Introduction

Breast cancer is the most common female malignancy and the second leading cause of cancer mortality worldwide (1). Invasion and metastasis are fundamental properties of malignant cancer cells and primary factors that promote cancer progression (2, 3). The invasion and metastasis of cancer cells involve multistep biological processes and various cytophysiological changes including the degradation of tissue barriers such as the extracellular matrix (ECM) and basement membrane (1, 3). ECM degradation and remodeling require the action of proteolytic enzymes, along with matrix metalloproteinases (MMPs) (1, 3). MMPs are a family of highly homologous zinc-dependent, protein-degrading endopeptidases (4 – 6). Currently, 28 members of this family have been identified which include collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-7), matrilysins, and membrane-type MMPs (MMP-14, MMP-15, MMP-16, and MMP-17). MMP family members are important components of many normal biological processes including embryonic development, angiogenesis, and wound healing. They also participate in various pathological processes such as tumor invasion, atherosclerosis, inflammation, and rheumatoid arthritis (2, 4 – 6). In particular, MMP-2 (gelatinase-A, 72 kDa) and MMP-9 (gelatinase-B, 92 kDa) are known to be involved in the degradation of type IV collagen and to have structural and catalytic similarities. MMP-2 is constitutively expressed in tissues and is mainly regulated at the post-transcriptional level through interaction with the tissue inhibitor of metalloproteinase-2. In contrast, the expression of MMP-9 is largely controlled at the transcriptional level and can be induced by various physical stimulators, growth factors (fibroblast growth factor-2,
epidermal growth factor, and hepatocyte growth factor), cytokines (tumor necrosis factor-alpha; TNF-α), or chemicals such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (7). Among these stimulators, TPA can act as a tumor promoter that induces MMP-9 expression in certain cancer cells. MMP-9 is also known to be activated by transcription factors such as nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) through the PI3K/Akt, c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK signaling pathways (3 – 6). Cadin-2-en-1-β-epoxide, was isolated from cultures of Catharanthus roseus hairy roots (8). C. roseus is a widely used ornamental and medicinal plant that belongs to the family Apocynaceae and is cultivated mainly for its alkaloids. C. roseus has been reported to have pharmacologic properties such as anti-cancer (9), enzymatic anti-oxidant (10), and anti-diabetic (11) effects. However, the anti-effects of CR4-1 have not been fully characterized. The purpose of this study was to evaluate the effects of CR4-1 on the TPA-induced expression of MMP-9, urokinase-type plasminogen activator (u-PA), and interleukin (IL)-8, three factors that are involved in tumor invasion and metastasis. The results from our study suggest that CR4-1 inhibits TPA-induced ERK phosphorylation and prevents TPA-induced MMP-9 and IL-8 expression in MCF-7 human breast adenocarcinoma cells. Taken together, these results indicate that CR4-1 may be an effective anti-cancer reagent that prevents cancer metastasis through the down-regulation of MMP-9 in breast cancer cells.

Materials and Methods

Reagents and antibodies

CR4-1 was isolated from the root of Catharanthus roseus (Apocynaceae). The powdered hairy roots of C. roseus (200 g) were immersed in methanol (1.5 l) for three days at room temperature and then the supernatant was concentrated under vacuum to yield 22.5 g of the extract, which was suspended in water and extracted with ethyl acetate and n-butanol successively to produce 11.2 g of ethyl acetate and 7.4 g of n-butanol extract. The entire ethyl acetate extract was subjected to normal phase column chromatography over silica gel (400 g) to yield fraction 15 (250 ml) with ethyl acetate: methanol (95:5, V:V). This fraction was examined by TLC. Fraction 15 was sequentially performed with methanol containing V:V). This fraction was examined by TLC. Fraction 15 (250 ml) with ethyl acetate: methanol (9.5:0.5, V:V). This fraction was examined by TLC. Fraction 15 (250 ml) with ethyl acetate: methanol (90:10, V:V). This fraction was examined by TLC. 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ated using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. MCF-7 cells (1 × 10^4 cells/well) were seeded in a 96-well plate and grown overnight. The cells were pretreated with 20, 40, and 80 μg/ml of CR4-1 for 1 h. After pretreatment, the cells were stimulated with 50 nM TPA in the presence or absence of CR4-1 for 24 h. The cell proliferation assay was repeated 6 times. The effect of CR4-1 on cell proliferation was assessed using the CellTiter 96® AQueous One Solution Assay (Promega). The solution reagent contained MTS and phenazine methosulfate (PMS), an electron-coupling reagent. Aliquots of 20 μl of the AQueous One Solution reagent were then added to each well, and the cells were incubated for another 1 h. Absorbance at 492 nm was determined with a microplate reader (Apple LB 9110; Berthold Technologies GmbH, Bad Wildbad, Germany).

