The anti-metastatic effects of the phytoestrogen arctigenin on human breast cancer cell lines regardless of the status of ER expression

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Abstract. Arctigenin is a plant lignan extracted from Arctium lappa that has been shown to have estrogenic properties. In spite of the health benefits of phytoestrogens reducing the risk of osteoporosis, heart disease, and menopausal symptoms, its benefits against the risk of breast cancer have not been fully elucidated. Thus, we investigated the effects of arctigenin on metastasis of breast cancer using both estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines to see if the effects are dependent on the status of ER expression. In ER-positive MCF-7 cells, arctigenin efficiently inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell migration and invasion. The activity of crucial metastatic protease matrix metalloproteinase (MMP)-9 in gelatin zymography was also efficiently decreased by arctigenin, as well as its mRNA expression. Notably, arctigenin exhibited similar anti-metastatic effects even in ER-negative MDA-MB-231 cells, suggesting that the anti-metastatic effects of arctigenin were not exerted via the ER. The upstream signaling pathways involved in the regulation of MMP-9 and urokinase plasminogen activator (uPA) were analyzed using western blotting. The activation of Akt, NF-κB and MAPK (ERK 1/2 and JNK 1/2) was found to be inhibited. Taken together, these data suggest that arctigenin confers anti-metastatic effects by inhibiting MMP-9 and uPA via the Akt, NF-κB and MAPK signaling pathways on breast cancer, regardless of ER expression. Therefore, we propose that the intake of arctigenin could be an effective supplement for breast cancer patients.

Introduction

Arctigenin is a phenylpropanoid dibenzyl butyrolactone lignan (Fig. 1) found in members of the Asteraceae family eg: Arctium lappa (1). Arctium lappa, commonly known as burdock, and its extracts have long been consumed in Europe, North America and Asia. It is also traditionally used in folk medicine for treating sore throat and various infections. Several studies have shown that arctigenin possesses therapeutic effects for anti-viral (2-4), anti-inflammatory (5,6), immune modulation (7-10) and antitumor activities. Particularly, the antitumor activity of arctigenin has drawn much scientific interest and it has been tested in various human cancer cell lines, showing promising results in cancer cells of the stomach (11,12), liver (12), pancreas (13,14), intestine (15-17), lung (12,18,19), bladder (20) and ovaries (21). Regarding the effects of arctigenin in breast cancers, Hsieh et al (22) showed that arctigenin markedly inhibited the growth of ER-negative MDA-MB-231 cells by triggering the mitochondrial caspase-independent apoptotic pathway. However, as lignans have been identified as phytoestrogens, arctigenin has also been reported to have estrogenic properties (23), making it an interesting candidate as breast and other hormone responsive cancer treatments.

When breast cancers express estrogen receptors, estrogen is considered to promote tumor growth. Since phytoestrogens are plant-derived chemicals that are structurally and/or functionally similar to estrogen, the role of phytoestrogens in breast cancer therapy is a highly debated topic (24,25). Although their affinities to estrogen receptors are lower than that of estrogen, it is still of concern that a high intake of phytoestrogen may promote estrogen-dependent transcription, leading to increased risk in breast cancer patients, especially those treated with hormone therapy to reduce estrogen effects.
within the breast tissue. However, there is growing evidence that phytoestrogens have a protective effect on the initiation of breast cancers by inhibiting the enzymes involved in the synthesis of estrogen, resulting in the reduced production of estrogen (26-28). Moreover, the low rates of breast cancer in Asia imply the health benefit of soy phytoestrogen, which is abundant in traditional Asian diet, in reducing the risk of breast cancer. Thus, it is still questionable whether phytoestrogens have beneficial effects against the risk of breast cancers, and determine the necessity for breast cancer patients to increase the intake of arctigenin or arctigenin-enriched foods.

