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Aromatic-Turmerone Attenuates Invasion and Expression of MMP-9 and COX-2 Through Inhibition of NF-κB Activation in TPA-Induced Breast Cancer Cells

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ABSTRACT

Recent evidence suggests that breast cancer is one of the most common forms of malignancy in females, and metastasis from the primary cancer site is the main cause of death. Aromatic (ar)-turmerone is present in *Curcuma longa* and is a common remedy and food. In the present study, we investigated the inhibitory effects of ar-turmerone on expression and enzymatic activity levels of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced matrix metalloproteinase (MMP)-9 and cyclooxygenaase-2 (COX-2) in breast cancer cells. Our data indicated that ar-turmerone treatment significantly inhibited enzymatic activity and expression of MMP-9 and COX-2 at non-cytotoxic concentrations. However, the expression of tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, MMP-2, and COX-1 did not change upon ar-turmerone treatment. We found that ar-turmerone inhibited the activation of NF-κB, whereas it did not affect AP-1 activation. Moreover, The ChIP assay revealed that in vivo binding activities of NF-κB to the MMP-9 and COX-2 promoter were significantly inhibited by ar-turmerone. Our data showed that ar-turmerone reduced the phosphorylation of PI3K/Akt and ERK1/2 signaling, whereas it did not affect phosphorylation of JNK or p38 MAPK. Thus, transfection of breast cancer cells with PI3K/Akt and ERK1/2 siRNAs significantly decreased TPA-induced MMP-9 and COX-2 expression. These results suggest that ar-turmerone suppressed the TPA-induced up-regulation of MMP-9 and COX-2 expression by blocking NF-κB, PI3K/Akt, and ERK1/2 signaling in human breast cancer cells. Furthermore, ar-turmerone significantly inhibited TPA-induced invasion, migration, and colony formation in human breast cancer cells. J. Cell. Biochem. 113: 3653–3662, 2012.

KEY WORDS: AR-TURMERONE; INVASION; TPA; MMP-9; NF-κB

Breast cancer is the second leading cause of metastasis-related deaths among females worldwide. Prognosis of breast cancer patients is strongly associated with the stage of the cancer at its time of initial discovery. If the breast cancer is detected when there is an invasive state, then the prognosis is poorer. In fact, metastasis is the main cause of mortality in breast cancer patients. Accordingly, the major focus of breast cancer treatment is to identify chemo-preventive drugs for the treatment of breast cancer metastasis [Jemal et al., 2010; Zwiefel and Janni, 2011].

Cancer metastasis is a multi-step process involving the detachment of cancer cells from the primary cancer, followed by adhesion, migration, and invasion into the bloodstream or lymphatic vessels, extravasation out of the lymphatic vessels mediated by MMPs, and interactions with target tissues. Cancer invasion and metastasis are associated with extracellular matrix (ECM) degradation as well as MMP activity. MMP-9 is considered to be one of the critical MMPs involved in cancer invasion and has been found to be directly associated with metastasis and poor prognosis of breast cancer [Brinckerhoff and Matrisian, 2002; Velinov et al., 2010]. Therefore, inhibition of MMP-9 activity is important for reducing metastasis, which is the key to cancer progression. Expression of MMP-9 can be stimulated by various agents, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), inflammatory cytokine TNF-α, or growth factor EGF, during various pathological processes such as cancer invasion, atherosclerosis, inflammation, and rheumatoid arthritis. Consequently, inhibition of MMP-9 expression and/or its upstream

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regulatory pathways is critical in treating malignant cancers, including breast cancer [Shin et al., 2007; Weng et al., 2008].

Inappropriate up-regulation of MMPs and COX-2 frequently occurs in various types of cancer cells, with recent studies implicating the aberrant up-regulation of MMPs and COX-2 in the development of breast cancer. Furthermore, in breast cancer, high expression of MMPs and COX-2 may serve as a prognostic biomarker for the presence of breast cancer [Larkins et al., 2006; Roy et al., 2009]. A recent article also reported the over-expression of MMP-9 and COX-2 in highly invasive, estrogen-independent breast cancer MBA-MB-231 cells [Kim et al., 2006]. COX-2 is present in many solid cancers, such as breast and colon cancers, and may play a critical role in cancer progression. Up-regulation of COX-2 blocks apoptosis, promotes angiogenesis, induces invasion, and increases metastasis in human cancer cells. The up-regulation of COX-2 and PGE2 may be involved in cancer cell invasion by stimulating the expression of MMPs [Ishizaki et al., 2006; Kwak et al., 2007].

Metastasis is the organized response of cancer progression and involves the up-regulation of many genes, of which NF-κB is a critical transcription factor. The promoter regions of MMP-9 and COX-2 are highly conserved and have been shown to contain NF-κB-binding sites [Takada and Aggarwal, 2003]. Lines of evidence have shown that NF-κB regulates the expression of MMP-9 and COX-2 through the PI3K/Akt and MAPK signaling pathways, which play key roles in regulating metastasis. It is predominantly a heterodimeric complex of p65/Rel A and p50, and it is present as an inactive homodimer or heterodimer within the cytoplasm. As a dimer, NF-κB associates through its Rel homology domain with an inhibitory molecule, IκBα, a member of the IκB protein family. NF-κB then translocates to the nucleus where it binds to the NF-κB-binding sites present in the promoter regions of MMP-9 and COX-2 [Shin et al., 2007; Ralhan et al., 2009]. Therefore, inhibition of NF-κB activation may have a potential therapeutic effect in cancer metastasis.

Recent attention has focused on identifying cancer chemo-preventive phytochemicals derived from food and medicinal plants. The turmeric oil isolated from *Curcuma longa*, has been used for this purpose for centuries in Southeast Asia as both a remedy and a food. Its major constituents are aromatic-turmerone, alpha-turmerone, beta-turmerone, alpha-santalene, and aromatic-curcumene [Singh et al., 2010]. The turmeric oil possesses anti-tumor, anti-inflammatory, anti-fungal, and anti-oxidative activities [Wuthi-udomlert et al., 2000; Aratanechemuge et al., 2002; Singh et al., 2010]. Aromatic-turmerone (ar-turmerone) also has various biological activities such as anti-oxidant and anti-platelet effects [Lee, 2006]. However, the effects of ar-turmerone from *C. longa* on cancer metastasis have not been investigated. Elucidating the signaling pathways that mediate inflammation may help identify novel inhibitory compounds with low toxicity and high selectivity for killing metastatic cancer cells. In the present study, we investigated the possible inhibitory activity of ar-turmerone on TPA-induced up-regulation of MMP-9 and COX-2 in human breast MDA-MB-231 cells. Here, we provide evidence that ar-turmerone inhibits TPA-induced MMP-9 and COX-2 expression by blocking NF-κB, PI3K/Akt, and ERK1/2 signaling. Furthermore, we show that ar-turmerone inhibits colony formation, migration, and invasion of breast cancer cells. Together, these findings reveal new mechanisms through which ar-turmerone mediates its anti-invasive and anti-metastatic activities. These findings may be useful in developing novel therapeutic strategies against cancer metastasis.