**Matrigel invasion assay**

To measure cell invasion through Matrigel (Sigma), the upper chamber of a Transwell (8 μm pore; Millipore, Billerica, MA, USA) was coated with 100 μg/cm² of Matrigel for 15 min at 37°C and then left for 10 min room temperature. Cells (2 × 10^5) were treated with 20 – 80 μg/ml of CR4-1 and TPA (50 nM) and added to the upper well; the lower wells were filled with culture media. The plates were then incubated for 24 h and cells on the upper side of the membrane were removed with cotton swabs. The membrane was fixed in methanol and cells on the membrane were stained with hematoxylin and eosin. Pictures of the membrane were taken in five random fields (100 × magnification) and the cells were counted.

**Wound-healing assay**

A wound-healing assay was performed using the procedure described by Chen (12) with minor modifications. MCF-7 cells were seeded in a 6-well plate and grown overnight to 80% – 90% confluence. The cell monolayer was scratched with a 200-μl pipette tip. The cells were then washed twice with serum-free culture media to remove floating cells and fresh medium without serum was added. Cells were pretreated with 20 – 80 μg/ml of CR4-1 for 1 h and then stimulated with 50 nM TPA in the presence of CR4-1 for 24 h; DMSO was used as a vehicle control. Cells migrating from the leading edge of the wound were photographed at 0 and 24 h.

**Western blotting**

MCF-7 cells (3 × 10^5 cell/well) were seeded in a 6-well plate and incubated overnight. The cells were then washed three times with DMEM and pretreated with 40 and 80 μg/ml of CR4-1 for 1 h. After pretreatment, the cells were stimulated with 50 nM TPA in the presence of CR4-1 for 30 min; DMSO was used as a vehicle control. MCF-7 cells were then washed three times with ice-cold PBS and scraped on ice. Total cell lysates were prepared by incubating cells in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% SDC, 1 mM EDTA, 1 mM EGTA, 1 mM orthovanadate, aprotinin (10 μg/ml) and 0.4 mM phenylmethylsulfonyl fluoride (PMSF)] at 4°C for 30 min; then the lysates were centrifuged at 11,290 × g for 30 min at 4°C, and the supernatant was collected. Equal amounts of protein (30 μg) were subjected to 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated with a blocking buffer [5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST)] for 1 h at room temperature. Membranes were then incubated with the indicated antibodies overnight at 4°C and washed three times (10 min per wash) with PBST. Membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. After washing with PBST, proteins were detected with an enhanced chemiluminescence (ECL) solution (Amersham Bioscience, Buckinghamshire, UK) and X-ray film.

**Gelatin zymogram**

MCF-7 cells (3 × 10^5 cell/well) were seeded in a 6-well plate and incubated overnight. MCF-7 cells were washed three times with DMEM. Cells were treated with various concentrations of CR4-1 followed by TPA (50 nM) treatment for 24 h. MMP-9 activity in the supernatant was assayed by performing substrate gel electrophoresis as previously described (13). Briefly, 20 μl of culture supernatant was loaded on a Zymogram gel supplemented with 0.1% gelatin under non-denaturing conditions to detect the presence of MMP-9. Electrophoresis was performed at a constant voltage of 120 V for 90 min. Gels were washed several times with 2.5% Triton X-100 for 30 min at room temperature and then incubated in zymography incubation buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 10 mM CaCl_2) at 37°C for 18 h. The gels were stained with Coomassie brilliant blue R-250 (Sigma) for 30 min and then destained for 1 h in a solution of 10% acetic acid and 10% methanol. Areas of gelatinolytic degradation appeared as transparent bands on the blue background.

