Frondoside A has an anti-invasive effect by inhibiting TPA-induced MMP-9 activation via NF-κB and AP-1 signaling in human breast cancer cells

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Abstract. Metastasis and invasion are among the main causes of death in patients with malignant tumors. The aim of this study was to determine the anti-invasive activity of frondoside A against human breast cancer cells. We investigated the inhibitory effect of frondoside A on cell clonogenicity, invasion and migration in TPA-stimulated human breast cancer cells at non-cytotoxic concentrations. Frondoside A significantly attenuated TPA-induced colony formation, invasion and migration in MBA-MB-231 human breast cancer cells. Induction of MMP-9 is especially important for the metastasis of many cancer tumor cell types. Additionally, we found that frondoside A suppresses TPA-induced MMP-9 enzymatic activity, secretion and expression. This effect was associated with reduced activation of AP-1 and NF-κB, and correlated with enhanced expression of TIMP-1 and TIMP-2. Frondoside A significantly inhibited the TPA-induced MMP-9 expression possibly via the suppression of AP-1 and NF-κB signaling pathways. Frondoside A reduces the activation of the PI3K/Akt, ERK1/2 and p38 MAPK signals. These results suggest that the anti-metastatic effects of frondoside A on human breast cancer cells might result from inhibited TPA activation of AP-1 and NF-κB and reduced TPA activation of PI3K/Akt, ERK1/2 and p38 MAPK signals, ultimately leading to downregulation of MMP-9 expression. These results indicate the role of frondoside A in metastasis and its underlying molecular mechanisms, thus, suggesting frondoside A as a chemopreventive agent for metastatic breast cancer.

Introduction

Worldwide, breast cancer is one of the most common forms of malignancy in females. Most breast cancer deaths are caused by distant metastasis from the primary tumor site and breast cancer is a leading cause of mortality from such metastases (1). Despite recent advances in breast cancer treatment, subsequent metastasis can still occur through blood vessels or lymphatic channels. In recent years, attention has been focused on the anticancer properties of natural products, which play an important role in the prevention of disease. Thus, effective chemo-preventive treatments for breast cancer metastasis would have an important impact on breast cancer morbidity and mortality (2).

In metastasis, malignant tumor cells move from the primary tumor to a secondary distant organ through a complex multistage process involving the coordination of several signaling pathways that allow changes in cell migration and invasion (3). ECM interactions, disconnection of intercellular adhesion, degradation of the ECM, and the invasion of lymph and blood vessels are important steps in cancer invasion and metastasis. The degradation of ECM, mediated by the concerted action of matrix metalloproteinases (MMPs), plays an important role in tumor invasion and metastasis (4,5). The MMPs play a substantial role in several physiologic processes, including tissue development, remodeling, and wound healing. However, they are also involved in some tissue-destruction diseases, such as arthritis, inflammation, cardiovascular diseases, invasion, and metastasis. The MMP family of zinc- and calcium-dependent endoproteinases is divided into four subclasses based on their substrates. These include collagenase, gelatinase, stromelysin, and membrane-associated MMPs (6). MMPs process a broad spectrum of cell surface molecules and function in several important biological processes. Activating MMPs enables the degradation of ECM by cancer cells, allowing vasculature access, invasion, and migration into organs. MMP-2 (also known as gelatinase A and 72-kDa type IV collagenase) and MMP-9 (also known as gelatinase B and 92-kDa type IV collagenase) are the most important enzymes in the degradation of the basement membrane due to their ability to degrade type IV collagen. Therefore, they are of pivotal importance for cancer invasion and metastasis (7). Increased expression of MMP-9 is associated with the progression and invasion of tumors and MMP-2 is constitutively overexpressed in highly metastatic tumors. MMP-9 is strongly expressed in invasive breast cancer and may be used as a marker for the metastatic potential of breast cancer (8). Interestingly, the activity of MMPs tends to inhibit the endogenous tissue inhibitors of metalloproteinases

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(TIMPs), which are specific physiological inhibitors of MMPs. In particular, TIMP-1 binds to the hemopexin domain of MMP-9. Disturbance in the balance between MMPs and TIMPs may contribute to degradation or deposition of ECM. Therefore, TIMP activators may be useful chemo-preventive treatments for malignant cancer (7,9,10).

The expression of MMP-9 can be stimulated by various agents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), inflammatory cytokines, or growth factors, through the activation of various intracellular signaling pathways. TPA is one of the most commonly utilized agents for studying the mechanisms of carcinogenesis (11,12). In addition to carcinogenesis, TPA induces MMP-9 expression via the PI3K/Akt and MAPK signaling pathways, increasing the invasiveness of cell lines. PI3K activation leads to phosphorylation of phosphatidylinositides, which then activates Akt. Akt appears to play various important roles in regulating cellular growth, inflammatory reactions, and metastasis. Mitogen-activated protein kinases (MAPKs) are members of a highly conserved family of protein serine/threonine kinases that includes extracellular signal-regulated kinases (ERK1/2), c-jun N-terminal or stress-regulated protein kinases (JNK/SAPK), and p38 MAPKs. These kinases are involved in numerous cellular activities and are important regulators of inflammatory reactions and metastasis (13-15).

