypothesized that SME may exhibit anticancer properties through the inhibition of cell invasion. Therefore, the molecular mechanism by which SME affects the invasiveness of the breast cancer MCF-7 cell line was investigated. SME reduced TPA-induced cell invasion via the mitogen-activated protein kinase (MAPK)/AP-1 signaling pathways, and decreased MMP-9 expression was associated with the extent of the inhibition of breast cancer cell invasion.

Materials and methods

Cell and reagents. MCF-7 cells were acquired from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% antibiotics (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin B) and 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37˚C in an incubator with 5% CO_2 saturation. DAPI, TPA, dimethyl sulfoxide and anti-β-actin antibody were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies against MMP-9 (catalog no. 12759), inhibitory κ B kinase (IKK)α (catalog no. 2682), IKKβ (catalog no. 2678), proliferating cell nuclear antigen (PCNA; catalog no. 7907), IκBα (catalog no. 371), transcription factor p65 (catalog no. 372), and horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (catalog no. 2004, 2005) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against c-Jun N-terminal kinase (JNK; catalog no. 9252), p38 (catalog no. 9212), extracellular signal-regulated kinase (ERK; catalog no. 9102), phosphorylated (p)-JNK (catalog no. 9252), p-p38 (catalog no. 9211), p-ERK (catalog no. 9101), p-c-Jun (catalog no. 9261), p-IκBα (catalog no. 2859) and p-IKKα/β (catalog no. 2697) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat anti-rabbit Alexa Fluor 568 [IgG heavy and light chains (H&L)] (catalog no. A-11036) was obtained from Invitrogen (Thermo Fisher Scientific, Inc.).

Preparation of S. miltiorrhiza extract. Dried roots of *S. miltiorrhiza* Bunge were purchased from Kwangmyungdang Medicinal Herbs Co., Ltd. (Ulsan, Korea) and authenticated by Professor Guem-San Lee (Department of Herbology, Wonkwang University School of Korean Medicine, Iksan, Korea): A voucher specimen (WKU120302-SM201406A) was deposited at the Department of Herbology, College of Korean Medicine, Wonkwang University (Iksan, Korea). *S. miltiorrhiza Radix* powder (50 g) was extracted by sonication in 500 ml of 70% aqueous ethanol for 2 h, and then filtered through paper. This procedure was repeated twice. Liquid from the extracted solution was evaporated under 40 mmHg by rotary evaporator, and the resulting product was freeze-dried. The final extraction yield was 6.54% (w/w).

Determination of cell viability. MCF-7 cells were seeded on 96-well plates ($1.5x10^4$ cells/well) and treated with 1, 5, 10, 25 and 50 μg/ml SME for 24 h at 37˚C. Then, 100 μl EZ-CyTox assay reagent (Daeil Lab Service Co., Ltd., Seoul, Republic of Korea) was added 100 times diluted to the plate wells. Next, the cells were incubated for 30 min at 37˚C prior to measuring the absorbance with a 540-nm filter in an ELISA reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Zymography. To analyze the proteolytic activity of MMP-9, zymography was performed as previously described (35). Areas of gelatinase activity were detected as a white zone in a dark blue field.

Western blot analysis. Total protein extracts were prepared using an ice-cold M-PER Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Proteins were quantified using a BioSpec-nano Micro-volume Spectrophotometer (Shimadzu; Columbia, USA). For each lane, 20 μg protein was used. The protein samples were separated using SDS-PAGE (10% gel) and transferred to Hybond™ polyvinylidene fluoride membranes. The membranes were blocked with 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA) or 5% skim milk for 2 h at 4˚C. Membranes were then incubated overnight at 4˚C with primary antibodies. β-actin (catalog no. A2228) was purchased from Sigma-Aldrich (Merck KGaA). MMP-9 (catalog no. 12759), IKKα (catalog no. 2682), IKKβ (catalog no. 2678), PCNA (catalog no. 7907), IκBα (catalog no. 371) and p65 (catalog no. 372) were obtained from Santa Cruz Biotechnology, Inc. JNK (catalog no. 9252), p38 (catalog no. 9212), ERK (catalog no. 9102), phosphorylated (p)-JNK (catalog no. 9252), p-p38 (catalog no. 9211), p-ERK (catalog no. 9101), p-c-Jun (catalog no. 9261), p-IκBα (catalog no. 2859) and p-IKKα/β (catalog no. 2697) were purchased from Cell Signaling Technology, Inc. All Antibodies used were diluted at 1:2,000. Protein expression levels were measured by Mini HD6 image analyzer using Alliance 1D software (UVItec, Cambridge, UK) with Immobilon™ Western Chemilumi nescent HRP Substrate (enhanced chemiluminescence) kit (EMD Millipore, Billerica, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA isolation from cells was performed using the FastPure™ RNA kit (Takara Bio, Inc., Otsu, Japan). Complementary DNA was synthesized using a PrimeScript™ RT Reagent kit (Takara Bio, Inc.). qPCR analysis was performed using the StepOnePlus™ Real-Time PCR System and SYBR Green (both Applied Biosystems; Thermo Fisher Scientific, Inc.) to determine mRNA levels. The thermocycling conditions were as follows: 50˚C for 2 min and 95˚C for 10 min,

