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3-Caffeoyl, 4-dihydrocaffeoylquinic acid from *Salicornia herbacea* inhibits tumor cell invasion by regulating protein kinase C-δ-dependent matrix metalloproteinase-9 expression

Yong Pil Hwang[a,b], Hyo Jeong Yun[a,c], Jae Ho Choi[a,c], Hyo Kon Chun[d], Young Chul Chung[e], Sang Kyum Kim[a], Bong-Hee Kim[a], Kwang-il Kwon[a], Tae Cheon Jeong[f], Kwang Youl Lee[b,**], Hye Gwang Jeong[a,*]

[a] *Department of Toxicology, College of Pharmacy, Chungnam National University, Daejeon 305-764, South Korea*
[b] *College of Pharmacy, Chonnam National University, Gwangju, South Korea*
[c] *College of Pharmacy, Chosun University, Gwangju, South Korea*
[d] *Korea Research Institute of Bioscience and Biotechnology, Daejon, South Korea*
[e] *Division of Food Science, Korea International University, Jinju, South Korea*
[f] *College of Pharmacy, Yeungnam University, Kyungsan, South Korea*

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ABSTRACT

In this study, we determined the effects of a novel chlorogenic acid, 3-caffeoyl, 4-dicaffeoylquinic acid (CDCQ) isolated from *Salicornia herbacea*, on tumor invasion and migration in human fibrosarcoma HT-1080 cells and investigated the possible mechanism(s) involved. CDCQ reduced the phorbol myristate acetate (PMA)-induced activation of matrix metalloproteinase (MMP)-9 and MMP-2 and inhibited cell invasion and migration. CDCQ suppressed PMA-induced expression of MMP-9 mRNA and protein by suppressing the transcription factor AP-1, without changing the level of tissue inhibitor of metalloproteinase (TIMP)-1. CDCQ-inhibited PMA-induced MMP-2 expression by suppressing membrane-type 1 MMP (MT1-MMP), but did not alter the TIMP-2 level. CDCQ also inhibited the PMA-induced nuclear translocation of c-Jun and c-Fos, which are upstream of PMA-induced MMP-9 expression. Furthermore, CDCQ strongly repressed PMA-induced phosphorylation of ERK, p38 MAPK, and JNK, which are dependent on the PKCδ pathway. In conclusion, we demonstrated that the anti-invasive effects of CDCQ occur through the inhibition of AP-1 and signaling pathways involving PKCδ and three MAPKs, leading to the downregulation of MMP-9 expression. Thus, CDCQ is an effective anti-metastatic agent that functions by downregulating MMP-9 gene expression.

1. Introduction

Epidemiological and laboratory studies have shown that dietary agents can be important factors in reducing cancer risk (Sporn and Suh, 2000; Lippman and Hong, 2002). Herbal medicines derived from plants are increasingly being used to treat a variety of clinical diseases, although relatively little is known about their modes of action. *Salicornia herbacea* is a halophyte that grows in salt marshes and muddy seashores along the western coast of Korea (Han et al., 2003). It has been used as a seasonal vegetable in coastal areas (Chinnusamy et al., 2006) and as a folk medicine for disorders such as constipation, obesity, diabetes, and cancer (Bang et al., 2002). A number of investigators have reported anti-oxidative, immunomodulatory, anti-hyperglycemic, and anti-cancer activities of *S. herbacea* (Lee et al., 2006; Kong et al., 2008; Ryu et al., 2009). In our previous studies, we isolated a new chlorogenic acid derivative, 3-caffeoyl, 4-dihydrocaffeoylquinic acid (CDCQ), from *S. herbacea* and demonstrated its anti-oxidative and anti-inflammatory properties (Chung et al., 2005; Hwang et al., 2009; Han et al., 2010). Although various bioactivity studies of CDCQ have been carried out, the molecular mechanism(s) by which CDCQ acts on the expression of matrix metalloproteinase (MMP)-9 and the invasiveness of HT-1080 cells are still unclear.

