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Novel suppressive effects of cardamonin on the activity and expression of transglutaminase-2 lead to blocking the migration and invasion of cancer cells

Mi Kyung Park [a,1], Seung Ho Jo [b,1], Hye Ja Lee [a], June Hee Kang [a], You Ri Kim [a], Hyun Ji Kim [a], Eun Ji Lee [a], Jae Young Koh [c], Kyung Ok Ahn [b], Kyung Chae Jung [b], Seung Hyun Oh [d], Soo Youl Kim [b], Chang Hoon Lee [a,*]

[a] College of Pharmacy, Dongguk University, Seoul 100–715, Republic of Korea
[b] National Cancer Center, 809 Madu 1-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do 410–769, Republic of Korea
[c] R&D Center, AmorePacific Corporation, 314–1 Boradong, Giheung-gu, Yongin-si, Gyeonggi-do 449–729, Republic of Korea
[d] College of Pharmacy, Gachon University of Medicine and Science, 7–45 Songdo-dong, Yeonsu-gu, Incheon 406–840, Republic of Korea

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ABSTRACT

Aims: Alpinia katsumadai was recently found in our previous study to have anti-migratory and anti-invasion activities against HT-1080 cells. However, the study did not demonstrate the exact component of *Alpinia katsumadai* with anti-migratory and anti-invasive activities. We tested the effects and relevant mechanism of cardamonin (CDN) on the migration and invasion of cancer cells.

Main methods: Migration and invasion of cancer cells were measured using multi-well chambers. Zymography and Western blots were used to examine the effects of CDN on the activities of matrix metalloproteinases (MMPs) and expression of transglutaminase-2 (Tgase-2).

Key findings: CDN, but not alpinetin, dose-dependently suppressed the migration and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced invasion of HT-1080 sarcoma cells. CDN suppressed the expression of Tgase-2, MMP-2, NF-κB and MMP-9 in HT-1080 cells, and suppressed MMP-2 and MMP-9 activities. Gene silencing of Tgase-2 suppressed the migration and invasion of HT-1080 cells and suppressed the activities of MMP-2 and MMP-9. Migration of various cancer cells having high levels of Tgase-2 were also inhibited by CDN. CDN and *Alpinia katsumadai* extracts also directly inhibited the activity of Tgase-2.

Significance: CDN inhibits migration of several cancer cell lines expressing Tgase-2 via suppression of Tgase-2 expression and inhibition of Tgase-2 activity. The finding that CDN has Tgase-2 inhibitory activity will give us a new scaffold or clue of pharmacophore for the development of more effective Tgase-2 inhibitors.

Introduction

Metastasis is the ability of cancer cells to spread from its origin to distant locations within the body and to continue its growth (Valastyan and Weinberg, 2011). The cause of death for the vast majority of cancer patients is the development of metastatic lesions at sites distant from that of the primary tumor (Mendoza and Khanna, 2009). The high mortality rates associated with cancer are caused by the metastatic spread of tumor cells away from the site of their origin. In fact, metastases are the cause of 90% of cancer deaths (Steeg, 2006).

Due to the importance of metastasis, many research studies on the mechanism of metastasis are underway in an attempt to develop a therapy protocol that can modulate the metastasis of cancer cells (Jung et al., 2012; Mazzocca and Carloni, 2009).

Transglutaminase 2 (Tgase-2) is a multifunctional enzyme, which may act as a protein cross-linking enzyme in cancer and inflammation (Kim, 2011; Park et al., 2010). Recently, Tgase-2 is reported to be involved in epithelial mesenchymal transition, a key step in cancer metastasis (Lin et al., 2011). Furthermore, Tgase-2 is overexpressed in highly aggressive and chemo-resistant human brain, breast, lung, and colorectal cancers and is involved in migration and invasion of cancer cells such as breast cancer cells and neuroblastomas (Antonyak et al., 2009; Choi et al., 2011; Joshi et al., 2006; Miyoshi et al., 2010; Oh et al., 2011; Yuan et al., 2005).

Recently, we found that *Alpinia katsumadai* (AK) suppresses migration and 12-O-tetradecanoyl-13-acetate-induced invasion of HT-1080 cells through suppression of Tgase-2 (Park and Lee, 2011). AK has been widely used in traditional Chinese and Korean medicine to treat various diseases including emesis and gastric disorders. Also, AK is known to have anti-pruritic activity, anti-inflammatory activity, and antinociceptive activity (Choi et al., 2009; Choi et al., 2010; Yang et al., 2009).

Cardamonin (CDN: 2′,4′-dihydroxy-6′-methoxychalcone) and alpinetin (7-hydroxy-5-methoxyflavanone) are two major and

* Corresponding author at: College of Pharmacy, Dongguk University, Seoul 100–715, Republic of Korea. Tel.: +82 31 961 5213; fax: +82 31 961 5206.
E-mail address: uatheone@dongguk.edu (C.H. Lee).
[1] Equally Contributed.

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well-known components of AK (Wang et al., 2007). CDN especially has several biological activities including anti-tumor and anti-inflammatory activities (Chow et al., 2012; Yadav et al., 2012). However, the anti-migratory and anti-invasive activities of CDN and its relevant mechanisms have not been reported.

Therefore, we examined the effects of CDN on the migration and invasion of cancer cells including HT-1080, MD-MB231, and SH-SY5Y cells. Here, we demonstrate that CDN has anti-migratory and anti-invasive effects against HT-1080 cells and these effects are mediated by suppressing the expression and activity of Tgase-2.