Despite the successful treatment of the primary tumor, metastasis is a major cause of lethality in cancer patients. It was reported that 30-75% of patients undergoing surgery and adjuvant treatment developed recurrent metastatic disease and the median survival of patients with metastatic breast cancer is approximately 2 years (29,30). Zhang et al (15) reported that arctigenin inhibited HCT116 colon cancer cell migration and invasion through the regulation of the H1F4A and Wnt/β catenin pathways. However, to the best of our knowledge, the anti-metastatic effect of arctigenin has not been clearly evaluated on breast cancer cells to date. In this study, we assessed the anti-metastatic effects of arctigenin on breast cancer cells. In order to check if the effects of the phytoestrogen arctigenin depended on the status of ER expression we employed both estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines.

Materials and methods

Cell culture. MCF-7 and MDA-MB-231 human breast cancer cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 µg/ml insulin (all from Welgene Inc., Daegu, Korea). MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS only. All media also contained 1% antibiotic-antimycotic solution (Welgene).

Wound healing assay. The cells were seeded in 6-well plates coated with collagen type I (Corning Life Sciences, Bedford, MA, USA) and grown to reach 100% confluence. The monolayers of cells were scratched with a 200 µl pipette tip to create a wound. The cells were then treated with conditioned media containing 0, 10, 50 and 200 µM arctigenin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). A total of 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich, St. Louis, MO, USA) was used to induce cell migration in MCF-7 cells, while the treatment of TPA was omitted in MDA-MB-231 cells due to their inherent invasive properties. Two independent experiments were done in duplicate wells and optical microscopic images were captured from two different areas of each well at 0 and 24 h. The area between the wound edges was measured at each time-point using ImageJ software as described before (31). Wound closure was quantified as previously described (32).

Matrigel invasion assay. The effect of arctigenin on the invasiveness of human breast cancer cells were determined using Matrigel and Transwell chambers (Corning Life Sciences) with 8.0-µm pore polycarbonate filter inserts in 24-well plates. The lower face of the polycarbonate filter (Transwell insert) was coated with Matrigel for 1 h at 37°C. After coating, 3x10⁴ cells were seeded into the Transwell chamber and 750 µl culture media was added to the lower chamber. After 24 h, cells were treated with conditioned media containing 2% FBS and 0, 10, 50 and 200 µM arctigenin (Santa Cruz Biotechnology). TPA (100 nM) was used to induce only MCF-7 cells and not the innately invasive MDA-MB-231 cells. The media in the bottom chamber was also changed to media containing 10% FBS and incubated at 37°C in 5% CO₂ atmosphere for 24 h. Cells that migrated across the membrane were then fixed and stained using hematoxylin and eosin (H&E) staining and photographed under an inverted microscope using x200 magnification.

Protein extraction and western blot analysis. The cells were treated with conditioned media containing 0, 10, 25, 50, 100 and 200 µM arctigenin for 24 h. For induction of invasive characteristics, 100 nM TPA was added for 1 h and then cells were harvested for protein extraction. The cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.5 with 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate-SDS and 2 mM EDTA) containing phosphatase and protease inhibitor cocktail (GenDEPOT, Barker, TX, USA). Lysates were cleared of debris at 13,000 rpm for 20 min at 4°C and protein concentrations were determined using bicinchoninic acid reagent (BCA; Sigma-Aldrich). The proteins were separated by SDS-PAGE and transferred onto the polyvinylidene fluoride (PVDF) membranes at 100 V for 40 min. The membranes were blocked in 5% skim milk in Tris-buffered saline Tween-20 buffer (10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature. All primary antibodies were from Cell Signaling Technology (Beverly, MA, USA), were diluted 3,000 times and incubated with blots overnight at 4°C: phospho (#4060) and total (#4691) Akt, phospho (#3033) and total (#8242) NF-κB, phospho (#4370) and total (#4668) ERK1/2, phospho (#9258) and total (#9165) c-jun, phospho (#4668) and total (#9258) INK 1/2, c-jun (#9165), c-fos (#2250) and GAPDH (#5174). Corresponding HRP-conjugated secondary antibody (#sc-2004; Santa Cruz Biotechnology) diluted 5,000 times was incubated with blots for 1 h at room temperature. The blots were developed and imaged using Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Densitometric analysis was performed using Scion Image (Scion Corp., Frederick, MD, USA) with data from three independent experiments.
**Zymography.** Conditioned media were collected and electrophoresed on 8% SDS-PAGE containing 0.1% (v/v) gelatin under non-reducing conditions. To remove SDS, the gel was washed with 2.5% Triton X-100 for 1 h at room temperature. Gelatinase reaction was carried out at 37˚C in reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl$_2$, 0.04% NaN$_3$) for 18 h in the case of MDA-MB-231 cells and 48 h in the case of MCF-7 cells. The gel was then stained with staining solution (0.05% Coomassie brilliant blue in 45% methanol and 10% acetic acid) and destained with destaining solution (10% acetic acid) and methanol and 10% acetic acid. Proteolysis was detected as clear bands in a dark blue field. Densitometric analysis was performed using Scion Image with data from three independent experiments.