MATERIALS AND METHODS

MATERIALS

BD BioCoat™ Matrigel™ Invasion Chambers were obtained from BD Biosciences (San Jose, CA). Cell culture medium, RPMI 1640, and fetal bovine serum (FBS) were purchased from Gibco BRL (now part of Invitrogen Corporation, Carlsbad, CA). FuGENE-6 transfection reagent and X-treme GENE siRNA Transfection Reagent were purchased from Roche Applied Science (Indianapolis, IN). Antibodies against phosphorylated p38 (p-p38), p-JNK, p-ERK, p-IκB α, MMP-2, and MMP-9 were purchased from Cell Signaling Technology (Beverly, MA). AKT 2, ERK 1 small interfering RNA (siRNA), and antibodies against COX-1, COX-2, ERK, JNK, p38, c-Jun, c-Fos, and NF-κBand Histone H1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ar-turmerone and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

Human breast cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin at 37°C in a humidified incubator in a 5% CO_2 atmosphere.

DETERMINATION OF CELL VIABILITY

The effect of ar-turmerone on MDA-MB-231 cell viability was determined by MTT assay. Briefly, cells were seeded in wells (5×10^4 cells/24-wells) and incubated at 37°C for 24 h to allow attachment. The attached cells were untreated or treated with 1, 2, 5, 10, and 20 μM ar-turmerone for 24 h at 37°C. Cells were washed with PBS prior to the addition of MTT (0.5 mg/ml of PBS) and incubated at 37°C for 30 min. Formazan crystals were dissolved with dimethylsulfoxide (150 μl/well) and detected at 570 nm using a microplate reader (Wallac 1420; PerkinElmer Life Sciences, Boston, MA).

CLONOGENIC ASSAY USING SOFT AGAR COLONY FORMATION

Colony formation ability was examined by anchorage-independent soft agar assay. Briefly, 0.5% agar gel with 10% FBS and 1% penicillin–streptomycin in DMEM was prepared and added to the wells of a six-well culture dish as base agar. MDA-MB-231 cells were plated for anchorage-independent growth analysis in 0.4% agar gel with 10% FBS and 1% penicillin–streptomycin in DMEM supplemented with the target treatment on top of the base agar. The medium was replaced once every 2 days. Cell colonies were then counted at after 2 and 3 weeks with a microscope and digital camera.

CELL INVASION ASSAY

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 of DMEM for

30 min immediately before the experiments. Cells (5×10^4) suspended in 0.5 ml of serum-free medium were added to the upper chamber of the Matrigel-coated filter inserts. After treatment with ar-turmerone for 1 h, 0.5 ml of serum-free medium containing 50 nM of TPA was added to the bottom well as a chemoattractant. The chambers were then 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 at 10× objective.

IN VITRO WOUND-HEALING REPAIR ASSAY

For 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, after which the cells were incubated in medium without serum. The migrated cells were then 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, after which closure of the wounded area was calculated.

GELATIN ZYMOGRAPHY ASSAY

The enzyme activities of MMP-2 and MMP-9 in conditioned medium were determined by gelatin zymography protease assay. Briefly, cells (2×10^5) were seeded in six-well plates and allowed to grow to 80% confluency. The cells were then maintained in serum-free medium for 12 h prior to designated treatments with ar-turmerone and PMA for 24 h. Conditioned media were collected, cleared by centrifugation, and mixed with 2× SDS sample buffer (Invitrogen Corporation), followed by electrophoresis in polyacrylamide gel containing 0.1% (w/v) gelatin. Following electrophoresis, the gels were incubated in renaturing buffer (2.5% Triton X-100) with gentle agitation to remove SDS, followed by incubation in 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% sodium dodecyl sulfate (SDS). The protein content of the cell lysates was then determined using Bradford reagent (Bio-Rad, Hercules, CA). Proteins in each sample (50 μg of 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 then visualized by enhanced chemiluminescence detection (Amersham Biosciences, Piscataway, NJ) using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. Images were acquired using an ImageQuant 350 analyzer (Amersham Biosciences).

RT-PCR AND REAL-TIME PCR

Total cellular RNA was isolated using RNA Spin Mini RNA isolation kits (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using Maxime RT PreMix (Intron Biotechnology, Seongnam, Korea) and anchored oligo-dT_{15}-primers. The amplification sequence protocol consisted of 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were then subjected to 1.5% agarose gel electrophoresis, and images were captured using an ImageQuant 350 analyzer (Amersham Biosciences). Real-time PCR was performed with SYBR Green Master Mix (Applied Biosystems, Foster City, CA) using a Chromo4 instrument (Bio-Rad). The relative amount of target mRNA was determined using the C_t method by normalizing target mRNA C_t values to those for GAPDH (ΔC_t). The real-time PCR cycling conditions were 95°C for 5 min, 40 cycles for 30 s at 95°C, 20 s at 55°C, and 30 s at 72°C, and then fluorescence measurement. The primer sequences used were as follows: MMP-9-sense (5′-ttccctggagacctgagaacc-3′), MMP-9-anti-sense (5′-cggcaagtcttccgagtagttt-3′), COX-2-sense (5′-tacaagcagtggcaaaggc-3′), COX-2-anti-sense (5′-agatcatctctgcctgagtatctt-3′), GAPDH-sense (5′-aggtggtctcctctgacttc-3′), and GAPDH-anti-sense (5′-taccaggaaatgagcttgac-3′).

MEASUREMENT OF PROSTAGLANDIN E_2 CONCENTRATION

The cells were incubated first with various concentrations of ar-turmerone for 1 h and then with TPA for 23 h. Prostaglandin E_2 levels were quantified in the culture media using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions.

IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

MCF-7 and MDA-MB-231 cells were cultured directly on glass cover-slips in 35-mm diameter dishes. Cells were then 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 cells with a 1:100 dilution of polyclonal antibody against NF-κB for 24 h. After extensive washing with PBS, cells were further incubated with a 1:1,000 dilution of secondary fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG antibody for 4 h at room temperature. Cell nuclei were then 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 vivo association of nuclear proteins with the human MMP-9 promoter, chromatin immunoprecipitation (ChIP) analysis was conducted as described previously [Johnson and Bresnick, 2002], with some modifications. Briefly, 2×10^7 MDA-MB-231 cells were incubated in 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 mononucleosomal size. Fragmented chromatin was reacted with antibodies for 3 h at

4°C. Protein–DNA complexes were recovered using protein A agarose beads, washed, and then eluted with elution buffer. Cross-links 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 DNAs were isolated using a DNA purification kit (QIAGEN). Immunoprecipitated DNA was used for each PCR. PCR primers for the MMP-9 promoter (373 bp including NF-κB cluster, Gene Bank accession no., AF538844) were as follows: sense (5′-CACTTCAAAGTGGTAAGA-3′), anti-sense (5′-GAAAGTGATGGAAGACTCC-3′) and COX-2 promoter (420 bp including NF-κB cluster) were as follows: sense (5′-TCCCGACGT-GACTTCCTCGA-3′), anti-sense (5′-GGAGAG GAGGGAAAA ATT TG-3′).

TRANSIENT TRANSFECTION AND DUAL LUCIFERASE ASSAY

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

STATISTICAL ANALYSIS

All results are expressed as mean ± SE. Each experiment was repeated at least three times. Statistical significances were compared between each treated group and analyzed by paired Student's *t*-test. Data with $P < 0.05$ were considered statistically significant.

RESULTS

AR-TURMERONE SUPPRESSES TPA-INDUCED MMP-9 AND COX-2 EXPRESSION IN HUMAN BREAST CANCER MDA-MB-231 CELLS

We carried out the effects of ar-turmerone on MMP-9, MMP-2, COX-1, COX-2, TIMP-1, and TIMP-2 expression in estrogen receptor-negative and aggressive human breast cancer MDA-MB-231 cells. TPA treatment resulted in an increase in MMP-9, COX-2, TIMP-1, and TIMP-2 expression, whereas TPA had little effect on the expression of MMP-2 or COX-1. Pretreatment with ar-turmerone notably inhibited MMP-9 and COX-2 expression in comparison with TPA-treated control cells, whereas ar-turmerone had no effect on TIMP-1 or TIMP-2 expression. We further examined the inhibitory effects of ar-turmerone against TPA-induced MMP-9 and COX-2 enzyme activity. Cells were treated with ar-turmerone 1 h prior to TPA addition for another 24 h. Conditioned medium was collected, and the enzyme activity of MMP-9 and COX-2 was analyzed using gelatin zymography and prostaglandin E_2 (PGE_2) ELISA kit. Treatment with TPA for 24 h dramatically induced MMP-9 enzyme activity, whereas MMP-2 enzyme activity was not affected by TPA. Treatment of MDA-MB-231 cells with ar-turmerone suppressed TPA-induced MMP-9 activity in a dose-dependent manner in the concentration range of 10–30 μM. Furthermore, treatment of MDA-MB-231 cells with ar-turmerone decreased TPA-stimulated secretion of MMP-9 in a dose-dependent manner (Fig. 1A). As shown in Figure 1B, treatment of MDA-MB-231 cells with TPA also resulted in an increase in PGE_2 released in comparison with the control. However, ar-turmerone inhibited TPA-mediated PGE_2 production in a dose-dependent manner. To determine whether or not the inhibition of MMP-9 and COX-2 expression by ar-turmerone is due to reduced transcription, we performed real-time RT-PCR. Treatment of breast cancer cells with ar-turmerone significantly inhibited TPA-induced MMP-9 and COX-2 mRNA expression in a dose-dependent manner (Fig. 2A,B). However, ar-turmerone showed no inhibitory effect on MMP-9 and COX-2 expression and enzymatic activity at 10–40 μM in estrogen receptor-positive and non-aggressive human breast cancer MCF-7 cells (Fig. 1A,B). Thus, to confirm that the inhibitory activity of ar-turmerone is mediated by direct cytotoxic action in human breast cancer cells, we examined the cytotoxicity of ar-turmerone in breast cancer cells. Ar-turmerone at concentrations lower than 30 μM showed no cytotoxic effects on human breast cancer cells (Fig. 1C), indicating that ar-turmerone was not toxic to human breast cancer cells. These results suggest that ar-turmerone inhibits MMP-9 and COX-2 expression and enzymatic activity in the estrogen receptor-negative and aggressive human breast cancer MDA-MB-231cell at non-cytotoxic concentrations.

AR-TURMERONE INHIBITS MMP-9 AND COX-2 ACTIVITY THROUGH SUPPRESSION OF NF-KB ACTIVITY

Expression of MMP-9 and COX-2 is known to be regulated by transcription factors such as AP-1 and NF-κB that interact with their respective binding elements in the MMP-9 and COX-2 gene promoters [Chou et al., 2010; Ratovitski, 2010]. To further elucidate whether or not inhibition of MMP-9 and COX-2 activity and expression by ar-turmerone is mediated through inhibition of AP-1 and NF-κB signaling, we used inhibitors specific for AP-1 (Curcumin) and NF-κB (Bay-11-7802) [O-charoenrat et al., 2004; Wang et al., 2009]. Human breast cancer MDA-MB-231cell were pretreated with Curcumin or Bay-11-7802 or ar-turmerone for 1 h and then stimulated with TPA for 24 h. According to the results, Curcumin or Bay-11-7082 inhibited TPA-induced MMP-9 and COX-2 expression (Fig. 3A). These results indicate that the suppression of NF-κB and AP-1 activity may be responsible for inhibition of MMP-9 and COX-2 expression. Since ar-turmerone decreased the expression of MMP-9 and COX-2, we examined whether or not these transcription factors are regulated by ar-turmerone in TPA-stimulated breast cancer cells. For this, cells were transiently transfected with NF-κB or AP-1-Luc reporter plasmids, which contain the luciferase gene driven by NF-κB or AP-1-responsive elements, respectively. Transfected breast cancer cells were treated with ar-turmerone in the presence of TPA. As shown in Figure 3B, the TPA-induced increase in NF-κB responsive promoter activation was suppressed by ar-turmerone in a dose-dependent manner. However, in cells treated with ar-turmerone in the presence of TPA, the AP-1 responsive promoter activity was not affected, suggesting that ar-turmerone regulated the transcriptional activation of MMP-9 via inhibition of TPA-stimulated NF-κB activity but