Materials and methods

Materials

Chemicals and reagents were purchased from Sigma-Aldrich Co., unless specified otherwise. Radioactive [1,4-^{14}C] putrescine dihydrochloride was purchased from GE Healthcare Co., and the stock solution was diluted with distilled water until the radiological dosage reached 20 μCi/ml. Tgase-2 was purchased from Sigma-Aldrich and dissolved in distilled water to a final concentration of 1 unit/ml. All aqueous solutions were stored in a deep freezer before use. CDN and alpinetin were kindly gifted by Dr. Koh (Amore Pacific Co.).

Cell migration assay

Migration assays were performed using a multi-well chamber (Neuroprobe Inc. Gaithersburg, MD) coated with 10 μg/ml fibronectin as a chemoattractant (Park et al., 2011). Briefly, HT-1080 cells were suspended in DMEM at 1×10^6 cells/ml, and a 25 μl aliquot of this suspension was placed into the upper well of the chamber. Next, the aliquot was separated by a 8 μm polyhydrocarbon filter from the 3% serum-containing lower well. After incubation for 4 h at 37 °C, nonmigrated cells on the upper surface of the membrane were scrapped off, and the migrated cells on the lower surface were stained by Diff-quick, which were subsequently counted under five randomly chosen high power fields (400×).

Cell invasion assay

Invasion assays were performed using a 24-well Transwell unit with polycarbonate filters having a diameter of 6.5 mm and a pore size of 8.0 mm (Corning Costar). Briefly, a fixed number of cells (5×10^4 cells/chamber) were used for each invasion assay. The lower and upper parts of Transwell were coated with 20 ml of a 1:2 mixture of Matrigel:DMEM. Cells were plated on the Matrigel-coated Transwell in the presence of various concentrations of CDN. The medium in the lower chambers contained 0.1 mg/ml of bovine serum albumin. After incubation for 24 h at 37 °C, cells invading the lower surface of the membrane were fixed with methanol, and stained with hematoxylin and eosin (H&E stain) which were subsequently counted under five randomly selected fields (400×).

Gelatin zymography

Matrix metalloproteinase (MMP)-2 and MMP-9 enzymatic activities were assayed by gelatin zymography (Park and Lee, 2011). Samples of serum-free conditioned medium were electrophoresed on a 10% SDS-polyacryamide gel. After electrophoresis, the gel was washed in 2.5% Triton X-100 for 1 h and incubated at 37 °C for 24 h in activation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3). After staining with Coomassie brilliant blue R-250, enzymatic activities were detected as clear bands against the blue background.

Tgase-2 gene silencing by small interfering RNA

A small interfering RNA (siRNA) duplex targeting human Tgase-2, 5′-AAGAGCGAGAUGAUCUGGAAC-3′ (Invitrogen), was introduced into the cells using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were harvested, and cell lysates were prepared in order to analyze the level of Tgase-2 by Western blotting. Cells incubated with Lipofectamine RNAiMAX and Stealth Negative control (Invitrogen) were employed as the negative control.

In vitro Tgase-2 inhibition assay

The inhibitory effect of each compound was determined by measuring the incorporation of [1, 4-^{14}C] putrescine into succinylated casein. Following 10 min of pre-incubation of 2.5 milliunits (mU) of Tgase-2 from the guinea pig liver with each concentration of chemicals in 0.1 ml of reaction buffer solution without 10 mM $CaCl_2$, we added 0.4 ml of substrate solution containing 5 mg of succinylated casein and 100 nCi of [1,4-^{14}C] putrescine. After further incubation at 37 °C for 1 h, the reaction was terminated by the addition of 4 ml of cold (4 °C) 7.5% (w/v) TCA. TCA-insoluble precipitates were collected in GF/A glass fiber filters (Millipore Co.), washed with cold 5% (w/v) TCA, dried and assessed for incorporation of radiolabel using a scintillation counter (Beckman Coulter Co.). The IC_{50} value was determined through a logistic linear regression method. The resultant data represent the means of three independent experiments.

Statistical analysis

All data are expressed as percentages of the control and shown as means ± SD. Values of $p<0.05$ were considered significant.

Results

Effects of CDN on the migration and invasion of Tgase-2 expressed cancer cells

We examined the effects of CDN on other cancer cells highly expressing Tgase-2 such as MDA-MB-231 cells and ATRA-treated SH-SY5Y cells. At first, we confirmed the expression of Tgase-2 in these cells by Western blots (Fig. 1A). CDN dose-dependently suppressed the migration of MDA-MB-231 cells (Fig. 1B and C). One day treatment of ATRA induced Tgase-2 expression (Fig. 1A) and induced migration of SH-SY5Y cells (Fig. 1D and E) (Lee et al., 2012). HT-1080 cells also expressed a high level of Tgase-2 (Fig. 1A). The effects of CDN were examined on the migration of HT-1080 cells. The migration of HT-1080 cells was significantly reduced by treatment with 5 μM CDN and was further strongly inhibited with an increased concentration (25 μM) of CDN (Fig. 1F and G). TPA induced the invasion of HT-1080 cells (Park and Lee, 2011) and treatment with increasing concentrations of CDN subsequently decreased the TPA-induced invasion of HT-1080 cells (Fig. 1H and I). These results suggested that CDN is an effective anti-migratory or anti-invasive compound against cancer cells by highly expressing Tgase-2.

Suppression of MMP-2, MMP-9, and Tgase-2 by CDN

Next, we examined whether CDN suppresses the expression of MMP-2, MMP-9, NF-κB p65, IκBα, and Tgase-2 since the extracts of *Alpinia katsumadai* are reported to suppress the expression of MMPs and Tgase-2 (Park and Lee, 2011). Treatment of HT-1080 cells with TPA resulted in an increased level of MMP-2, MMP-9, phospho p65 (pNF-κB p65), and Tgase-2 expression and a decreased expression of IκBα with or without TPA (Fig. 2A and B). However, pretreatment with CDN decreased the expression of MMP-2, MMP-9, pNF-κB p65,