### Table I. Primer sequences and thermal cycling parameters used for RT-PCR.

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<th>Gene</th>
<th>Primer sequence</th>
<th>PCR conditions (temperature and time)</th>
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| MMP-9     | Forward: 5'-TTC ATC TTC CAA GGC CAA TC-3'  
Reverse: 5'-CTT GAC GCT GTC AAA GTT CG-3' | Denaturation 94˚C, 30 sec  
Annealing 55˚C, 30 sec  
Extension 72˚C, 30 sec |
| uPA       | Forward: 5'-CCA ATT AGG AAG TGT AAC AGC-3'  
Reverse: 5'-GCC AAG AAA GGG ACA TCT ATG-3' | 94˚C, 30 sec  
55˚C, 30 sec  
72˚C, 45 sec |
| uPAR      | Forward: 5'-CAT GCA GTG TAA GAC CAA CCG GGA-3'  
Reverse: 5'-AAT AGG TGA CAG CCC GGC CAG AGT-3' | 94˚C, 30 sec  
65˚C, 30 sec  
72˚C, 45 sec |
| GAPDH     | Forward: 5'-ATC CCA TCA CCA TCT CCC AG-3'  
Reverse: 5'-TTC TAG ACG GCA GGT CAG GT-3' | 94˚C, 30 sec  
58˚C, 30 sec  
72˚C, 60 sec |
| 18S rRNA  | Forward: 5'-GTA ACC CGT TGA ACC CCA TT-3'  
Reverse: 5'-CCA TCC AAT CGG TAG TAG CG-3' | 94˚C, 30 sec  
58˚C, 30 sec  
72˚C, 30 sec |

Arctigenin inhibits cell migration and invasion of human breast cancer cells. The effects of arctigenin against metastasis were assessed by the ability of the cells to move and close the gap after creating uniform wounds with a 200 µl pipette in the wound healing assay. Since MCF-7 cells are non-invasive ER-positive breast cancer cells, representing the ER/PR-positive luminal subtype, they were stimulated with the tumor promoter TPA to induce cell migration. However, treatment of TPA was omitted in MDA-MB-231 cells due to their inherent invasive properties. Our results show that arctigenin inhibited cell migration in both cell types. ER-positive MCF-7 control cells showed almost no cell migration. On TPA treatment, however, cell migration and almost complete wound closure were observed within 24 h (Fig. 2A). This TPA-induced cell migration was inhibited by arctigenin in a dose-dependent manner; exerting almost 90% inhibition of cell migration with 200 µM arctigenin treatment (Fig. 2B). MDA-MB-231 cells exhibited high migratory characteristics, showing rapid wound closure, even in the control cells (Fig. 3A). However, similar to the effect of artigenin on MCF-7 cells, arctigenin inhibited the cell migration of MDA-MB-231 cells in a dose-dependent manner (Fig. 3B). The ability of cancer cells to invade and move across matrices were also evaluated using the Matrigel invasion assay. Arctigenin inhibited the TPA-induced invasive characteristics of MCF-7 cells (Fig. 2C) as well as the innate invasiveness of MDA-MB-231 cells (Fig. 3C) in a dose-dependent manner. Thus, our data suggested that arctigenin inhibited cell migration and invasion of human breast cancer cells regardless of the presence of ER.