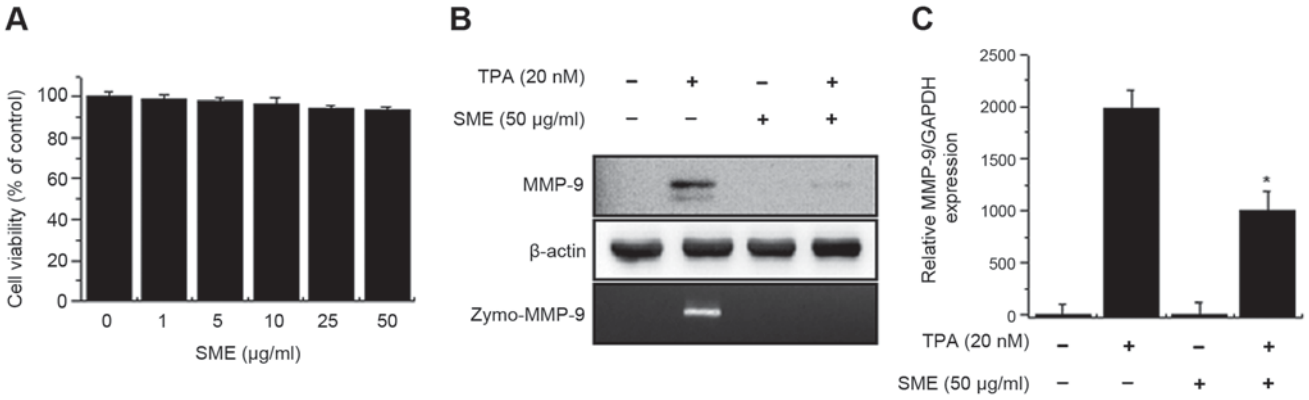

Figure 1. SME inhibits TPA-induced MMP-9 expression in MCF-7 cells. (A) SME was treated at various concentrations (0, 1, 5, 10, 25 and 50 μg/ml) for 24 h. An MTT assay was used to measure cell viability. (B) MCF-7 cells in a monolayer were treated with the indicated SME concentrations in the presence of TPA for 24 h. Cell lysates were analyzed by western blotting with an anti-MMP-9 antibody. The blot was re-probed with an anti-β-actin antibody to confirm equal loading amounts. Conditioned medium was prepared and used for gelatin zymography. (C) MMP-9 mRNA levels were analyzed by quantitative polymerase chain reaction using GAPDH mRNA as an internal control. Each value represents the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. TPA only. SME, *Salvia miltiorrhiza* extract; TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP-9, matrix metalloproteinase-9; Zymo, zymography.

followed by 50 cycles of 95˚C for 15 sec and 60˚C for 1 min. The primers used were as follows: MMP-9 (NM 004994) sense, 5'-CCTGGAGACCTGAGAACCAATCT-3' and antisense, 5'-CCACCCGA GTGTAACCATAGC-3'; and GAPDH (NM 002046) sense, 5'-ATGGAAATCCCATCACCATCT T-3' and antisense, 5'-CGCCCCACTTGATTTTGG-3'. The mRNA levels were normalized to the GAPDH housekeeping gene expression levels. The method of quantification was $2^{-\Delta\Delta Cq}$ method (36).

Automated image acquisition and processing. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min, and then washed three times with cold PBS. Next, the cells were incubated for 45 min at room temperature with blocking buffer to prevent nonspecific antibody binding. Then, anti-p-c-Jun (red) antibodies (dilution, 1:1,000) were added and the cells were incubated for 24 h at 4˚C. Subsequently, the cells were washed three times and then incubated for 1 h at room temperature with DAPI (blue) and 1:1,000 diluted goat anti-rabbit Alexa Fluor 568 (IgG H&L) in 0.1% triton X-100 for nuclear and p-c-Jun staining. Images were captured by an ArrayScan™ VTI system using Cellomics VHCS: View Software, version 1.6.30 (Cellomics, Inc., Pittsburgh, PA, USA).