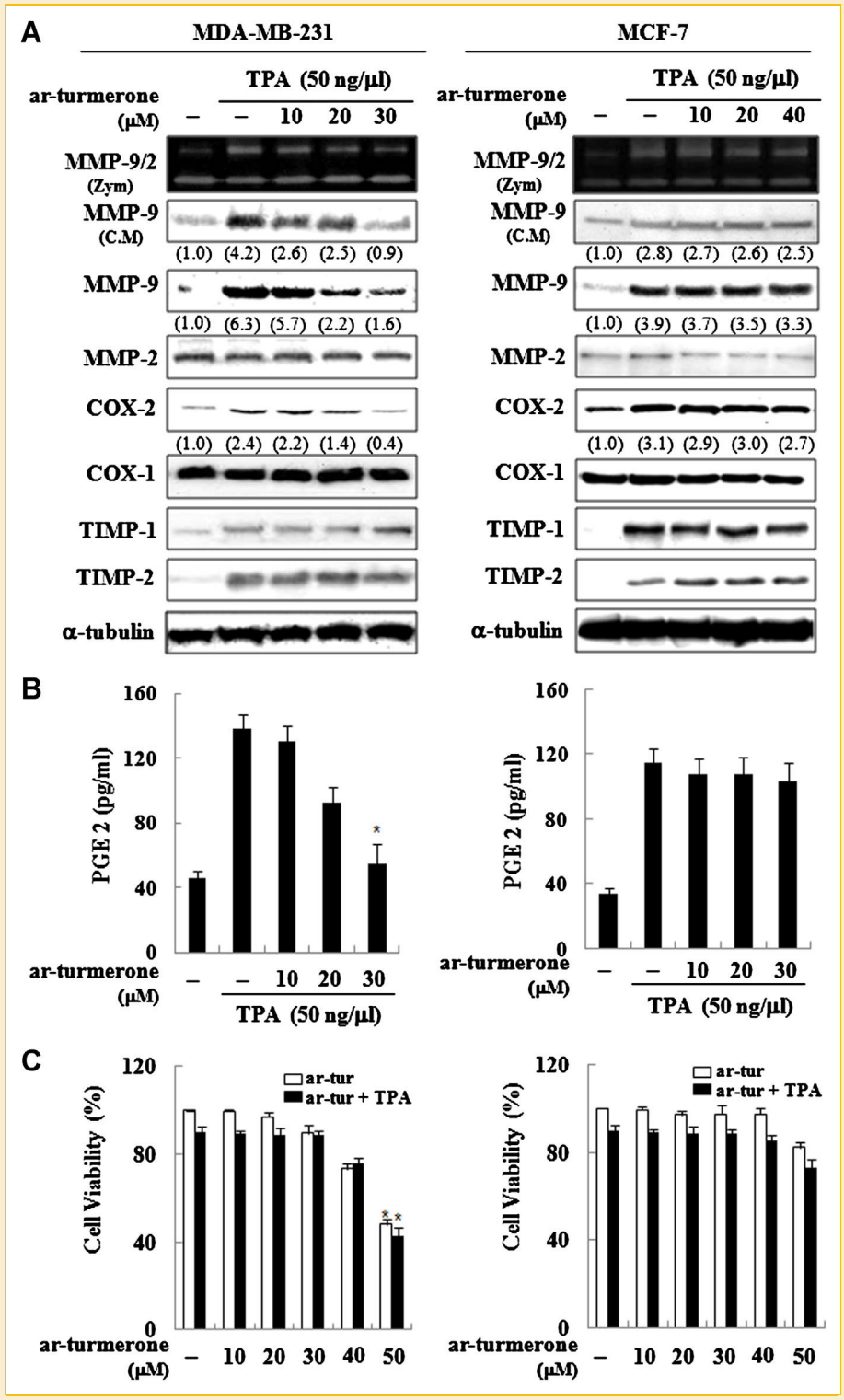

Fig. 1. Effects of ar-turmerone on MMP-9, MMP-2, COX-1, COX-2, TIMP-1, and TIMP-2 expression in human breast cancer cells. A: MDA-MB-231 and MCF-7 cells were treated with ar-turmerone for 1 h, followed by TPA (50 ng/ml) treatment for 24 h. MMP-9 and MMP-2 enzymatic activities were analyzed by gelatin zymography (Zym), secretion by Western blotting (Conditioned medium: C.M.), and intracellular protein expression by Western blotting. Protein levels of MMP-9, MMP-2, COX-1, COX-2, TIMP-1, and TIMP-2 were evaluated by Western blotting. Western blot detection of α-tubulin was performed using a protein-loading control for each lane. Numbers at bottom are expressed as relative intensity of band (fold of control) estimated by using Image Quant TL software. B: PGE_2 was measured in the cultured supernatant by using an ELISA kit. C: Effect of ar-turmerone on cell viability. Cells were treated with indicated concentration of ar-turmerone in the presence of TPA (50 ng/ml) for 24 h. Cell viabilities were determined by MTT assay. Each bar represents the mean ± SE from three independent experiments in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus TPA-treated group.