**Arctigenin inhibits the crucial proteolytic enzyme MMP-9.** For the cancer cells to metastasize, it has to first cross the physical barriers, i.e. the basement membrane and extracellular matrix. This is made possible by several protease enzymes, primarily the matrix metalloproteases (MMPs) that are capable of disrupting the extracellular matrix. The gelatinase MMP-9 is a highly regulated enzyme that is upregulated in the case of invasive breast cancers (34). Therefore, we looked at the effect of arctigenin on MMP-9, at both the activity and transcriptional levels. Since the synthesis and secretion of MMP-9 are induced by a variety of stimuli, including cytokines and TPA, both cell lines were treated with 100 nM TPA for 24 h to induce detectable levels of MMP-9. First, we measured the activity of secreted MMP-9 in the conditioned media using zymography.
The collected media after 24 h treatment with various doses of arctigenin was run on SDS-PAGE gels containing 0.1% gelatin and the gelatinolytic activity could be quantified from the clear bands due to proteolysis after staining the gel with Coomassie Blue. The collected media after 24 h treatment with various doses of arctigenin was run on SDS-PAGE gels containing 0.1% gelatin and the gelatinolytic activity could be quantified from the clear bands due to proteolysis after staining the gel with Coomassie Blue.

Figure 2. Arctigenin inhibits cell migration in ER-positive human breast cancer cell lines. (A) Optical microscopic images of TPA-induced wound healing in ER-positive MCF-7 human breast cancer cells 0 and 24 h after the creation of the wound. (B) Quantification of cell migration confirmed that 100 nM TPA induced cell migration in MCF-7 cells which was significantly inhibited by 200 µM arctigenin. (C) Optical microscopic images of invaded cells after invasion assay confirmed that arctigenin inhibits TPA-induced cell invasiveness of MCF-7 cells. Graphs represent the mean ± SD of two independent experiments done in duplicate wells and images captured from two different areas of each well (n=8). P-values were calculated with respect to TPA treated controls. *P<0.05 and **P<0.01.

Figure 3. Arctigenin inhibits cell migration in ER-negative MDA-MB-231 human breast cancer cells. (A) Optical microscopic images of wound healing in ER-negative MDA-MB-231 human breast cancer cells. (B) Quantification of cell migration shows that arctigenin significantly inhibited cell migration in MDA-MB-231 cells. (C) Optical microscopic images of invaded cells after invasion assay confirm that arctigenin inhibits innate invasive characteristics of MDA-MB-231 cells. Graphs represent the mean ± SD of two independent experiments done in duplicate wells and images captured from two different areas of each well (n=8). P-values were calculated with respect to controls without TPA. **P<0.01.
brilliant blue. The endogenous level of MMP-9 activities were barely detectable in MCF-7 or MDA-MB-231 cells, while TPA treatment amplified MMP-9 activity ~20 times in both MCF-7 (Fig. 4A) and MDA-MB-231 (Fig. 4B) cell lines. However, arctigenin exerted a dose-dependent inhibitory effect in both cell lines, exhibiting almost complete inhibition at 200 µM arctigenin treatment (Fig. 4A and B). The gene expression of MMP-9 at the transcription level was also studied using RT-PCR. TPA treatment amplified MMP-9 mRNA levels ~3 times in both MCF-7 (Fig. 4C) and MDA-MB-231 (Fig. 4D) cell lines. Similar to the effect of arctigenin on MMP-9 activity, the treatment with arctigenin showed a clear inhibitory effect on the expression of MMP-9 gene; in particular, the cells treated with conditioned media containing 200 µM arctigenin showed almost basal level expression of MMP-9 in both cell lines (Fig. 4C and D). Therefore, our data suggested that arctigenin inhibited cell migration of human breast cancer cells by regulating the activity of MMP-9 and its gene expression regardless of the ER status.

