Suppression of TPA-induced cancer cell invasion by Peucedanum japonicum Thunb. extract through the inhibition of PKCα/NF-κB-dependent MMP-9 expression in MCF-7 cells

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Abstract. Metastatic cancers spread from their site of origin (the primary site) to other parts of the body. Matrix metalloproteinase-9 (MMP-9), which degrades the extracellular matrix, is important in metastatic cancers as it plays a major role in cancer cell invasion. The present study examined the inhibitory effect of an ethanol extract of Peucedanum japonicum Thunb. (PJT) on MMP-9 expression and the invasion of MCF-7 breast cancer cells induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). Western blot analysis, gelatin zymography, and reverse transcription-quantitative PCR revealed that PJT significantly suppressed MMP-9 expression and activation in a dose-dependent manner. Furthermore, PJT attenuated TPA-induced nuclear translocation and the transcriptional activation of nuclear factor (NF)-κB. The results indicated that the PJT-mediated inhibition of TPA-induced MMP-9 expression and cell invasion involved the suppression of the PKCα/NF-κB pathway in MCF-7 cells. Thus, the inhibition of MMP-9 expression by PJT may have potential value as a therapy for restricting the invasiveness of breast cancer.

Introduction

Breast cancer is one of the most common malignancies affecting women worldwide, and the second leading cause of cancer-related mortality in women (1). The majority of breast cancer-related deaths are caused by distant metastases from the primary tumor site. Treatment failure mainly arises from cancer cell proliferation, invasion and metastasis, which ultimately lead to patient mortality. Invasion and metastasis are the fundamental characteristics and major causes of morbidity and mortality in patients with breast cancer. The metastatic process is initiated by cancer cell invasion, which involves changes in cell adhesion, the proteolytic degradation of the extracellular matrix (ECM) and the migration of cancer cells through tissue (2). The ECM consists of type IV collagen, laminin, heparan sulfate proteoglycan, nidogen, and fibronectin (3). ECM degradation requires extracellular proteinases, of which the matrix metalloproteinasases (MMPs) play an essential role in cancer metastasis (4,5).

The MMPs are a family of structurally conserved, zinc-dependent endopeptidases, which are known to be involved in the proteolytic degradation of the ECM. MMPs are divided into four subclasses as follows: collagensases, gelatinases, stromelysins and membrane-associated MMPs, based on their substrates (6). MMP-9 in particular, is considered to be one of the critical MMPs involved in cancer cell invasion and has been found to be directly associated with the invasion, metastasis and poor prognosis of breast cancer (7,8). Thus, inhibiting MMP-9 expression and/or its upstream regulatory pathways, may prove to be critical in the treatment of malignant tumors, including breast cancer. A variety of stimuli, including growth factors (e.g., fibroblast growth factor-2, epidermal growth factor and hepatocyte growth factor), cytokines (e.g., tumor necrosis factor-α), oncogenes (e.g., Ras) and 12-O-tetradecanoylphorbol-13-acetate (TPA) are all known to induce MMP-9 expression (9-13). Among these stimuli, TPA is a well known, selective activator of protein kinase C (PKC) (10) which stimulates MMP-9 synthesis and
secretion during breast cancer cell invasion (14,15). Cytokine and TPA treatments have been shown to induce MMP-9 expression through the activation of transcription factors, such as nuclear factor (NF)-κB (16,17), which possess binding sites on the MMP-9 gene promoter (18). The NF-κB element is centrally involved in the induction of MMP-9 gene expression by TPA (16,19).

*Peucedanum japonicum* Thunb. (PJT) is a medicinal plant which belongs to the Umbeliferae family. PJT leaves are traditionally consumed as a medicinal herb to treat coughs in Japan and Korea. The PJT root has also been used as a folk medicine for colds and neuralgia in Taiwan. Previous *in vitro* studies have reported that PJT possesses antioxidant activity (20), inhibits tyrosinases (21), possesses anti-obesity properties (22) and opposes platelet aggregation (23). Furthermore, it has been hypothesized that PJT may have anti-metastatic properties based on its possible inhibition of cell invasion (24,25). However, the mechanisms through which PJT exerts anti-invasive effects remain poorly understood.