The promoter region of MMP-9 is highly conserved and contains AP-1 and NF-κB binding sites. TPA regulates the expression of MMP-9 by modulating the activation of AP-1 and NF-κB through PI3K/Akt and MAPK signaling pathways. AP-1 and NF-κB are inducible transcription factors that play a central role in regulating the expression of a wide variety of genes associated with cell proliferation, immune response, inflammation and malignant transformation (14,15). Several authors have reported that the human MMP-9 promoter contains a *cis*-acting regulatory element and NF-κB and AP-1 binding sites. These sites regulate transcription of the MMP-9 gene. NF-κB is predominantly a heterodimeric complex of p65/Rel A and p50. The NF-κB transcription factor is found in the cytoplasm as an inactive homodimer or heterodimer. The dimer associates through the Rel homology domain with an inhibitory molecule, IκBα, a member of the IκB protein family. Stimulation by TPA causes IκBα to dissociate from NF-κB and phosphorylation and degradation via the ubiquitin/proteaseome-dependent pathway. AP-1 consists of homodimers and heterodimers of members from Fos and Jun families. After activation, AP-1 and NF-κB move into the nucleus and promote metastasis (11,16).

Recent studies of new anti-metastatic agents have demonstrated that some natural compounds with chemopreventive capability attenuate the metastasis of several types of cancer. Frondoside A is a triterpenoid saponin, the major component of sea cucumbers, which is known to exhibit a variety of biological activities. Sea cucumbers have been traditionally used for food delicacy in East Asia, as well as a dietary health supplement in United States and Canada. Recently the anti-tumor and anti-angiogenic activities of sea cucumbers have attracted considerable attention (17-19). In addition, some authors have reported that frondoside A exerts anti-proliferative and apoptotic effects on the growth of cancer cells (20-22). Although various bioactivity studies of frondoside A have been performed, the molecular mechanisms by which frondoside A affects the expression of MMP-9 and the invasion of breast cancer cells remain unclear. In this study, human breast cancer MDA-MB-231 cell line was used to investigate both the effect of frondoside A on TPA-induced MMP-9 expression and the underlying molecular mechanism of that effect. We show that frondoside A inhibits the colony formation, migration, and invasion of breast cancer cells by suppressing MMP-9 expression and blocking the activation of AP-1 and NF-κB transcription factors and PI3K/Akt , ERK1/2 and p38 MAPK signaling.

Materials and methods

Materials. BD BioCoat™ Matrigel™ Invasion Chambers were obtained from BD Biosciences (San Jose, CA, USA). Cell culture medium, RPMI-1640, and fetal bovine serum (FBS) were purchased from Gibco-BRL (now part of Invitrogen Corporation, Carlsbad, CA, USA). FuGENE 6 transfection reagent and X-treme GENE siRNA Transfection Reagent were purchased from Roche (Indianapolis, IN, USA). Antibodies against for phosphorylated p38 (p-p38), p-JNK, p-ERK, p-IκBα MMP-2, and MMP-9 were purchased from Cell Signaling Technology (Beverly, MA, USA). MMP-9 small interfering RNA (siRNA), and antibodies for ERK, JNK, p38, c-Jun, c-Fos, NF-κB, IκBα, and Histone H1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Frondoside A and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Human breast cell lines MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37˚C in a humidified incubator with 5% CO_2.

Clonogenic assay using soft agar colony formation. Colony formation ability was examined by an anchorage-independent soft agar assay. Briefly, 0.5% agar gel with 10% FBS and 1% penicillin-streptomycin in RPMI-1640 was prepared and added as a base agar to each well in a six-well culture dish. MDA-MB-231 cells were plated for anchorage-independent growth analysis in 0.4% agar gel with 10% FBS and 1% penicillin-streptomycin in RPMI-1640 that was supplemented with the target treatment. Plated cells were placed on top of the base agar. The medium was replaced once every day. Cell colonies were counted at weeks two and three using a microscope and digital camera.

Cell invasion assay. The cell invasion assay was conducted using BioCoat™ Matrigel™ Invasion Chambers according to the manufacturer's instructions. Briefly, the Matrigel coating was re-hydrated in 0.5 ml RPMI-1640 for 30 min immediately before the experiments. Cells ($5x10^4$) suspended in 0.5 ml of serum-free medium were added to the upper chamber of Matrigel-coated filter inserts. After treatment with frondoside A for 1 h, 0.5 ml of serum-free medium containing 50 nM of PMA was added to the bottom well as chemoattractant. The chambers were incubated for 24 h. After incubation, cells on the upper side of the chamber were removed using cotton swabs, and cells that had migrated were fixed and stained with 2% ethanol containing 0.2% crystal violet powder. Invading cells were enumerated under a light microscope using a x10 objective.

In vitro wound-healing repair assay. For the cell migration assay, the cells were seeded into a 24-well culture dish until 90% confluent. The cells were then maintained in serum-free medium for 12 h. The monolayers were carefully scratched using a 200-μl pipette tip. Cellular debris was removed by washing with PBS, and then the cells were incubated in medium without serum. The migrated cells were fixed in cold 75% methanol for 30 min and washed three times with PBS. The cultures were photographed at 0 and 24 h to monitor the migration of cells into the wounded area, and the closure of wounded area was calculated.