Cytoplasmic and nuclear fractionation. MCF-7 cells ($2x10^6$) were treated with SME and/or TPA for 4 h at 37˚C. Following this, the cells were centrifuged at 1,500 x g for 5 min at 4˚C. Nuclear and cytoplasmic extracts were separated by using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Invasion and migration assays. The invasion (35) and migration assays (37) were performed as previously described. Cell growth medium (0.5 ml) with cells was added to the upper chamber, and medium with TPA alone or with SME was added to bottom wells.

Statistical analysis. The data were analyzed by one-way analysis of variance and Duncan's multiple range tests using SAS software, version 9.1; (SAS Institute Inc., Cary, NC, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of SME on TPA-induced MMP-9 expression and secretion in MCF-7 cells. The treatment of cells with SME did not affect cell viability until concentrations reached 50 μg/ml (Fig. 1A). On that basis, an SME concentration of 50 μg/ml was used for subsequent experiments. In order to investigate the effect of SME on the expression of MMP-9, cells were pretreated with SME for 1 h and then incubated with TPA for 24 h at 37˚C. TPA-mediated expression/secretion of MMP-9 was decreased by pre-treatment with SME (Fig. 1B). qPCR analysis revealed that SME significantly reduced the levels of MMP-9 mRNA expression induced by TPA (Fig. 1C).

Effects of SME on the TPA-mediated MAPK signaling pathway. To confirm whether SME is involved in MAPK activation, western blot analysis was performed. Exposure to TPA for 15 or 30 min markedly elevated the phosphorylation levels of p38/JNK/ERK, and pre-treatment with SME markedly decreased them (Fig. 2).

Effects of SME on TPA-induced NF-κB and AP-1 activation. To confirm this TPA-induced activation of NF-κB (p65/p50) and AP-1 (c-Jun/c-Fos), immunofluorescence and western blotting were used. TPA induced substantial p-c-Jun expression, whereas pre-treatment with SME blocked it (Fig. 3A and B). Pre-treatment with SME did not affect the translocation of p65 into the nucleus by TPA (Fig. 3B). Additionally, SME did not affect the levels of p-IKKαβ, p-IκBα or IκBα induced by TPA (Fig. 3C). These results suggested that SME inhibited TPA-induced MMP-9 expression by blocking AP-1 activation.

Effect of SME on TPA-induced MCF-7 cell invasion/migration in vitro. To demonstrate whether SME inhibits the invasion and migration abilities of MCF-7 cells, *in vitro* invasion and migration assays were performed. Subsequent to treatment of

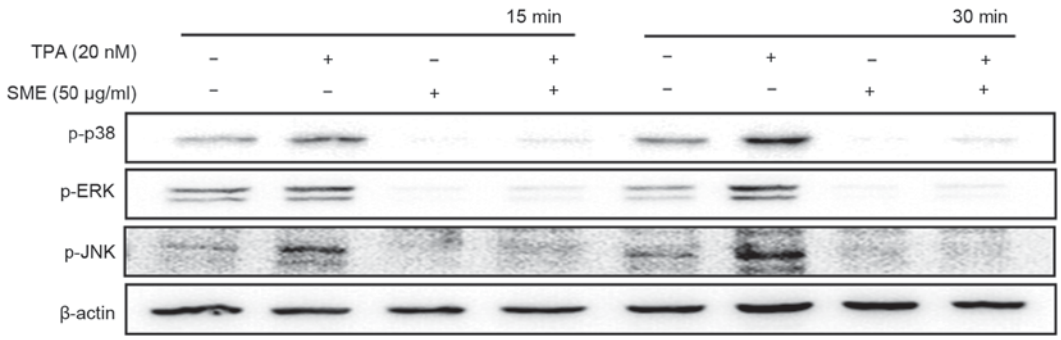

Figure 2. SME inhibits TPA-induced mitogen-activated protein kinase activation in MCF-7 cells. Cells were pretreated with SME for 1 h and then stimulated with TPA for 15 and 30 min. Western blotting for p-p38, p-ERK and p-JNK was performed as described in the Materials and methods section. SME, *Salvia miltiorrhiza* extract; TPA, 12-O-tetradecanoylphorbol-13-acetate; p, phosphorylated; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase.