Arctigenin suppressed the expression of uPA. It is well known that the activation of MMP-9 is mediated by the active serine protease urokinase-type plasminogen activator (uPA) and its receptor, uPAR (35). The cleavage of plasminogen to generate active plasmin was catalyzed by uPA/uPAR, which facilitates the release of several proteolytic enzymes, including MMPs. Thus, we tested whether the inhibitory effects of arctigenin on MMP-9 enzyme results from the suppressed expression of uPA and uPAR. TPA significantly induced both uPA and uPAR expressions in MCF-7 cells (Fig. 5A and C), while the endogenous levels in invasive MDA-MB-231 cells were relatively high (Fig. 5B and D) and the effect of TPA in inducing uPA and uPAR expression was very mild compared with MCF-7 cells. However, both cell lines showed that the expression of uPA was inhibited by high concentrations of arctigenin (Fig. 5A and B), whereas the uPAR levels remained unchanged (Fig. 5C and D).

Arctigenin inhibits TPA-induced Akt/NF-κB and MAPK signaling in MCF-7 human breast cancer cells. Our data so far showed that arctigenin inhibited cell migration in human breast cancer cells by inhibiting MMP-9 and uPA. The expression of MMP-9 and uPA can be modulated by various intracellular upstream signaling cascades, particularly the PI3K/Akt pathway and MAPK pathways (36). The activation of protein kinase C (PKC) by TPA induces the activation of two transcription factors, NF-κB and AP-1; of which, activations are regulated by the PI3K/Akt and MAPK pathways. To understand the mechanism underlying the suppression of MMP-9 and uPA by arctigenin, we examined whether
the treatment with arctigenin alter the PI3K/Akt and MAPK pathways. Since Akt activates the NF-κB transcription factor that translocates into the nucleus and initiates the transcription of MMP-9 and uPA, we first tested the inhibitory effect of arctigenin on the phosphorylation of Akt and NF-κB. As shown in Fig. 6A, the activated/phosphorylated forms of both Akt and NF-κB, which increased with the TPA treatment, were efficiently inhibited by arctigenin.

The MAPK pathway consists of the ERK 1/2, JNK 1/2 and p38 signaling pathways. Its activation leads to the translocation of c-fos and c-jun into the nucleus to form the transcription factor AP-1, resulting in increased transcription of uPA and MMP-9. As shown in Fig. 6B, the phosphorylation of ERK 1/2 and JNK 1/2 was significantly attenuated by arctigenin treatment in a dose-dependent manner, subsequently leading to a decrease in the expression of c-fos and c-jun. However, the phosphorylation of p38 was not altered by the treatment with arctigenin, suggesting that AP-1 transcription activity is suppressed by arctigenin through ERK 1/2 and JNK 1/2 MAPK pathways, rather than through p38 MAPK pathway.

Taken together, our data suggested that the inhibitory effect of arctigenin on MMP-9 and uPA appears to be mediated through the inhibition of AP-1 and NF-κB activity, via suppressing the phosphorylation of Akt, ERK 1/2 and JNK 1/2 (Fig. 7).

Discussion

According to their chemical structures, phytoestrogens are classified into isoflavones and lignans. Isoflavones are major constituents in soy products while lignans are widely distributed in fruits and vegetables. Arctigenin is a lignan phytoestrogen extracted from greater burdock and is consumed worldwide as foods and beverages. It is also used in traditional medicines for its health benefits. Several scientific studies report the beneficial effects of arctigenin and these include anti-oxidant, anti-inflammatory and antitumor activities. It has been shown that arctigenin inhibits the growth of various cancer cells by inhibiting the mitochondrial respiration (18), induction of apoptosis (11,14,16,20-22) and cell cycle arrest (11,19-21). Studies also show that it kills cancer cells, specifically while sparing the normal cells (37). However, the effect of arctigenin on breast cancer cells has not been fully understood, although arctigenin, as a phytoestrogen, has been reported to have estrogenic properties. Recent studies reported that arctigenin possesses anticancer effect
on ER-negative MDA-MB-231 cells by the mitochondrial caspase-independent apoptotic pathway (22). However, they observed that arctigenin showed neither a proliferative nor an anti-proliferative effects on ER-positive MCF-7 cells, suggesting that the antitumor effect of arctigenin may not be related to the ER signaling pathway.