The MMPs are key enzymes in extracellular matrix degradation and play a substantial role in pathological processes, including inflammation, arthritis, cardiovascular diseases, pulmonary dis-

* Corresponding author. Department of Toxicology, College of Pharmacy, Chungnam National University, Daejeon 305-764, South Korea. Tel.: +82 42 821 5936.
** Corresponding author at: Tel.: +82 62 530 2949.
E-mail addresses: KwangLee@chonnam.ac.kr (K.Y. Lee), hgjeong@cnu.ac.kr (H.G. Jeong).

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eases, and cancer cell metastasis (Cho et al., 2007; Kajanne et al., 2007). Among the human MMPs reported to date, MMP-2 and -9 are the primary enzymes involved in degrading type-I and -IV collagens and extracellular matrix (Westermarck and Kahari, 1999; Chang and Werb, 2001). Both MMP-2 and -9, which are abundantly expressed in various malignant tumors, contribute to cancer cell invasion and metastasis (Kupferman et al., 2000).

Generally, MMP-2 is constitutive and is overexpressed in highly metastatic tumors, whereas MMP-9 can be stimulated by cytokines, growth factors, and phorbol esters, through the activation of different intracellular signaling pathways (Cho et al., 2007; Kajanne et al., 2007). MMP activation is mediated by membrane-type matrix metalloproteinases (MT-MMPs) such as MT1-MMP (Egeblad and Werb, 2002). The concerted action of highly expressed MT1-MMP and adequately expressed tissue inhibitor of metalloproteinase (TIMP) leads to the activation of MMPs (Visse and Nagase, 2003). TIMPs are physiological inhibitors of MMPs. In particular, TIMP-1 binds the hemopexin domain of MMP-9. By inhibiting MMPs, TIMPs can block extracellular matrix degradation, tumor invasion, and metastasis (Kruger et al., 1998).

The major signaling pathways that regulate tumor cell invasion and MMP expression are the mitogen-activated protein kinase (MAPK) (Eberhardt et al., 2000), phosphoinositide 3-kinase (PI3K) (Hennessy et al., 2005), and protein kinase C (PKC) (Shin et al., 2007) signaling pathways. Furthermore, stimulators such as cytokines and phorbol myristate acetate (PMA) control the expression of MMP-9 through the activation of transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), and through the MAPK and PI3K/AKT signaling pathways (Eberhardt et al., 2000; Cho et al., 2007). MMP-9 has binding sites for AP-1 and NF-κB, which are well-known transcription factors that regulate the expression of a number of genes involved in metastasis, tumorigenesis, and inflammation (Garg and Aggarwal, 2002; Jung et al., 2002).

Several studies have indicated that the inhibition of MMP-9 activity may affect tumorigenesis in many ways, including the inhibition of invasion, metastasis, and angiogenesis (Blackburn et al., 2007; Kunigal et al., 2007). Recently, researchers have focused on finding biologically active compounds from natural sources that can inhibit MMP-9 activity and would thus have potential as candidates for therapeutics and functional foods (Ngameni et al., 2006; Yoon et al., 2006). In the present study, we identified the chlorogenic acid derivative CDCQ from *S. herbacea* as an inhibitor of MMP-9 activity. Mechanistic studies revealed that CDCQ potently inhibits MMP-9 expression and tumor cell invasion through the inhibition of AP-1 and signaling pathways involving PKCδ and three MAPKs.

2. Materials and methods

2.1. Materials

CDCQ (Fig. 1A) was isolated from *S. herbacea* as described previously (Chung et al., 2005). Rottlerin (Rot), U1026, SB203580 (SB), and SP600125 (SP) were obtained from Calbiochem (La Jolla, CA, USA). Phorbol-12-myristate-13-acetate (PMA) and curcumin were purchased from Sigma Chemical (St. Louis, MO, USA). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide]-based colorimetric assay kit was purchased from Roche (Indianapolis, IN, USA). Fetal bovine serum (FBS), RPMI1640, sodium pyruvate, and Trizol were from Gibco BRL (Grand Island, NY, USA). Antibodies against phospho-MAP kinase, phospho-PKCδ, MMP-2, and MMP-9 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against c-Jun, c-Fos, lamin B, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The MMP-9 promoter vector was kindly provided by Dr. W. Eberhardt (Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany) (Eberhardt et al., 2002). The pNF-κB-Luc and pAP-1-Luc reporter plasmid were from Stratagene (La Jolla, CA, USA). Other chemicals and reagents were of analytical grade.