Gelatin zymography assay. The activity of MMP-2 and MMP-9 in the conditioned medium was determined by gelatin zymography protease assay. Briefly, cells ($2x10^5$) were seeded in 6-well plates and allowed to grow to 80% confluence. The cells were then maintained in serum-free medium for 12 h prior to designated treatments with frondoside A and TPA for 24 h. Conditioned media were collected, cleared by centrifugation and mixed with 2X sodium dodecyl sulfate (SDS) sample buffer (Invitrogen Corporation), and electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. Following electrophoresis, the gels were incubated in a renaturing buffer (2.5% Triton X-100) with gentle agitation to remove SDS and then incubated in a developing buffer (50 mM Tris-HCl buffer, pH 7.4, and 10 mM $CaCl_2$) overnight at 37˚C to allow digestion of the gelatin. Gels were then stained with SimplyBlue SafeStain (Invitrogen Corporation) until clear bands suggestive of gelatin digestion appeared.

Western blot analysis. Cells were harvested in ice-cold lysis buffer consisting of 1% Triton X-100, 1% deoxycholate, and 0.1% SDS. The protein content of the cell lysates was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA). Proteins in each sample (50 μg total proteins) were resolved by 12% SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and exposed to the appropriate antibodies. The proteins were visualized by the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. Images were acquired using an ImageQuant 350 analyzer (Amersham Biosciences).

Immunofluorescence confocal microscopy. MDA-MB-231 cells were cultured directly on glass cover-slips in 35 mm-diameter dishes. Cells were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 100% methanol for 10 min. To investigate the cellular localization of NF-κB, we treated cell with a 1:100 dilution of polyclonal antibody against NF-κB for 24 h. After extensive washing with PBS, cells were further incubated for 4 h at room temperature with a 1:1000 dilution of secondary fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG antibody. Cell nuclei were stained with 1 μg/ml of 4',6-diamidino-2-phenylindole (DAPI), and then analyzed by confocal microscopy using an LSM 510 Meta microscope (Zeiss, Jena, Germany).

Chromatin immunoprecipitation assay. To detect the *in vitro* association of nuclear proteins with human MMP-9 promoter, chromatin immunoprecipitation (ChIP) analysis was conducted as described previously (23) with some modifications. Briefly, $2x10^7$ MBA-MB-231 cells were incubated in a culture medium containing 1% formaldehyde for 10 min at room temperature, and the cross-linking reaction was quenched by adding glycine to 0.125 M. Isolated nuclei were digested with 200 U of MNase at 37˚C for 15 min followed by sonication to produce chromatin of primarily mononucleosome size. Fragmented chromatin was reacted with antibodies for 3 h at 4˚C. Protein-DNA complexes were recovered with protein A agarose beads, washed, and then eluted with elution buffer. Crosslinks were reversed at 65˚C in 0.25 M NaCl overnight and DNA was digested with proteinase K for 2 h at 50˚C. The DNA was isolated using a DNA purification kit (Qiagen). Immunoprecipitated DNA was used for each PCR. PCR primers for the MMP-9 promoter (373 bp, including the NF-κB/AP-1 cluster, Gene Bank accession no. AF538844) were: sense (5'-CACTTCAAAGTGGTAAGA-3'), antisense (5'-GAAAGTGATGGAAGACTCC-3').

Transient transfection and dual luciferase assay. To determine promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI). MBA-MB-231 cells were transfected with NF-κB luciferase reporter plasmid and AP-1 luciferase reporter plasmid (Stratagene, Grand Island, NY) using FuGENE 6 reagent (Roche Applied Science) by according to the manufacturer's instructions. A *Renilla* luciferase control plasmid pRL-CMV (Promega) was cotransfected as an internal control for transfection efficiency. Twenty-four hours after transfection, the cells were incubated with indicated reagents for 1 h and then treated with TPA for 24 h. Luciferase activity was assayed using a dual-luciferase assay kit (Promega) according to the manufacturer's instructions. Luminescence was measured using a GloMax™ 96-microplate luminometer (Promega).

Transient transfection of siRNA. Transfection of MDA-MB-231 cells with siRNA was performed using X-treme GENE siRNA Transfection Reagent (Roche Applied Science, Basel, Switzerland), according to the manufacturer's instructions. Commercially available human MMP-9-specific siRNAs, and negative control siRNAs were used for transfection. In brief, X-treme GENE siRNA transfection reagent (10 μl) was added to 100 μl serum-free medium containing 2 μg of each siRNA oligo, and was incubated for 20 min at room temperature. Gene silencing was measured after 72 h by western blotting.

Statistical analysis. The results are expressed as the means ± SE. Each experiment was repeated at least three times. Differences between treatment groups were compared using paired Student's t-tests. Comparisons with $p<0.05$ were considered statistically significant.