In the present study, we demonstrated that arctigenin confers anti-metastatic effects on human breast cancer cells regardless of the presence of ER. Arctigenin efficiently inhibited cell migration in highly invasive MDA-MB-231 cells and also the TPA-induced cell migration in MCF-7 cells. This inhibition was shown to be dose-dependent, with
Figure 7. Proposed action mechanism of arctigenin for the inhibition of cell migration and invasion via the Wnt/β-catenin pathway in breast cancer cells. Arctigenin inhibited the two main regulatory pathways for MMP-9 expression, the MAPK/AP-1 and Akt/NF-κB pathways. This inhibited downstream MMP-9 synthesis at the transcription level, and a decreased proteolytic activity led to the impairment of cell migration.

Our data show that arctigenin decreased MMP-9 at the transcriptional level with a corresponding suppression in its activity level, which was observed with gelatinase zymography. The other metastatic protease uPA was also inhibited by arctigenin, whereas the uPAR levels remained unchanged. Zhang et al. (15) have previously reported the inhibition of cell migration and invasion via the Wnt/β-catenin pathway in human colon cancer cells. They also showed that arctigenin inhibited MMP-9 along with MMP-2. The activity of MMP-9 in various tumor cells is tightly controlled, with regulation occurring primarily at the transcription level. The promoter of human MMP-9 contains cis-acting regulatory elements that bind with transcription factors such as nuclear factor-kappa B (NF-κB) or activator protein-1 (AP-1) (36). NF-κB and AP-1 are ubiquitous eukaryotic transcription factors that can be induced by multiple stimuli. NF-κB, a heterodimer of p50 and p65, is sequestered in the cytoplasm under normal conditions due to its association with the inhibitory protein, IκBα. Stimulation by inflammatory cytokines or tumor promoters leads to the dissociation of IκBα from NF-κB and the activated NF-κB translocates into the nucleus, binding to the promoter region of MMP-9 and leading to gene expression. AP-1 is a nuclear transcription factor that is comprised of homodimers and heterodimers of the members of the Fos and Jun family proteins. NF-κB and AP-1-dependent MMP-9 expression is regulated by Akt and MAPKs, respectively. In this study, we proved that arctigenin can inhibit metastasis of breast cancer cells through these pathways (Fig. 7). The activated Akt and NF-κB, within 1 h of TPA treatment, was significantly inhibited by arctigenin. Furthermore, the phosphorylation of ERK 1/2 and JNK 1/2 were significantly attenuated by the treatment with arctigenin in a dose-dependent manner, which subsequently led to a decrease in the expression of c-fos and c-jun. However, the phosphorylation of p38 was not altered by the treatment with arctigenin. Although TPA is known to activate all three MAPKs i.e. ERK 1/2, JNK1/2 and p38, several studies showed that the activation of p38 depends on concentration or time-points of TPA treatment or cell lines (40,41). Since activation of p38 was not observed in this study, we presumed that TPA treatment for 24 h was unable to induce p38 signaling in MCF-7 human breast cancer cells. This suggests that the AP-1 transcription activity is suppressed by arctigenin through ERK 1/2 and JNK 1/2 MAPK pathways, rather than through the p38 MAPK pathway.

Taken together, these data suggest that arctigenin confers anti-metastatic effects by inhibiting MMP-9 and uPA via the Akt, NF-κB and MAPK signaling pathways on breast cancers, regardless of ER expression. Therefore, we propose that consumption of burdock tea and arctigenin-enriched food products may be a safe supplement for breast cancer patients in preventing metastasis.

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References