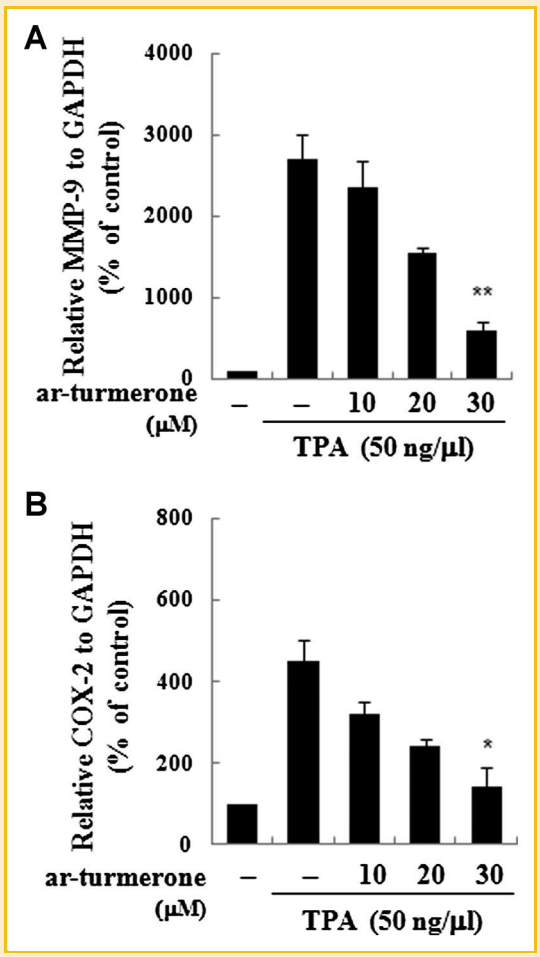

Fig. 2. Effects of ar-turmerone on MMP-9 and COX-2 mRNA expression in human breast cancer MDA-MB-231 cells. MDA-MB-231 cells were treated with TPA only or with ar-turmerone for 6 h. Relative MMP-9 (A) and COX-2 (B) mRNAs expression ($2^{-\Delta C_t}$) was performed by real-time PCR and calculated by subtracting the C_t value for GAPDH from the C_t value for MMP-9 and COX-2 which were determined by real-time RT-PCR relative to GAPDH mRNA $\Delta C_t = C_t$ MMP-9 or COX-2–C_tGAPDH. Quantitative analyses of MMP-9 and COX-2 mRNA expression are expressed as mean ± SE from three independent experiments in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus TPA-treated group.

not by affecting AP-1 activity. Next, cells were treated with different concentrations of ar-turmerone in the presence of TPA for 1 h, and nuclear extracts were prepared and tested by Western blotting. TPA-induced nuclear translocation of AP-1 and NF-κB, whereas ar-turmerone inhibited the nuclear translocation of NF-κB but not AP-1 (Fig. 3C). Similar results were also found using confocal microscopy (Fig. 3D). As we identified one NF-κB-binding site (−600) in the MMP-9 promoter and one NF-κB-binding site (−500) in the COX-2 promoter, we used ChIP-PCR assay to determined whether or not NF-κB is involved in TPA-induced MMP-9 and COX-2 gene expression. Chromatin was extracted and immunoprecipitated using anti-NF-κB antibody, and the MMP-9 and COX-2 promoter regions were amplified by PCR. As shown in Figure 3E,F, in vivo binding of NF-κB to the MMP-9 and COX-2 promoters increased in response to TPA treatment, whereas TPA-induced NF-κB binding to the MMP-9 and COX-2 promoters was significantly inhibited by ar-turmerone. Our cumulative data suggest that the inhibition of MMP-9 and COX-2 promoter activation by ar-turmerone in breast cancer cells occurs through NF-κB inactivation.

AR-TURMERONE INHIBITS TPA-MEDIATED PI3K/Akt AND ERK SIGNALING

MMP-9 and COX-2 gene expression can be activated by a number of signal transduction pathways, including those involving PI3K/Akt and MAPKs, which are upstream modulators of NF-κB [Huang et al., 2005; Cho et al., 2007]. To evaluate the effects of ar-turmerone on these signaling pathways, we examined its effects on TPA-induced MAPK phosphorylation using Western blotting. MDA-MB-231 cells were pretreated with different concentrations of ar-turmerone for 1 h and then stimulated with TPA for 1 h. Whereas TPA increased the levels of PI-3K/Akt, ERK1/2, JNK, and p38 MAPK phosphorylation, ar-turmerone specifically inhibited TPA-induced phosphorylation of PI3K/Akt and ERK1/2 in a dose-dependent manner, but not that of JNK and p38 (Fig. 4A). Ar-turmerone alone did not affect the level of phosphorylation. The levels of non-phosphorylated PI3K/Akt, ERK1/2, JNK, and p38 were unaffected by either TPA or ar-turmerone treatment. To identify the PI3K/Akt and ERK1/2 signaling pathways involved in TPA-stimulated MMP-9 and COX-2 expression, the effect of Akt and ERK small interference RNA (siRNA) approach on TPA-stimulated MMP-9 and COX-2 expression were analyzed by Western blotting in Human breast cancer MDA-MB-231cell. TPA-induced MMP-9 and COX-2 expression were inhibited by Akt and ERK siRNA (Fig. 4B). These results suggest that ar-turmerone suppresses TPA-induced MMP-9 and COX-2 expression via modulation of PI3K/Akt and ERK1/2 signaling in breast cancer cells.

AR-TURMERONE INHIBITS TPA-INDUCED COLONY FORMATION, MIGRATION, AND INVASION OF HUMAN BREAST CANCER CELLS

Cancer metastasis is a multistep and complex process that includes cell proliferation, proteolytic digestion of the ECM, cell migration to the circulation system, and cancer growth at metastatic sites [Liotta et al., 1991]. To investigate the effects of ar-turmerone on the invasive potency of breast cancer cells, we carried out wound-healing, transwell invasion, and soft agar colony formation assays with human breast cancer MDA-MB-231 cells. The anti-invasive potential of ar-turmerone was determined by measuring its effect on the TPA-induced clonogenicity of breast cancer cells in a soft agar clonogenic assay. Figure 5A,D shows that ar-turmerone exhibited an inhibitory effect on the TPA-induced clonogenic ability of breast cancer cells. We next assessed the effect of ar-turmerone on cell invasion in a transwell invasion assay. According to quantitative assessment, treatment with 30 μM ar-turmerone inhibited cell migration across Matrigel by 53% after 24 h (Fig. 5B,D). In the same part of the experiment, wound-healing assay indicated that TPA-induced migration of breast cancer cells was inhibited by ar-turmerone (Fig. 5C). To examine whether TPA-induced invasion by regulating the MMP-9 and COX-2, we were performed using a MMP-9 and COX-2 small interference RNA (siRNA) approach. Knockdown of endogenous MMP-9 and COX-2 in MDA-MB-231