Results

Frondoside A inhibited TPA-induced colony formation, migration and invasion of human breast cancer cells. In vitro invasion and migration assays including trans-well, soft agar, and wound-healing assays were used to investigate the differential inhibitory effects of frondoside A on the human breast cancer MDA-MB-231 cells. The anti-invasive potential of frondoside A was first investigated by studying its effects on

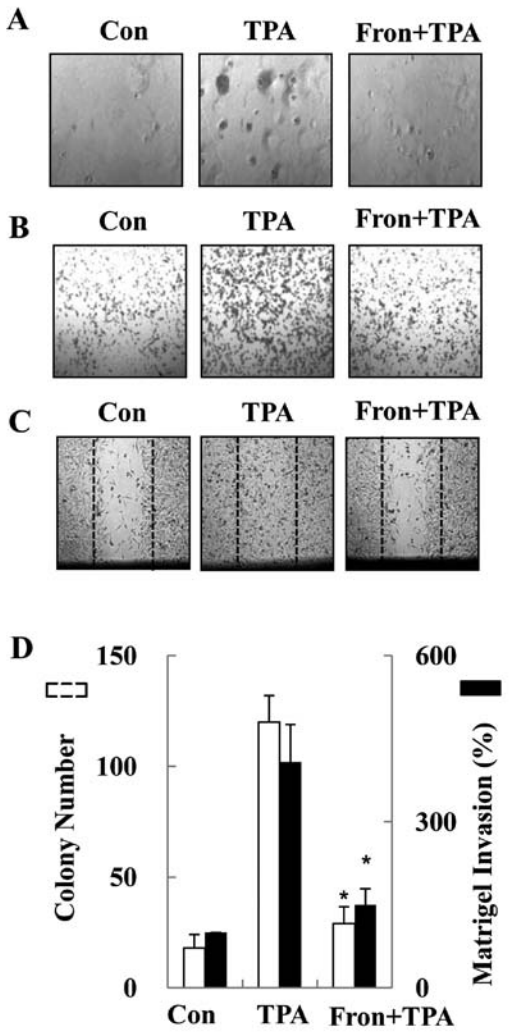

Figure 1. Inhibitory effect of frondoside A on colony formation, migration and invasion of human breast cancer cells. (A) Clonogenicity of MDA-MB-231 was determined by a soft agar colony formation assay as described in Materials and methods. (B) MDA-MB-231 cells were treated with frondoside A (1 μM) followed by TPA (50 ng/ml) treatment for 24 h. Cells invading the bottom side of the filter were stained with crystal violet and counted under a microscope. (C) Cells were scratched with a pipette tip and then treated with (1 μM) followed by TPA (50 ng/ml) treatment for 24 h. Migrating cells were photographed under phase-contrast microscopy. (D) Quantification of invasive ability and clonogenicity is expressed as the number of invasive cells relative to that of invasion by TPA-untreated cells. Means ± SE of 3 independent experiments are shown in columns; *P<0.05 versus TPA-only group.

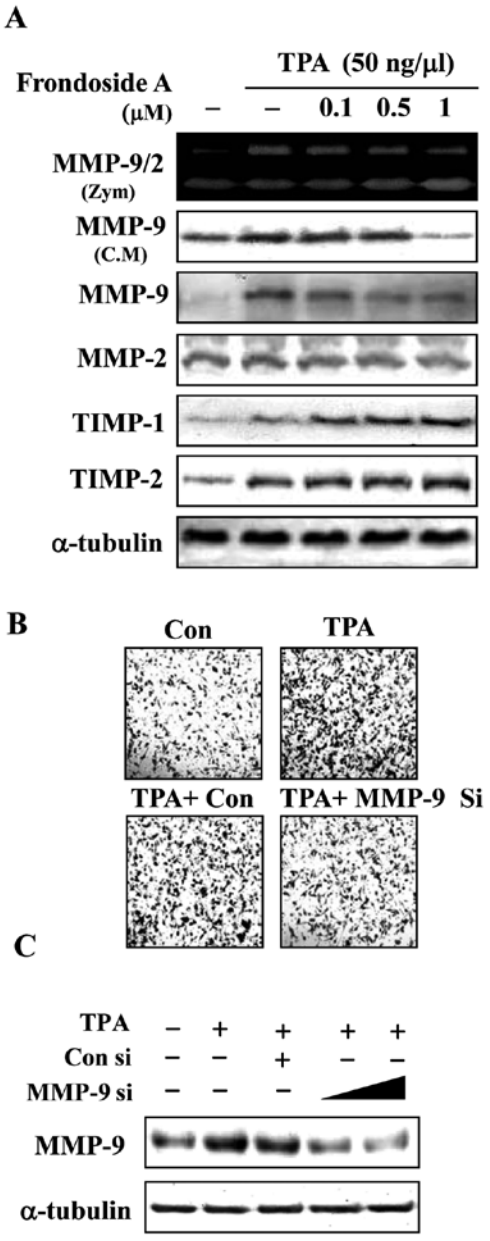

Figure 2. The effect of frondoside A on MMP-9, MMP-2, TIMP-1 and TIMP-2 expression in human breast cancer cells. (A) MDA-MB-231 cells were treated with frondoside A followed by TPA (50 ng/ml) treatment for 24 h. MMP-9 and MMP-2 enzymatic activity was analyzed by gelatin zymography (Zym). Secretion and intracellular protein expression were analyzed by western blotting (conditioned medium, C.M). MDA-MB-231 cells were treated with frondoside A followed by the addition of TPA. The TIMP-1 and TIMP-2 protein levels were evaluated by western blotting. Western blot detection of α-tubulin was performed using a protein-loading control for each lane. (B and C) Cells were transfected with the MMP-9 si or Con si and then incubated with TPA for 24 h. Cells invading the bottom side of the filter were stained with crystal violet and counted under a microscope. The expression of MMP-9 protein were examined by western blotting.