In this study, we addressed this hypothesis by assessing the potential effects of PJT on TPA-induced cell invasion and MMP-9 expression in MCF-7 human breast cancer cells, and exploring the related molecular mechanisms. Our findings demonstrated that PJT suppressed TPA-induced MMP-9 expression by blocking NF-κB through PKCα, and that the suppression of MMP-9 expression correlated with the inhibition of cell invasion.

Materials and methods

**Cells and materials.** MCF-7 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a 5% CO₂ incubator. PJT, TPA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anti-phosphorylated (p)-IκBα (Cat. no. #2859) and p-IKKα (Cat. no. #2679) and IKKε (Cat. no. #2682) and IKKβ (Cat. no. #2678) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against MMP-9 (Cat. no. SC-12759), p50 (Cat. no. SC-7178), p65 (Cat. no. SC-372) and proliferating cell nuclear antigen (PCNA; Cat. no. SC-7907) and horseradish peroxidase (HRP)-conjugated IgG (Cat. sc-2004,2005) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Preparation of PJT extracts.** Roots of PJT were purchased from Kwangmyungdang Medicinal Herbs Co. Ltd. (Ulsan, Korea) and authenticated by Professor G.S. Lee, one of the authors of this article. A voucher specimen (WKU010107-PJ201305E) has been deposited at the Department of Herbolgy, College of Korean Medicine (Wonkwang University, Iksan, Korea). The powdered PJT (100 g) was extracted using sonication with 1,000 ml of 70% aqueous ethanol for 1 h. The extract was evaporated under 40 mmHg pressure using a rotary evaporator and then freeze-dried. The yield of the final extract was 12.02% (w/w).

**Determination of cell viability.** The effect of PJT on MCF-7 cell viability was determined using an established MTT assay. Briefly, 3x10⁴ cells were seeded in wells and incubated at 37°C for 24 h to allow attachment. The attached cells were left untreated or treated with 1, 10, 25, 50 and 100 µg/ml PJT for 24 h at 37°C. The cells were then washed with PBS prior to the addition of MTT (0.5 mg/ml in PBS) and incubated at 37°C for 30 min. Formazan crystals were dissolved with dimethylsulfoxide (100 µl/well) and detected at 570 nm using a Model 3550 microplate reader (Bio-Rad, Richmond, CA, USA).

**Western blot analysis.** The MCF-7 cells (5x10⁴) were treated with PJT (25, 50 and 100 µg/ml) for 1 h and then incubated with TPA for 24 h at 37°C. The cells were then lysed with ice-cold M-PER® Mammalian Protein Extraction Reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). The protein concentration in the lysate was determined using the Bradford method (26). Samples (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide and transferred onto Hybond™-polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Little Chalfont, UK) using a western blot apparatus. Each membrane was blocked for 2 h with 2% bovine serum albumin or 5% skim milk, and then incubated overnight at 4°C with 1 µg/ml of a 1:2,000 dilution of primary antibody. HRP-conjugated IgG (1:2,000 dilution) was used as the secondary antibody. Protein expression levels were determined by signal analysis using an image analyzer (FujiFilm, Tokyo, Japan).