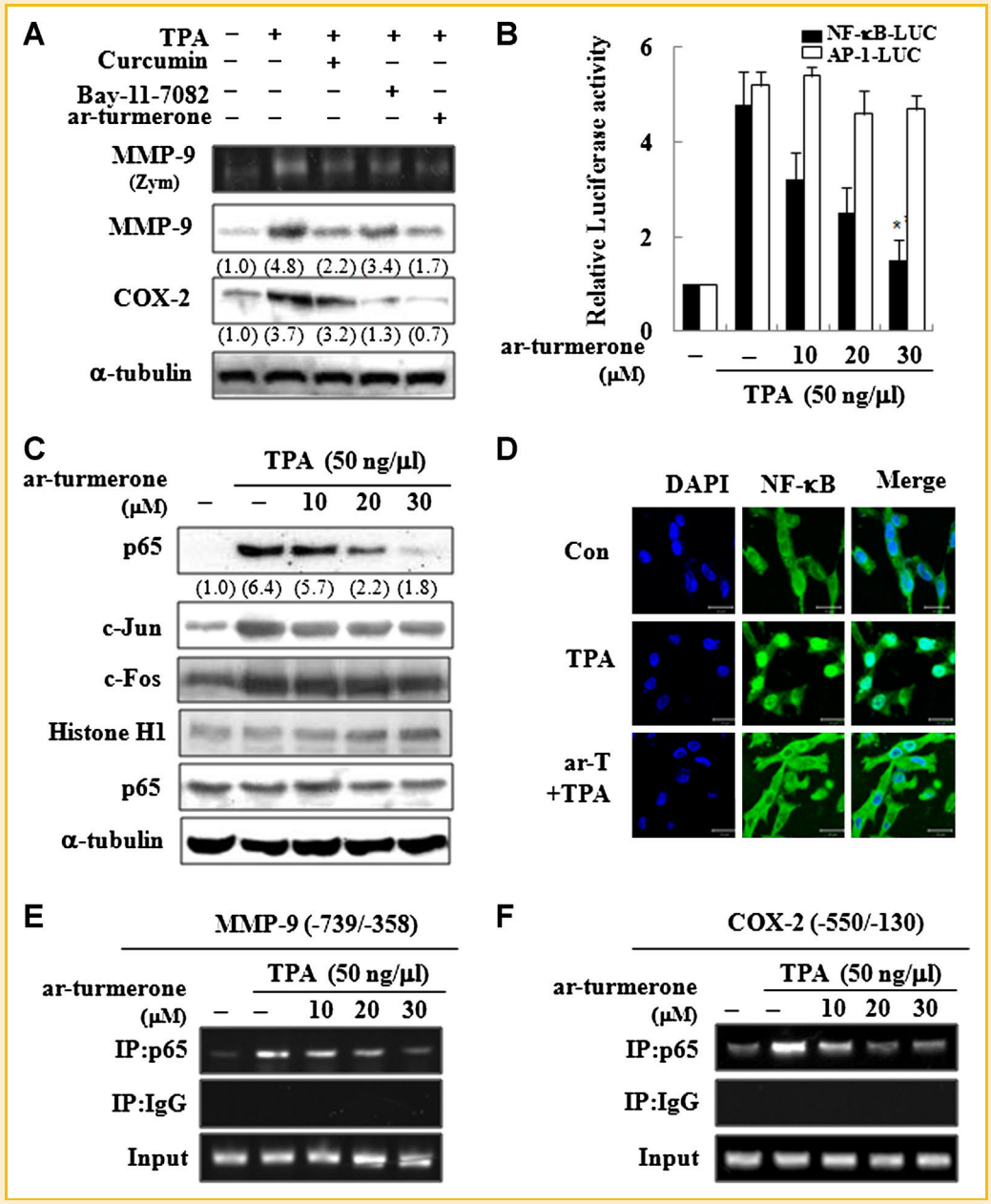

Fig. 3. NF-κB is involved in ar-turmerone-mediated down-regulation of MMP-9 and COX-2 expression. A: MDA-MB-231 Cells were treated with TPA (50 ng/ml) for 24 h in the presence of Curcumin (AP-1 inhibitor, 10 μM), Bay-11-7802 (NF-κB inhibitor, 10 μM) and ar-turmerone (30 μM). Subsequently, MMP-9 enzymatic activity was analyzed by gelatin zymography (Zym), secretion by Western blotting (conditioned medium: C.M.), and intracellular protein expression by Western blotting. Numbers at bottom are expressed as relative intensity of band (fold of control) estimated by using Image Quant TL software. B: Cells were transfected with NF-κB or AP-1-Luciferase reporter. After 24 h, cells were incubated with the indicated concentrations of ar-turmerone for 1 h, followed by stimulation 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 NF-κB or AP-1-luciferase activity. Each bar represents the mean ± SE from three independent experiments in each group. $^{*}P < 0.05$ versus TPA-treated group. C: The cells were treated with ar-turmerone for 1 h, followed by TPA (50 ng/ml) treatment for 1 h. Nuclear extracts and total cell extracts were prepared and analyzed by Western blotting by using anti- NF-κB p65, c-Jun and c-Fos antibody. For western blotting detection of Histone H1 and α-tubulin was used as a protein loading control for each lane. D: Nuclear translocation of NF-κB p65 was assessed by confocal microscopy. The cell were pre-treated with ar-turmerone for 1 h and stimulated with TPA 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 taken by confocal microscopy (bar is 20 μm). E,F: Cells were incubated with ar-turmerone for 1 h and then with TPA for 4 h. DNA immunoprecipitated with anti-NF-κB p65 antibody were purified as described in Materials and Methods Section. Precipitated MMP-9 promoter region (−739 to −358) and COX-2 promoter region (−550 to −130) were amplified by PCR. The input represents PCR products from chromatin pellets prior to immunoprecipitation.