the TPA-induced clonogenicity of breast cancer cells using a soft agar clonogenic assay. Frondoside A exhibited an inhibitory effect on the TPA-induced clonogenic ability of breast cancer cells (Fig. 1A and D). We next assessed the effect of frondoside A on invasion using a trans-well migration assay. Treatment with 1 μM of frondoside A inhibited 62% of cell invasion (Fig. 1B and D). The wound-healing assay also indicated that TPA-induced migration of breast cancer cells was inhibited by frondoside A (Fig. 1C). To confirm that the inhibitory activity of frondoside A was not due to direct cytotoxicity of frondoside A, we examined the toxicity of frondoside A in breast cancer cells. The breast cancer cells were treated with various concentrations (0.1, 0.5, 1 and 2 μM) of frondoside A for 24 h. Frondoside A had no cytotoxic effect on breast cancer cells at concentrations of 0.1-1 μM (data not shown), indicating that frondoside A was not toxic to breast cancer cells at these dosages. These results suggest cell clonogenicity, invasion and migration of human breast cancer cells are inhibited by frondoside A at non-cytotoxic concentrations.

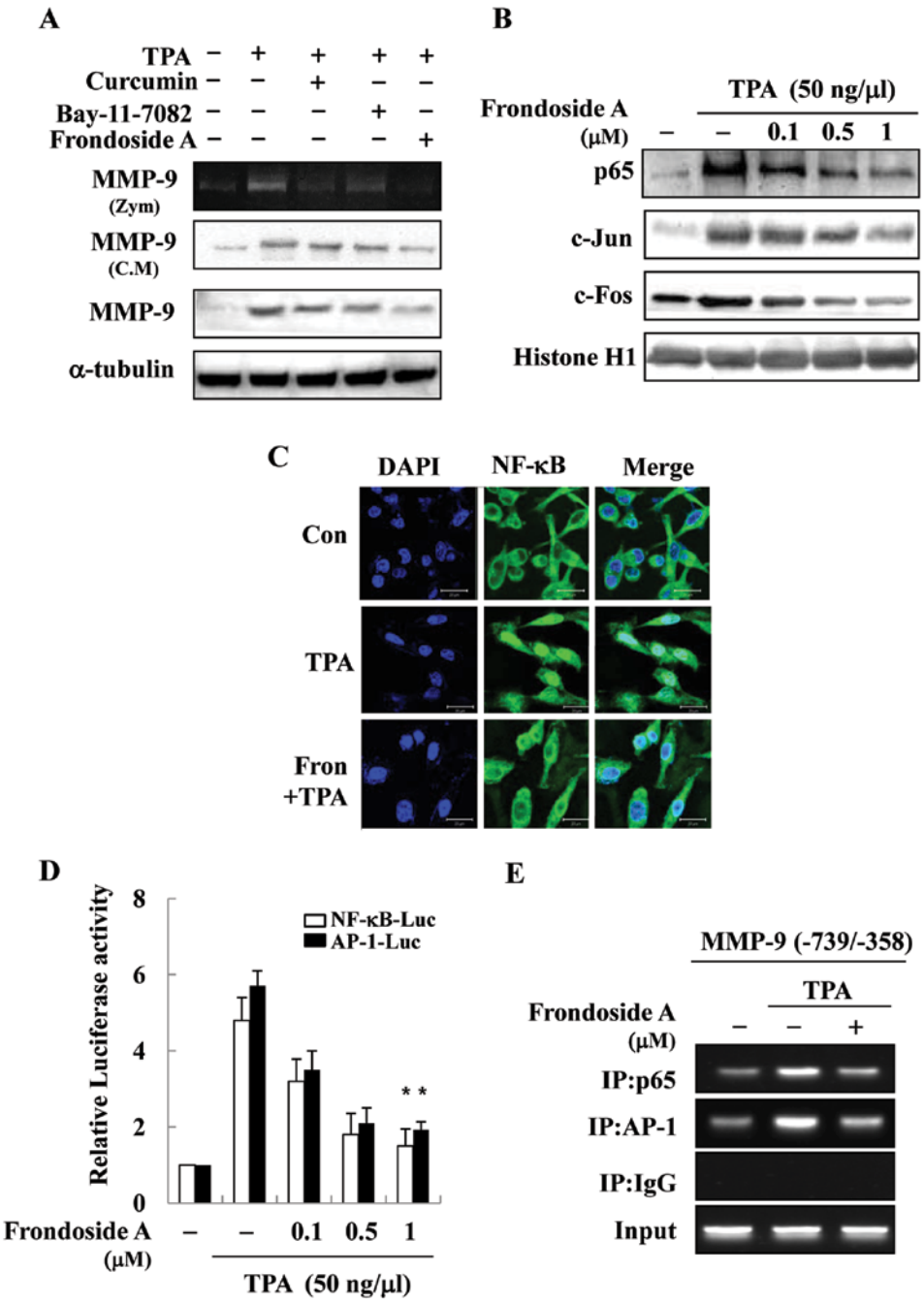

Figure 3. NF-κB and AP-1 are involved in frondoside A-mediated downregulation of MMP-9. (A) MDA-MB-231 cells were treated with TPA for 24 h in the presence of curcumin (AP-1 inhibitor, 10 μM), Bay-11-7802 (NF-κB inhibitor, 10 μM) and frondoside A (1 μM). Subsequently, MMP-9 enzymatic activity was analyzed by gelatin zymography (Zym). Secretion and intracellular protein expression were analyzed by western blotting (conditioned medium, C.M). (B) MDA-MB-231 cells were treated with frondoside A followed by TPA (50 ng/ml) treatment for 1 h. Nuclear translocation of NF-κB and AP-1 complex (c-jun and c-fos) was confirmed by western blotting. The nuclear extracts were prepared and analyzed by western blotting. (C) Nuclear translocation of NF-κB was assessed by confocal microscopy. The cells were pre-treated with frondoside A (1 μM) for 1 h and stimulated with TPA (50 ng/ml) for 1 h. Fixed cells were stained with DAPI or anti-NF-κB p65 antibody, followed by incubation with FITC-conjugated anti-rabbit IgG antibody. Images were obtained using a confocal microscope. (D) Cells were co-transfected with the AP-1 reporter, the κB-luc reporter, and the control *Renilla* luciferase plasmid, pRL-CMV. After 24 h, cells were incubated with the indicated concentrations of frondoside A for 1 h, and then stimulated with TPA (50 ng/ml) for 24 h. Equal amounts of cell extract were assayed for dual-luciferase activity. Expression of the *Renilla* luciferase control was used to normalize