2.2. Cell culture and cell treatments

HT-1080 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in RPMI1640 supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. The cells were treated with different concentrations of CDCQ in the absence or presence of PMA (10 nM) for 24 h. The CDCQ had been dissolved in dimethyl sulfoxide (DMSO), but the final DMSO concentration in the cultures did not exceed 0.1%.

2.3. Measurement of cell viability

HT-1080 cells were plated at a density of 4×10^4 cells/500 μL in 48-well plates, and cell viability was determined by a conventional MTT reduction assay. Briefly, after incubation, the cells were treated with MTT solution (final concentration, 1 mg/mL) for 1 h. The dark blue formazan crystals that formed in intact cells were solubilized with DMSO, and the absorbance at 570 nm was measured with a microplate reader (Varioskan, Thermo Electron Co., Berthold, Germany).

2.4. In vitro wound-healing assay

HT-1080 cells were seeded in a 6-well plate and grown overnight to confluence. The cell monolayer was scratched with a 200-μL pipette tip to create a wound and washed twice with serum-free RPMI1640 to remove floating cells. Serum-free medium was added, and the rate of wound closure was assessed and photographed 24 h later. Each value is derived from three randomly selected fields.

2.5. Matrigel invasion assay

HT-1080 cells were incubated in RPMI1640 with 10% FBS and then collected by trypsinization. Cells (1×10^5 cells/mL) in serum-free medium were added to the inner cup of a 48-well Transwell chamber (Corning Life Sciences, Oneonta, NY, USA) that had been coated with 50 μL of Matrigel (1:10 dilution in serum-free medium; BD Biosciences, Franklin Lakes, NJ, USA). Medium supplemented with 10% serum or another indicated agent was added to the outer cup. After 24 h, the cells that had migrated through the Matrigel and the 8-μm pore-size membrane were fixed, stained, and counted under a light microscope. Each experiment was performed in triplicate.

2.6. RNA preparation, semi-quantitative RT-PCT and real-time PCR

Total RNA was isolated with an RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the concentration was measured spectrophotometrically. The RNA (2 μg) was converted to complementary DNA using a RT-PCR Bead kit (Amersham Pharmacia) according to the manufacturer's protocol. The PCR amplification protocol was 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. PCR product formation was monitored continuously during the reaction, using Sequence Detection System software (ver. 1.7; Applied Biosystems, Foster City, CA, USA). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR®). The mRNA levels of MMP-2 and MMP-9 in the treated cells were compared with the expression levels in control cells at each time point, using the comparative cycle threshold (Ct)-method (Johnson et al., 2000). The following primers were used: for MMP-2, forward 5′-AGT CTG AAG AGC GTG AAG-3′ and reverse 5′-CCA GGT AGG AGT GAG AAT G-3′; for MMP-9, forward 5′-TGA CAG CGA CAA GAA GTG-3′ and reverse 5′-CAG TGA AGC GGT ACA TAG G-3′; and for GAPDH, forward 5′-CCA CCC ATG GCA AAT TCC-3′ and reverse 5′-TGG GAT TTC CAT TGA TGA CAA-3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of GAPDH, a housekeeping gene.

2.7. Western blot analysis

After treatment, cells were collected, washed with phosphate-buffered saline (PBS), harvested, and then lysed on ice for 30 min in 100 μL of lysis buffer (120 mM NaCl, 40 mM Tris, pH 8, 0.1% NP40), followed by centrifugation (13,000 × *g*, 15 min). Supernatants were collected and protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in a 10% SDS-polyacrylamide gel. The resolved proteins were transferred to a PVDF membrane. The membrane was blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with specific primary antibodies for 3 h, followed by incubation with the appropriate alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h. Finally, the immunoreactive protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Pierce).