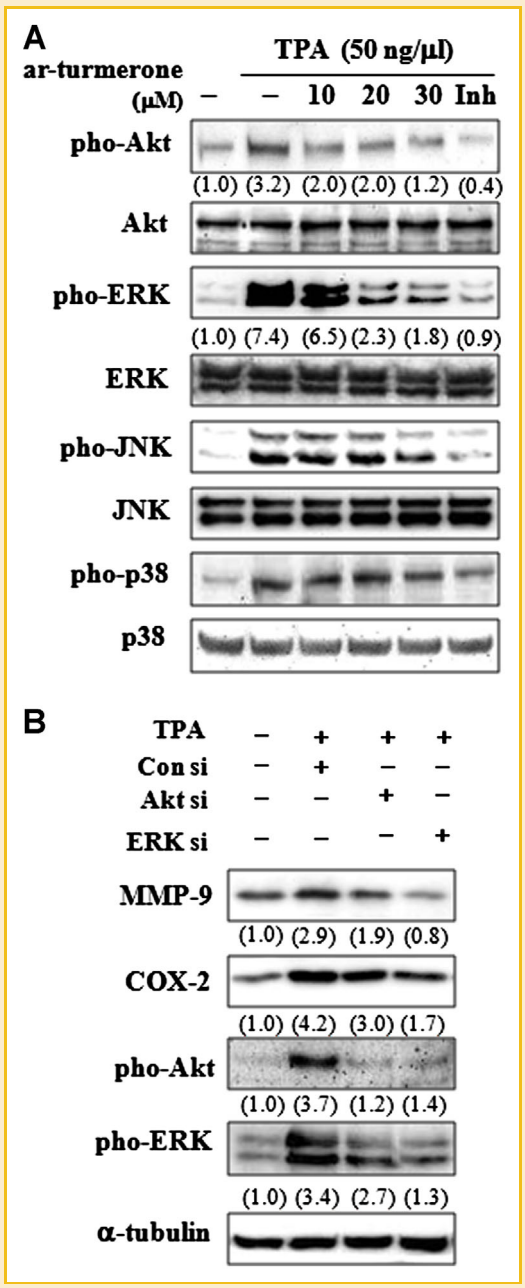

Fig. 4. Effect of ar-turmerone on TPA-induced activation of PI3K/Akt and MAPK signaling pathways. A: MDA-MB-231 cells were treated with the indicated concentrations of ar-turmerone or specific kinase inhibitors (LY294002 for Akt, SB203580 for p38, PD98059 for ERK and SP600125 for JNK, 10 μM, respectively) for 1 h, followed by stimulation with TPA (50 ng/ml) for 1 h. Equal amounts of cell extract were analyzed by Western blotting with anti-phospho-Akt, ERK1/2, JNK, and p38 antibodies. B: Cells were transfected with the siRNA for Akt or ERK or control siRNA (Con) using the X-treme GENE reagent according to the manufacturer's instruction. At 48 h after transfection, cell were treated with TPA (50 ng/ml) for 24 h. The expression of MMP-9, COX-2, phosphorylation of Akt and ERK protein were examined by Western blot. Numbers at bottom are expressed as relative intensity of band (fold of control) estimated by using Image Quant TL software.

cells suppressed TPA induce invasion when compared to control siRNA (Fig. 5E,F). These results that cell invasion are inhibited by ar-turmerone are consistent with the inhibition of MMP-9 and COX-2 expression by ar-turmerone. Therefore, these results suggest that the anti-metastatic effect of ar-turmerone is related to the inhibition of MMP-9 and COX-2 expression in breast cancer cells.

DISCUSSION

MMP-9 and COX-2 are associated with the pathophysiology of cancer progression as well as inflammatory disorders. Aberrant over-expression of MMP-9 and COX-2 has been observed in human cancers, such as breast, colon, and cervical cancers. Several studies have shown that abnormal MMP-9 and COX-2 expression may play a key role in inflammatory-related diseases [Kim et al., 2006, 2009; Jeong et al., 2007]. Based on these findings, certain natural anti-inflammatory compounds such as Curcumin and resveratrol have been found to exert anti-carcinogenic effects. Natural products having anti-carcinogenic effects obstruct the initiation, development, and progression of cancer by regulating various processes such as proliferation, differentiation, apoptosis, angiogenesis, and metastasis [Weng and Yen, 2012]. In recent years, much attention has been focused on the anti-cancer properties of natural products. Ar-turmerone is an abundant component of turmeric, which has been traditionally used in cooking, medicines, cosmetic formulations, and fabric dying for over 2,000 years in Asia. Ar-turmerone has been shown to be highly biologically active and possesses anti-oxidant, anti-inflammatory, and anti-cancer properties [Lee, 2006; Prakash et al., 2011]. Recently, the anti-metastatic effects of partially purified *C. longa* and Curcumin have been reported [Anand et al., 2008]. However, the effects of other components of this plant as well as the molecular mechanisms underlying its anti-metastatic effects have not been investigated. In this study, we demonstrated that ar-turmerone suppressed colony formation, invasion, and migration of breast cancer cells through inhibition of MMP-9 and COX-2 expression.

MMP-9 is an important regulatory molecule in cancer progression, and its over-expression is mostly associated with migration and invasion of various cancers. Thus, carcinoma could be prevented through the inhibition of MMP-9. The activity of MMP-9 is regulated at three steps: gene transcription, post-transcriptional activation of zymogens, and endogenous expression of TIMPs. Therefore, intense research has been undertaken in order to identify selective MMP-9 inhibitors. In the present study, we observed that ar-turmerone inhibited the activity of MMP-9 in TPA-stimulated breast cancer cells and down-regulated the enzymatic activity, secretion, and expression of MMP-9 at the transcriptional and translational levels [Egeblad and Werb, 2002]. However, we also found that ar-turmerone had no effect on TIMP-1 and TIMP-2 expression. COX-2 is associated with the inflammatory response, and its expression is up-regulated in human cancers, including breast cancer. Several studies have reported that COX-2 plays a central role as a regulator of chemotherapy resistance in cancer. Therefore, inhibitors of COX-2 have been developed as anti-inflammatory as well as anti-cancer drugs. Our data also show that