AP-1-luciferase activity and κB-luciferase activity. Each bar represents the mean ± SE from 3 independent experiments. *P<0.05 vs. the TPA-treated group. (E) Cells were incubated with frondoside A for 1 h and the incubated with TPA for 4 h. DNA immunoprecipitated by an anti-NF-κB p65 antibody was purified as described in Materials and methods. The precipitated MMP-9 promoter region (-739 to -358 bp) was amplified by PCR and real-time PCR. The input represents PCR products from chromatic pellets prior to immunoprecipitation.

The effect of frondoside A on MMP-9, TIMP-1 and TIMP-2 in human breast cancer cells. The upregulation of MMP-9 has been reported to play an essential role in invasion and metastasis in breast cancer cells (8). We therefore examined whether the inhibitory effect of frondoside A against breast cancer cell invasion was associated with regulation in MMP-9 enzyme activity

and secretion. Cells were treated with frondoside A 1 h prior to the addition of TPA, and incubation for an additional 24 h. Conditioned medium was collected and enzyme activity of MMP-9 was analyzed using gelatin zymography. Treatment with TPA for 24 h dramatically increased MMP-9 enzyme activity, whereas the MMP-2 enzyme activity was not affected by TPA. As shown in Fig. 2A, treatment of breast cancer cells with frondoside A at doses above 0.1 μM suppressed TPA-induced MMP-9 activity. Furthermore, treatment of breast cancer cells with frondoside A decreased TPA-stimulated secretion and intracellular expression of MMP-9 in a dose-dependent manner. Because physiological activity of MMP-9 is closely related to that of its specific endogenous inhibitors, TIMP-1 and TIMP-2 (7), we explored the potential effects of frondoside A on TIMP-1 and TIMP-2 expression. As shown in Fig. 2A, frondoside A increased the levels of TIMP-1 and TIMP-2 expression. TIMP-1 and TIMP-2 levels probably increase as a result of the inhibition of MMP-9 activation by frondoside A. These data indicate that TIMP-1 and TIMP-2 are involved in the downregulation of MMP in TPA-stimulated human breast cancer cells that have been treated with frondoside A. To examine whether TPA induced invasion by regulating the MMP-9, a small interference RNA (siRNA) approach was employed. Knockdown of endogenous MMP-9 in MDA-MB-231 cells suppressed TPA-induced MMP expression and invasion when compared to control siRNA (Fig. 2B and C). These results suggest frondoside A inhibits MMP-9 activation and are consistent with frondoside A inhibition of cell invasion. Further, the anti-metastatic effect of frondoside A may be related to the inhibition of the enzymatic ECM degradation processes in breast cancer cells.