**Reverse transcription–polymerase chain reaction (RT-PCR)**

MCF-7 cells (3 × 10^5 cell/well) were seeded in a 6-well plate and incubated overnight. MCF-7 cells were washed three times with DMEM. Cells were treated with various concentrations of CR4-1 followed by TPA (50 nM) treatment for 24 h. For RT-PCR, total RNA was isolated using...
an Easy-BLUE total RNA Extraction kit (iNtRon Biotechnology, Seoul, South Korea). Reverse transcription was conducted using ProSTAR™ (Stratagene, La Jolla, CA, USA). After cDNA was synthesized using Oligo (dT), the cDNA was amplified by PCR. The sequences of the primers used were as follows: MMP-9, 5′-aatctac cgagccgagct-3′ and 5′-ccaacctagtagacagtcg-3′; uPA, 5′-caccatgagagccctgctgg-3′ and 5′-ggtgtctggatgacgatgtc-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-tgagtcctgtctggatgacgatgtc-3′ and 5′-ctctt ggagccatgtaggccat-3′. PCR was performed using the following program: 95°C for 15 min; then 30 (MMP-9 and uPA) or 25 (GAPDH) cycles of 95°C for 30 s; 57°C for 30 s; and 72°C for 30 s; after PCR, the samples were heated (72°C for 10 min) and cooled to 4°C. The PCR products were separated on 1.2% agarose gels and detected by ethidium bromide staining.

**Enzyme-linked immunosorbent assay (ELISA)**

IL-8 levels in culture supernatant were measured by sandwich ELISA (R&D Systems, Minneapolis, MN, USA). To detect the concentration of IL-8 in MCF-7 cells, flat-bottom, 96-well plates were coated with mouse anti-human IL-8 mAb (720 μg/ml in PBS, R&D Systems). The wells were incubated overnight at room temperature; washed three times with 0.05% Tween-20 in PBS (wash buffer); and blocked with 300 μl 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The wells were washed again, and 100 μl of samples per well were added and incubated for 2 h at room temperature. Recombinant human IL-8 was used as standards. The wells were washed three times with wash buffer and incubated 2 h at room temperature with 100 μl biotinylated goat anti-human IL-8 at concentration of 20 ng/ml (18 μg/ml 1% BSA in PBS, R&D Systems). The wells were washed with wash buffer and incubated with Streptavidin–HRP in 0.1% BSA / 0.05% Tween-20 in PBS for 20 min at room temperature, avoiding direct light. The wells were washed again and 100 μl of tetramethylbenzidine was added to each well and incubated for 20 min at room temperature, avoiding direct light. To stop the reaction, 50 μl of 2 N H2SO4 was added into each well. Developed colors were detected by the Apollo LB 9110 microplate reader at 450 nm (corrected by absorption at 570 nm). The measurement was performed in triplicate.

**Statistical analyses**

Data obtained were analyzed using Student’s t-test and Microsoft® Excel 2007 (Microsoft, Redmond, WA, USA). The following P-values were used to indicate statistical significance: *P < 0.05, **P < 0.01, and ***P < 0.001.

**Results**

CR4-1 suppresses TPA-induced invasion and migration in MCF-7 cells

Prior to investigating the pharmacologic effects of CR4-1 (Fig. 1A) on TPA-induced MCF-7 cell invasion, we first determined the dose-dependent cell proliferation effects of CR4-1 on MCF-7 cells using the MTS assay. Repeated MTS assays revealed that TPA increased MCF-7 cell proliferation and CR4-1 attenuated TPA-induced MCF-7 cell proliferation. Moreover, there was no cytotoxicity of CR4-1 on MCF-7 cells even at the high concentration of 80 μg/ml (Fig. 1B). Cell invasion and wound healing assays were used to investigate the inhibitory effects of CR4-1 on the invasiveness of MCF-7 cells. As illustrated in Fig. 2A, cell invasion was increased among cells treated with TPA, and TPA-induced cell invasion was inhibited by treating cells with 40 or 80 μg/ml CR4-1. Furthermore, wound healing assays indicated that TPA-induced migration of MCF-7 cells was
inhibited by CR4-1 (Fig. 2B).