**Gelatin zymography.** Gelatin zymography was performed as previously described (27). Conditioned media were collected after 24 h of stimulation, mixed with non-reducing sample buffer, and electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was washed at room temperature for 30 min with 2.5% Triton X-100 solution, and subsequently incubated at 37°C for 16 h in 5 mM CaCl₂, 0.02% Brij and 50 mM Tris-HCl (pH 7.5). The gel was stained for 30 min with 0.25% (w/v) Coomassie Brilliant Blue (Sigma-Aldrich) in 40% (v/v) methanol and 7% (v/v) acetic acid, and photographed on an image analyzer (FujiFilm). Proteolysis was imaged as a white zone in a dark blue field. Densitometric analysis was performed using Multi Gauge Image Analysis software (FujiFilm).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RT-qPCR was performed as previously described (27). Total RNA was extracted from the MCF-7 breast cancer cells using a FastPure™ RNA kit (Takara Bio, Otsu, Japan). The RNA concentration and purity were determined by absorbance at 260/280 nm. cDNA was synthesized from 1 µg total RNA using a PrimeScript™ RT reagent kit (Takara Bio). MMP-9 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression were determined by quantitative (real-time) PCR (qPCR) using the ABI PRISM 7900 sequence detection system and SYBR®-Green (Applied Biosystems, Foster City, CA, USA). The following primers were used: MMP-9 (NM 004994) sense, CCTGGAGACCTGAGAACCAATCT and antisense, CCAGTAGTGAAACCATGCC; and GAPDH (NM 002046) sense, ATGGAAATCCCCATCACCATCTT and antisense,
CGCCCGACTTGATTTTGG. To control for variations in mRNA concentration, all results were normalized to those of the housekeeping gene, GAPDH. Relative quantification was performed using the comparative ΔΔCt method according to the manufacturer’s instructions.

**Preparation of nuclear extract.** Nuclear extract was prepared as previously described (27). The MCF-7 cells (2x10⁶) were treated with PJT in the presence or absence of TPA for 4 h. The cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.5) and pelleted at 1,500 x g for 3 min. Cytoplasmic and nuclear extracts were prepared from the cells using NE-PER® Nuclear and Cytoplasmic Extraction reagents (Pierce Biotechnology, Inc.).

**Membrane fractionation.** The MCF-7 cells (5x10⁵) were treated with PJT (100 μg/ml) for 1 h and then incubated with TPA for 1 h at 37°C. The cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.5), and pelleted at 4,000 rpm for 3 min. Cell lysis was carried out in homogenization buffer (20 mM Tris-HCl, 5 mM DTT, 2 mM EDTA, 5 mM EGTA, protease inhibitor, phosphatase inhibitors, pH 7.5) with brief sonication (5 times for 10 sec each at 10% amplitude) after incubating on ice for 15-30 min. Cell debris was removed by centrifuging the sample at 3,000 rpm for 10 min at 4°C. Cell lysate was centrifuged at 13,000 rpm for 30 min at 4°C to separate out soluble (cytosolic) and pellet (membrane) fractions. The pellet fraction was incubated in homogenization buffer containing 1% Triton X-100 for 30 min on ice, centrifuged at 50,000 rpm for 1 h and the supernatant was collected as the membrane fraction.

**Electrophoretic mobility shift assay (EMSA).** The activation of NF-κB was assayed using a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ-chain (κB, 5'-CCGGTTAACAGAGGGGCTTTCCGAG-3') binding site was synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [α-32P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 µg of nuclear extracts and binding buffer [10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% v/v glycerol, 100 ng poly (dI·dC) and 1 mM dithiothreitol] were then incubated for 30 min at room temperature in a final volume of 20 µl. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with 50-fold excess of cold κB oligonucleotide.

**Invasion assay.** Invasion assay was performed as previously described (27). The invasion assay was carried out in 24-well chambers (8-µm pore size) coated with 20 µl Matrigel diluted with DMEM. The Matrigel coating was rehydrated in 0.5 ml DMEM for 30 min immediately before the experiments. The MCF-7 cells (2x10⁵) were added to the upper chamber with chemoattractant in the bottom well. Conditioned medium (0.5 ml) was added to the lower compartment of the invasion chamber. The chambers were incubated for 24 h. Following incubation, the cells on the upper side of the chamber were removed using cotton swabs, and the cells that had migrated were fixed and stained with Toluidine blue (Sigma-Aldrich) solution. Invading cells were counted in 5 random areas of the membrane using a Leica DM IL LED Inverted Lab Microscope (Leica, Wetzlar, Germany). Analyzed data are the means ± SE from 3 independent experiments performed in triplicate.

**Statistical analysis.** Statistical data analysis was performed using analysis of variance (ANOVA) and Duncan’s test. A value of P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effect of PJT on of MCF-7 cell viability.** In order to investigate the cytotoxicity of PJT on MCF-7 cells, the cells were seeded into wells of 96-well culture plates at a density of 3x10⁴ cells/well. The toxicity of PJT on MCF-7 cells was determined by MTT assay. Treatment of the MCF-7 cells with all concentrations of PJT for 24 h did not cause any significant change in cell viability (Fig. 1). Thus, subsequent experiments utilized the optimal non-toxic concentration (100 μg/ml) of PJT.