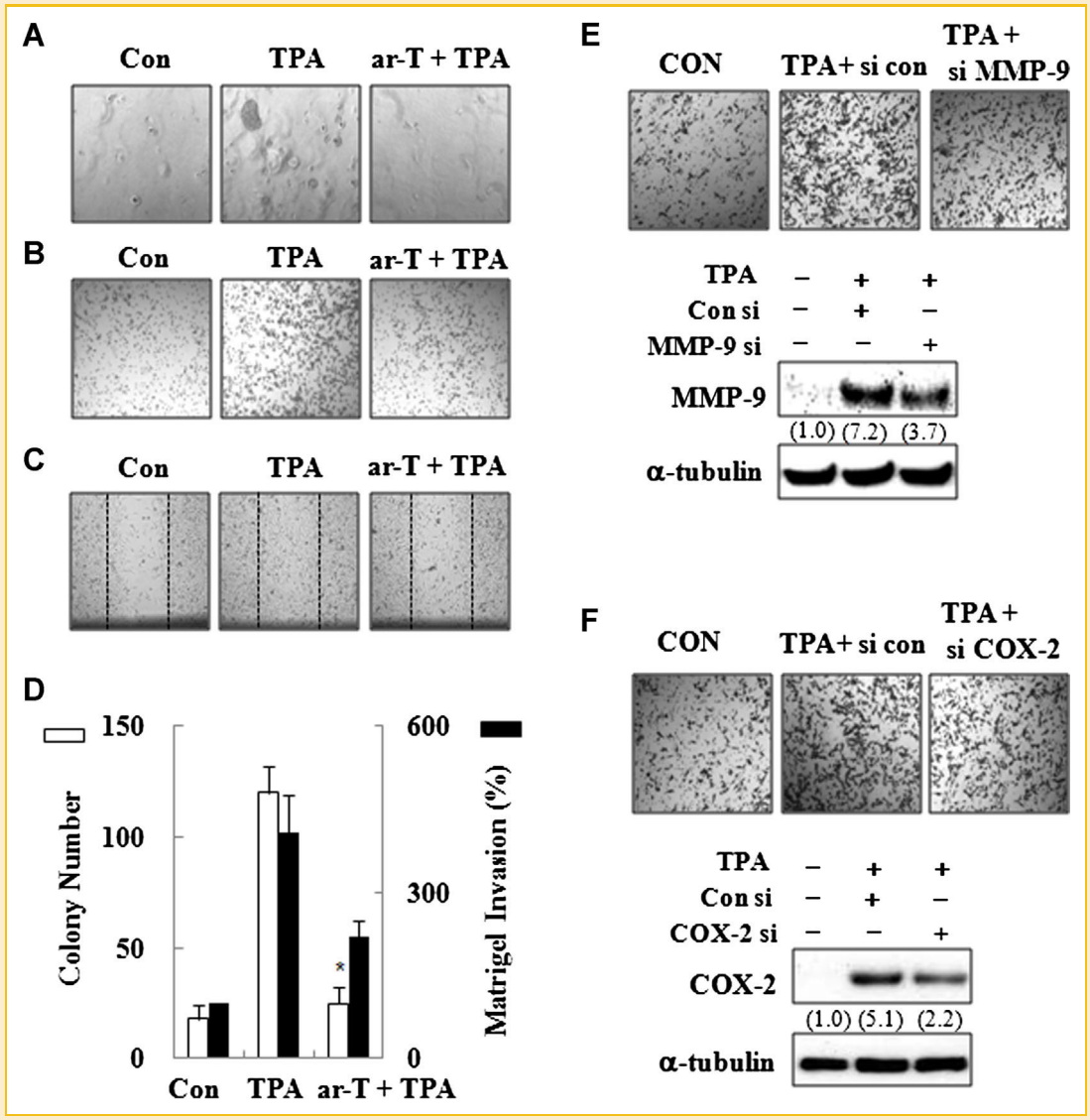

Fig. 5. Inhibitory effects of ar-turmerone on colony formation, migration, and invasion of human breast cancer cells. A: Clonogenicity of MDA-MB-231 cells were determined by a soft agar colony formation assay as described in Materials and Methods Section. B: MDA-MB-231 cells were treated with ar-turmerone (30 μM), followed by TPA (50 ng/ml) treatment for 24 h. Cells that invaded into 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 pretreated with ar-turmerone (30 μM), followed by TPA (50 ng/ml) treatment for 24 h. Migrating cells were photographed under phase contrast microscopy. D: Quantified levels of cell invasion and clonogenicity are expressed relative to that of basal invasion of TPA-untreated cells. Each bar represents the mean ± SE from three independent experiments in each group. $^{*}P < 0.05$ versus TPA-treated group. E,F: Cells were transfected with the MMP-9 si, COX-2 si or Con siRNA using the X-treme GENE reagent according to the manufacturer's instruction. At 48 h after transfection, cell were treated with TPA (50 ng/ml) for 24 h. Cells that invaded into the bottom side of the filter were stained with crystal violet and images were taken by microscope. The expression of MMP-9, COX-2, protein were examined by Western blot. Numbers at bottom are expressed as relative intensity of band (fold of control) estimated by using Image Quant TL software.

ar-turmerone inhibited TPA-induced expression of COX-2 at the transcriptional and translational levels.

In addition, we were able to conclude that the pharmacological actions of ar-turmerone are associated with inhibition of NF-κB activation. Activation of NF-κB plays a pivotal role in metastasis due to its ability to induce transcription of metastatic-related genes. NF-κB is suppressed in the cytoplasm as it is in a complex with an inhibitory IkB protein. Upon activation, NF-κB is free to translocate to the nucleus and activate target genes, including MMP-9 and COX-2. Therefore, many current anti-metastatic therapies seek to block NF-κB activity [Garg and Aggarwal, 2002]. In the present study, we demonstrated that ar-turmerone inhibited the increased activation and nuclear translocation of NF-κB, as assessed by Western blot analyses and promoter assay, respectively. Furthermore, we determined whether or not NF-κB endogenously binds to the MMP-9 and COX-2 promoters in human breast cancer cells. We observed that ar-turmerone inhibited binding of NF-κB to the MMP-9 and COX-2 promoters in a ChIP assay. Taken together, these results argue the possibility that ar-turmerone disrupts the recruitment of NF-κB to the MMP-9 and COX-2 promoters. According to the results

of this study, ar-turmerone inhibits TPA-induced activation of MMP-9 and COX-2 by suppressing NF-κB activation in breast cancer cells.

In summary, the present data demonstrated that ar-turmerone suppresses TPA-induced MMP-9 and COX-2 expression by inhibiting the activation of NF-κB via PI-3K/Akt and ERK signaling in human breast cancer MDA-MB-231 cells. We were able to confirm that ar-turmerone inhibits TPA-stimulated invasion of human breast cancer MDA-MB-231 cells with consequent suppression of MMP-9 and COX-2 expression. Therefore, our results suggest that ar-turmerone could be used as a potential treatment for breast cancer metastasis in vivo.

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