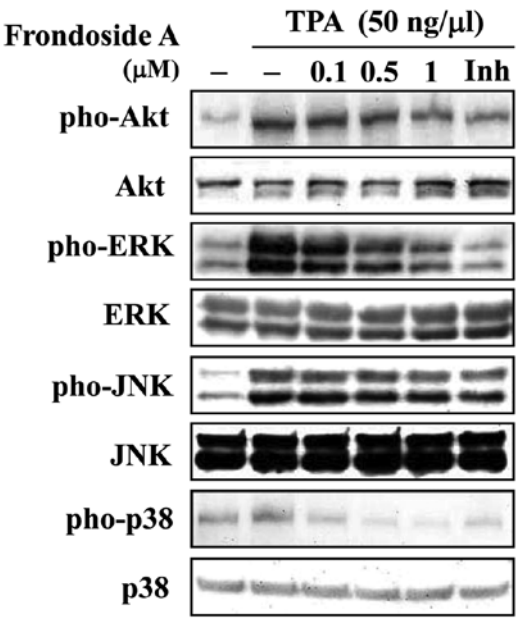

Figure 4. The effect of frondoside A on TPA-induced activation of PI3K/Akt and MAPK signaling pathways. (A) MDA-MB-231 cells were treated with the indicated concentrations of frondoside A or specific kinase inhibitors (1 μM) for 1 h and stimulated with TPA (50 ng/ml) for 1 h. Equal amounts of cell extracts were analyzed by western blotting with anti-phospho-Akt, anti-phospho-ERK1/2, anti-phospho-JNK, or anti-phospho-p38 antibody. Western blot detection of Akt, ERK1/2, JNK, or p38 was estimated from a protein-loading control for each lane.

Frondoside A inhibits MMP-9 activity through suppression of TPA-stimulated NF-κB and AP-1 activity. Expression of MMP-9 is regulated by the interaction of transcription factors, such as AP-1 and NF-κB, with binding elements in the MMP-9 gene promoter. To further determine whether frondoside A inhibition of MMP-9 activity is mainly the result of AP-1 and NF-κB signaling pathway inhibition, we investigated the effects of specific inhibitors of AP-1 (curcumin) and of NF-κB (Bay-11-7802) (24,25). Breast cancer cells were pretreated with curcumin or Bay-11-7802 for 1 h and then stimulated with TPA in the presence or absence of frondoside A for 24 h. The culture media were subjected to gelatin zymography and western blotting. Treatment with either curcumin or Bay 11-7802 inhibited TPA-induced MMP-9 enzyme activity, secretion, and expression (Fig. 3A). Furthermore, the inhibitory effects of curcumin and Bay-11-7082 were confirmed by the prevention of TPA-stimulated AP-1 and NF-κB promoter activity and nuclear translocation. This, in turn, resulted in the reversal of TPA-stimulated AP-1 and NF-κB promoter activity and nuclear translocation (data not shown). Because frondoside A decreased the expression of MMP-9, we examined whether these transcription factors are regulated by frondoside A in TPA-stimulated breast cancer cells. The cells were treated with different concentrations of frondoside A in the presence of TPA for 1 h, and nuclear extracts were prepared for and analyzed by western blotting. TPA induced the nuclear translocation of AP-1 and NF-κB, and frondoside A inhibited the nuclear translocation of Ap-1 and NF-κB (Fig. 3B and C). Cells transiently transfected with AP-1-Luc reporter or κB-Luc reporter plasmids were treated with frondoside A in the presence of TPA. As shown in Fig. 3D, TPA treatment increased AP-1 and NF-κB promoter activity. AP-1 and NF-κB promoter activity was suppressed by frondoside A in a dose-dependent manner. We found two AP-1 binding sites (-79 and -533) and one NF-κB binding site (-600) in the MMP-9 promoter. We used a CHIP-PCR assay to determine whether NF-κB was involved in TPA-induced MMP-9 gene expression. Chromatin was extracted and immunoprecipitated using anti-NF-κB antibody, and the MMP-9 promoter regions (NF-κB/AP-1 cluster -739/-358) were amplified by PCR and real-time PCR. As shown in Fig. 3E, *in vitro* binding of NF-κB to the MMP-9 promoter was increased by TPA, but the increase was significantly inhibited by frondoside A. These results indicate that the inhibition of AP-l and NF-κB signals mediates the inhibitory effect of frondoside A on MMP-9 expression.

Frondoside A TPA-mediated invasion and migration activation is through PI3K/Akt and ERK signaling pathways. MMP-9 gene expression can be activated by a number of signal transduction pathways, including those involving PI3K/Akt and MAPKs, which are upstream modulators of AP-1 or NF-κB (12). We investigated whether frondoside A inhibited the activation of these signaling pathways. As shown in Fig. 4, phosphorylation of PI3k/Akt, ERK1/2, JNK and P38 was significantly increased by stimulation with TPA. Addition of frondoside A 1 h after TPA treatment inhibited the phosphorylation of PI3K/Akt, ERK1/2 and p38 MAPK, but not JNK, in a dose-dependent manner. These results suggest that frondoside A may be used to suppress MMP-9 activity via modulation of PI3K/Akt, ERK1/2 and p38 MAPK signals that have been stimulated by TPA.