**CR4-1 dose-dependently decreases TPA-induced MMP-9 and u-PA expressions in MCF-7 cells**

To investigate changes in MMP-9 expression induced by TPA, we treated cells with TPA at various concentrations (0, 25, 50, and 100 nM) for 24 h in serum-free media. These experiments revealed that TPA increased the levels of MMP-9 expression in a dose-dependent manner in MCF-7 cells (Fig. 3A). MMP-9 expression was significantly increased following treatment with 50 nM TPA. This result indicates that TPA may enhance tumor metastasis and inflammation through up-regulation of MMP-9 and u-PA expression in MCF-7 cells. To determine the effect of CR4-1 on TPA-induced MMP-9 and u-PA expression, cells were treated with various concentrations of CR4-1 followed by TPA (50 nM) treatment for 24 h. MMP-9 and u-PA expression was determined by RT-PCR (Fig. 3B) and zymography (Fig. 3C). Our result showed that the levels of MMP-9 and u-PA expression significantly increased in response to TPA in the MCF-7 cells (Fig. 3: B and C). However, the levels of TPA-induced MMP-9 and u-PA expression were decreased by CR4-1 in a dose-dependent manner (Fig. 3: B and C). These results demonstrated that CR4-1 may be a candidate therapeutic agent for treating tumor metastasis and inflammation by inhibiting TPA-induced MMP-9 and u-PA expressions.

**CR4-1 inhibits TPA-induced production of IL-8, a pro-inflammatory cytokine, in MCF-7 cells**

In many cancer cells, TPA up-regulates expression of IL-8 (14, 15) which plays a major role in tumor invasion and metastasis (16, 17). In the present study, we elucidated whether CR4-1 would inhibit TPA induced IL-8 expression in breast cancer cells. As shown in Fig. 4,
CR4-1 inhibited TPA-induced IL-8 production in a dose-dependent manner in MCF-7 cells.

Discussion

CR4-1, a sesquiterpene glycoside, was isolated from cultures of Catharanthus roseus (Apocynaceae) hairy roots (8). C. roseus is known to exert effective cancer preventive and therapeutic effects through inhibition of cell viability, cell cycle progression, and apoptosis in various cancer cell lines (3, 18, 19). However, the mechanism underlying the inhibitory effects of CR4-1 on tumor metastasis and angiogenesis in breast cancer has not been completely elucidated. Therefore, we investigated whether CR4-1 prevents TPA-induced MMP-9 and IL-8 expression and explored the mechanism by which CR4-1 regulates the invasiveness of MCF-7 breast cancer cells.

Tumor metastasis is a multistep and complex process that involves cell viability, proteolytic digestion of ECM, cell migration to the circulatory system, and tumor growth at the metastatic site. MMPs play a key role in the degradation of ECM and basement membranes. Enhancement of MMP expression is involved in tissue remodeling, tumor invasion, metastasis, and angiogenesis, and the inhibition of MMP expression may play an important part in cancer therapy. The expression and activity of MMPs have been widely investigated in various cell types including breast cancer cells (2, 4, 20). In particular, MMP-9 is known to specifically cleave type IV collagen, the major component of basement membranes. Our study revealed that CR4-1 directly inhibited the invasion and migratory abilities of MCF-7 cells via the suppression of MMP-9 expression (Figs. 2 and 3).

The MMP-9 promoter has several transcription factor-
binding motifs including ones for AP-1 and NF-κB. Furthermore, MAP kinases (including ERK, JNK, and p38) are known to be involved in the regulation of MMP-9 transcription (20). However, previous reports showed that TPA-induced MMP-9 expression is inhibited by the MEK1/2 inhibitor, but not by JNK and p38 inhibitors, in MCF-7 cells (3, 4, 18). Similar to these reports, the present study showed that CR4-1 effectively suppressed TPA-induced MMP-9 gene expression by reducing ERK (Fig. 5B) and IκB (Fig. 5A) expression; this resulted in decreased invasion and migration of MCF-7 cells. These results suggest that the inhibition of MMP-9 was specifically regulated by CR4-1. However, CR4-1 led to an increase in phosphorylation of p38/JNK (Fig. 5).

In many cancer cells, TPA up-regulates expression of IL-8 (14, 15), which is elevated in breast cancer cells and plays a major role in tumor invasion and metastasis (16, 17). In the present study, we elucidated whether CR4-1 would inhibit TPA induced IL-8 expression in breast cancer cells. CR4-1 inhibited TPA-induced IL-8 expression (Fig. 4). TPA treatment also led to similar increases in the expression of u-PA. This factor has important roles in matrix proteolysis that facilitates cell invasion and metastasis and is associated with poor prognosis of breast cancer (16, 17, 21 – 23).

In conclusion, CR4-1 inhibited TPA-induced MMP-9 and IL-8 expression and activity by inactivating NF-κB via the ERK signaling pathway in MCF-7 cells. These results indicate that CR4-1 is a potential anti-metastatic and anti-invasion agent. Future studies are needed to further explore the anti-cancer properties of CR4-1 and its potential clinical applications.

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**References**


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