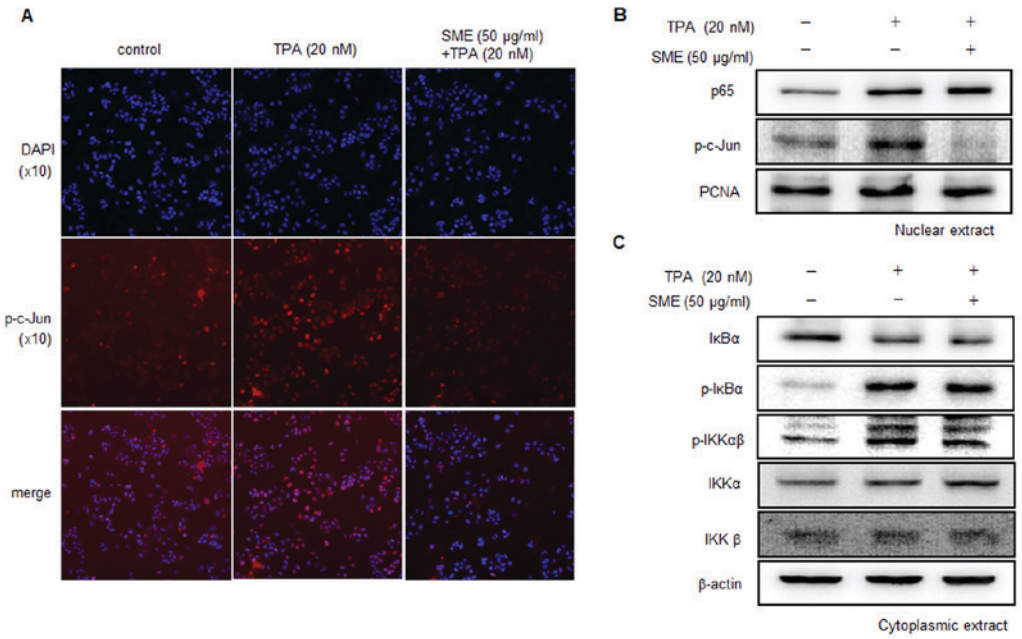

Figure 3. SME suppresses TPA-induced transcriptional activation of MMP-9 by inhibiting AP-1. (A) Cells were pretreated with SME in the presence of TPA. Following 4 h of incubation, the expression of p-c-Jun in the nucleus was assessed by immunofluorescence analysis (magnification, x10). (B) Cells were pretreated with SME for 1 h and then exposed to TPA for 4 h. Western blotting was performed to determine the nuclear levels of p65 and activator protein 1 (p-c-Jun) subunits. PCNA was used as loading control. (C) Cells were pretreated with SME for 1 h and then stimulated with TPA for 4 h. Western blotting was performed to determine the cytoplasmic levels of IκBα, p-IκBα, IKKα, IKKβ and p-IKKαβ. SME, *Salvia miltiorrhiza* extract; TPA, 12-O-tetradecanoylphorbol-13-acetate; p, phosphorylated; PCNA, proliferating cell nuclear antigen; IKK, inhibitory κ B kinase; IκBα, inhibitory κ B α; p65, nuclear factor-κB p65 subunit.

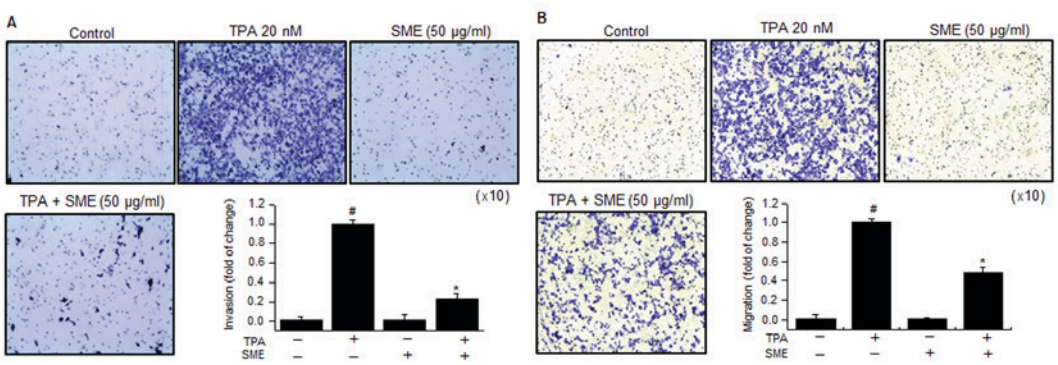

Figure 4. SME inhibits TPA-induced invasion and migration in MCF-7 cells. Change in (A) invasion and (B) migration in MCF-7 cells (magnification, x10). Each value represents the mean ± standard error of the mean of three independent experiments. $^{\#}$P<0.05 vs. control, *P<0.05 vs. TPA only. SME, *Salvia miltiorrhiza* extract; TPA, 12-O-tetradecanoylphorbol-13-acetate.

the cells with TPA, the levels of cell invasion and migration were significantly increased. However, the invasion and migration abilities of cells treated with 50 μg/ml SME and TPA was considerably lower compared with that of cells treated with TPA (Fig. 4).