**PJT suppresses the mRNA and protein expression as well as the secretion of MMP-9 induced by TPA in MCF-7 cells.** To examine the effects of PJT on TPA-induced MMP-9 expression, western blot analysis, RT-qPCR, and gelatin zymography were performed on the MCF-7 cells. Western blot analysis revealed that treatment of the MCF-7 cells with PJT blocked the upregulation of TPA-induced MMP-9 protein expression (Fig. 2A). RT-qPCR revealed a TPA-induced increase in MMP-9 expression levels in the MCF-7 cells, and that PJT blocked the TPA-induced MMP-9 upregulation in a dose-dependent manner (Fig. 2B). To determine the effects of PJT on TPA-induced MMP-9 secretion, gelatin zymography was performed, which demonstrated that treatment of the MCF-7 cells with TPA increased MMP-9 secretion. However, PJT significantly diminished TPA-induced MMP-9 secretion (Fig. 2C). These results demonstrated that PJT exerts potent inhibitory effects on TPA-induced MMP-9 expression in MCF-7 cells.
PKC isotypes in the TPA-treated MCF-7 cells, we measured the levels of PKCα, PKCβ and PKCδ in the cytosolic and membrane fractions. The TPA-induced membrane localization of PKCα was blocked by pre-treatment with PJT for 1 h (Fig. 3). These results suggested that TPA-induced MMP-9 expression involved PKCα activation in MCF-7 cells, and PJT inhibited TPA-induced PKCα activation.

**PJT inhibits TPA-induced NF-κB DNA binding activity.** To elucidate the mechanisms through which PJT inhibits MMP-9 expression, the effect of PJT on the TPA-induced activation of NF-κB was evaluated using EMSA. As shown in Fig. 4A, TPA substantially increased NF-κB binding activity. Pre-treatment with PJT inhibited the TPA-induced NF-κB binding activity; however, PJT alone had no effect on NF-κB binding activity. These results are consistent with the hypothesis that PJT specifically blocks NF-κB activation in MCF-7 cells. Western blot analysis revealed that PJT induced the phosphorylation of IKKαβ and IκBα, as well as that of IκKBα and IκKBβ in the cytoplasm and, thereby, the nuclear translocation of NF-κB subunits p50 and p65. These alterations were suppressed in the TPA MCF-7 cells treated with that had also been treated with PJT (Fig. 4B and C).

**Effect of PJT on TPA-induced MCF-7 cell invasion in vitro.** It has been previously reported that the upregulation of MMP-9 expression contributes to the invasive ability of cancer cells (22,23). Thus, in this study, we investigated the inhibitory effects of PJT on the invasive ability of MCF-7 breast cells by performing a Matrigel™ invasion assay. We treated the cells with 100 µg/ml PJT prior to treatment with 20 µM TPA for 1 h and subsequently incubated the cells for 24 h. The level of TPA-induced cell invasion was significantly increased compared with the untreated control cells. However, TPA-induced cell invasion was suppressed by PJT treatment (Fig. 5).

**Discussion**

Metastasis is the primary cause of breast cancer mortality. Tumor metastasis is a multistep process through which a subset of individual cancer cells disseminate from a primary tumor to distant secondary organs or tissues. This process involves cell proliferation, ECM degradation, cell migration, and tumor growth at metastatic sites (15,28). Cancer cell invasion is an early step in the metastatic cascade, representing the beginning of the transition from benign to malignant cancer. Tumor invasion is morphologically associated with a distorted edge of the primary tumor where individual or cohorts of cancer cells actively invade the ECM surrounding the primary tumor (29). MMP-9 is important in tumor metastasis as it is involved in basement membrane cleavage, which allows cells with a migratory phenotype to be more invasive and motile (23,25,30). In previous studies, inflammatory cytokines, growth factors and phorbol esters have been shown to stimulate MMP-9 by activating different intracellular signaling pathways in breast cancer cells (31-33). The inhibition of MMP-9 expression may thus prove to be an important therapeutic target for the development of therapies against tumor metastasis. This study provides the first evidence, to the best of our knowledge, that PJT inhibits TPA-induced cell invasion by suppressing MMP-9 expression in MCF-7 breast cancer cells.