Discussion

MMP-9 is an important regulatory molecule in a range of physiological processes. Recent studies show that MMP-9 is expressed differently in well-differentiated and poorly differentiated tissue samples and may play a key role during the development of breast cancer cells (26). Stimulation of breast cancer cells by the TPA-induced release of MMP-9 is an important intermediary of tumor metastasis. Therefore, the inhibition of MMP-9 would be an effective therapeutic approach for breast carcinomas. Many recent papers have reported the anti-metastatic effects of natural products that are useful therapeutic agents which disrupt the MMP-9 activation associated with metastatic cancer cells (27). Frondoside A is purified from sea cucumbers. Several studies have demonstrated the anticancer effects of frondoside A. We investigated frondoside A suppression of cell invasion via its inhibitory effect on MMP-9 expression. We also provided detailed molecular mechanisms for the first time, using TPA-treated breast cancer cells.

The activity of MMP-9 is regulated at three stages: gene transcription, post-transcriptional activation of zymogens, and endogenous expression of TIMPs (7). In our study, we showed that frondoside A inhibits the activity, secretion, and expression of MMP-9 in TPA-stimulated breast cancer cells at the transcriptional and translational levels. TIMP-1 and -2 inhibit the catalytic activity of MMP-9 by binding to activated MMP-9, controlling the degradation of ECM. We investigated the effect of frondoside A on the expression of TIMP-1 and TIMP-2 mRNA. TIMP-1 and TIMP-2 mRNA levels gradually increased with frondoside A concentration (data not shown). Additionally, the pharmacological actions of frondoside A are associated with prevention of AP-1 and NF-κB activation. The MMP-9 promoter region contains multiple DNA binding sites for transcription factors, such as AP-1 (-533 bp and -79 bp) and NF-κB (-600 bp). Upon stimulation by TPA, they are activated and bind to the promoter region to regulate MMP-9 expression (28,29). Activation of AP-1 and NF-κB plays a pivotal role in metastasis because they induce transcription of metastatic-related genes. NF-κB is then free to translocate to the nucleus and activate target genes, including MMP-9. c-Jun and c-Fos are members of the AP-1 family and translocate to the nucleus in TPA-stimulated breast cancer cells. AP-1 is an important transcription factor for MMP-9 expression. Therefore, many current anti-metastatic therapies seek to block AP-1 and NF-κB activity. The present study demonstrated that frondoside A inhibited nuclear translocation and transactivity of AP-1 and NF-κB, as assessed by western blot analyses and promoter assays. We investigated the functional significance of AP-1 and NF-κB transactivation in breast cancer cell MMP-9 activation. Treatment with curcumin and Bay-11-7802, potent inhibitors of AP-1 and NF-κB transcriptional activation, respectively, reduced the inductive effect of TPA on the activity, secretion, and protein expression of MMP-9. Bay-1101 and curcumin also reduced the TPA-induced transcriptional activity of AP-1 and NF-κB. These findings collectively suggest that frondoside A inhibits TPA-induced activation of MMP-9 by suppressing both AP-1 and NF-κB activation in breast cancer cells.

Frondoside A also significantly inhibited PI3K/Akt, ERK1/2 and p38 MAPK activation in TPA-stimulated breast cancer cells, indicating that frondoside A inhibits TPA-induced NF-κB and AP-1 activation via inactivation of the PI3K/Akt, ERK1/2 and p38 MAPK signaling pathways. Researchers have recently demonstrated that the PI3K/Akt and MAPK pathways are involved in the expression of MMP-9 in breast cancer cells by way of their role in the activation of AP-1 and NF-κB. Therefore, future experiments should be performed to determine whether frondoside A induces anti-metastatic effects in TPA-stimulated breast cancer cells through tight regulation of PI3K/Akt and MAPK expression. In response to TPA stimulation, PI3K/Akt and MAPKs are activated via phosphorylation of both tyrosine and threonine residues, and this phosphorylation leads to the activation of AP-1 and NF-κB. In this study, frondoside A inhibited the phosphorylation of PI3K/Akt, ERK1/2 and p38 MAPK, but had no effect on JNK phosphorylation. Our results indicate that in breast cancer cells, frondoside A is a potent inhibitor of the PI3K/Akt, ERK1/2 and p38 MAPK activation that results from TPA stimulation. This suggests that the anti-metastatic effects of frondoside A are due to inhibition of the PI3K/Akt, ERK1/2 and p38 MAPK signaling pathways.

In conclusion, our study focused on the frondoside A found in sea cucumbers. We evaluated the inhibitory effect of frondoside A on TPA-induced clonogenicity, invasion, and migration in breast cancer cells. Our data show that frondoside A inhibited TPA-induced cell clonogenicity, invasion, and migration. We demonstrated that frondoside A has an inhibitory effect on TPA-induced breast cancer cells through a reduction of MMP-9 enzymatic activity, secretion, and expression. Furthermore, frondoside A is a potent inhibitor of TPA-induced MMP-9 expression and blocks the NF-κB, AP-1, PI3K/Akt, ERK1/2 and p38 MAPK signaling pathways in breast cancer cells. Therefore, frondoside A is a potential agent for prevention of the metastasis of breast carcinoma *in vivo*.

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