Discussion

In the present study, it was revealed that SME blocked TPA-induced cell invasion and MMP-9 expression through inhibition of the MAPK/AP-1 signaling pathway. Concurrently, SME did not affect NF-κB signaling. These results suggest that SME blocks cell invasion by suppressing MMP-9 expression, mediated by the activity of the MAPK/AP-1 signaling pathway, in MCF-7 breast cancer cells.

The ECM provides biochemical and physical barriers to the proliferation and spread of cancer cells, and cancer cell invasion requires its degradation (38,39). Basement membranes consist of various proteins such as collagen, gelatin and other ECM components that are degraded by MMPs. The production of MMPs is tightly controlled by non-specific protease inhibitors or tissue-specific MMP inhibitors (40-43). When this regulation becomes impaired, MMP expression in cancer cells increases abnormally in response to various factors (21-24). In previous studies, this expression was increased by growth factors, inflammatory cytokines, phorbol esters and different signaling pathways in breast cancer cells (44-46). MMP-9 decomposes gelatin and accelerates breast cancer cell invasion (47). Therefore, regulation of MMP-9 expression may be important in treatment strategies for tumor metastasis. Songyou Yin, an herbal compound including *S. miltiorrhiza*, inhibits tumor invasion via the downregulation of MMPs in hepatocellular carcinoma (48). A previous study reported that SME extracts inhibit the proliferation of MCF-7 cells at a concentration of 5 μg/ml (49). However, the present study confirmed that the inhibitory effect of SME on cell invasion at a concentration of 50 μg/ml does not affect the toxicity of MCF-7 cells. The difference in the concentration of SME for cytotoxicity is considered to be caused by the change of the components depending on the difference of purification processes. The present study suggested that SME inhibited TPA-induced expression of MMP-9 in MCF-7 breast cancer cells (Fig. 1). These results demonstrated that SME has the ability to regulate MMPs.

It has been indicated in several previous studies that the active component of *S. miltiorrhiza* modulates the MAPK families, including ERK, JNK and p38 kinase. MAPK is involved in cell invasion and protease secretion processes (50,51). In MCF-7 breast cancer cells, the MAPK signaling pathway is activated by protein phosphorylation, which in turn is modulated by TPA (22,52). Previous studies have implicated MAPK in cancer metastasis (19,21,45). In the present study, SME suppressed the TPA-induced phosphorylation of ERK, JNK and p38 in MCF-7 breast cancer cells (Fig. 2).

MAPK families serve an important role in the activation of AP-1. ERK enhances AP-1 activation through c-Fos, whereas JNK leads to the phosphorylation of c-Jun (53). The overexpression of c-Jun enhances MMP-9 expression and *in vitro* chemo-invasion (54). In the present study, SME blocked TPA-induced c-Jun phosphorylation (Fig. 3). NF-κB is a transcription factor that serves a pivotal role in inducing the expression of MMP-9 (47,55). In the present study, treatment with SME did not inhibit TPA-induced phosphorylation of IKKαβ/IκBα, degradation of IκBα or nuclear translocation of p65NF-κB. These data suggest that SME specifically inhibits the MAPK/AP-1 signaling pathway in TPA-induced MCF-7 breast cancer cells. Previously, Tanshinone II-A demonstrated an inhibitory effect on the invasion and metastasis of human carcinoma cells (8,9). However, the mechanism for that effect was not known in breast cancer. Furthermore, Tanshinone II-A is an alcohol extract of the root of the traditional Chinese medicinal plant *S. miltiorrhiza* Bunge. These results indicate that *S. miltiorrhiza* may regulate the invasion abilities of breast cancer. Therefore, the present study assessed the effect of SME on TPA-stimulated cell metastasis in MCF-7 breast cancer cells, and the results indicated that SME inhibited cell invasion (Fig. 4). These findings demonstrated that SME attenuated TPA-induced MMP-9 expression and invasion in MCF-7 breast cancer cells by inhibiting MAPK/AP-1 activation, and revealed the potential use of SME as a traditional therapeutic agent in inhibiting breast cancer metastasis.

Acknowledgements

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