TPA increases the invasiveness of MCF-7 cells by activating MMP-9 through PKCs. The activation of PKCs has been shown to correlate significantly with an increased inva-
siveness in breast carcinomas (34). TPA activates conventional (α, βI, βII and γ) and novel (δ, ε, θ and η) PKCs by binding to the C1 domains of these isoforms (35). The activation of PKCs by TPA involves the translocation of PKC isoforms to the plasma membrane, resulting in proliferation, differentiation and malignant transformation, as well as tumor promotion and the progression in cancer cells. In a previous study by our group, TPA stimulation resulted in the translocation of PKCα

Figure 4. *Peucedanum japonicum* Thunb. (PJT) blocks 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced nuclear factor-kappa B (NF-κB) activation in MCF-7 cells. The cells were treated with PJT in the presence of TPA. Following a 4-h incubation, nuclear extracts were prepared as described in the Materials and methods. (A) NF-κB DNA binding was analyzed by electrophoretic mobility shift assay as described in Materials and methods. (B and C) Western blot analysis was performed to determine the nuclear levels of NF-κB (p50 and p65) subunits as well as the cytoplasmic levels of IκBα and p-IκBα, and IKKα, IKKβ and p-IKKαβ.

Figure 5. Effect of *Peucedanum japonicum* Thunb. (PJT) on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced Matrigel invasion of MCF-7 cells. The cells were seeded onto the upper chamber, and drugs were placed in the well. After a 24-h incubation, the cells on the bottom of the filter were fixed, stained with Toluidine blue solution, and counted under a light microscope. Each value represents the means ± SEM of 3 independent experiments. *P<0.01 vs. treatment with TPA alone.
and PKCδ from the cytosol to the cell membrane, although the translocation of PKCβ was not observed. Treatment with a non-toxic dose of a PKCδ inhibitor (rottlerin), a broad PKC inhibitor (GFI09203X) and a PKCα inhibitor (Gö6976) caused the marked inhibition of TPA-induced MMP-9 expression and secretion (36). These data indicated that the TPA-induced activation of PKCα and PKCδ mediated MMP-9 expression and secretion (36). In the present study, we demonstrated that PJT inhibited the TPA-induced membrane localization of PKCα, but not that of PKCδ (Fig. 3) in MCF-7 cells.

NF-κB, which has been reported to be regulated by PKC isoforms (37,38) is a transcription factor that regulates MMP-9 expression through binding sites on its promoter (18). NF-κB is an inducible, dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors and consists of two major polypeptides, p65 and p50 (39). NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB, an inhibitory factor of NF-κB. Various inducers, such as TPA, cytokines and stress cause the dissociation of this complex, presumably through IκB phosphorylation, which results in NF-κB being released from the complex. NF-κB is then translocated to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription. NF-κB elements are centrally involved in MMP-9 gene induction by TPA (16,40), and our results demonstrated that PJT inhibited MMP-9 expression by suppressing NF-κB in breast cancer cells treated with TPA.

Our experiments confirmed that TPA-induced cell invasion was suppressed by PJT. This was demonstrated by the Matrigel invasion assay showing the inhibition of the TPA-induced invasion potential of MCF-7 cells by PJT (Fig. 5).

In conclusion, our results demonstrated that PJT is a potent inhibitor of TPA-induced MMP-9 expression and strongly blocked the action of the NF-κB signaling pathway through PKCα in MCF-7 cells. This is the first study, to the best of our knowledge, to demonstrate that TPA-induced MCF-7 cell invasion was suppressed by PJT through the inhibition of MMP-9 expression and through the suppression of PKCα/NF-κB in MCF-7 cells. The findings of this study suggest that PJT may potentially be developed into a potent chemopreventive agent for the prevention of breast cancer metastasis.

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