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Decursinol angelate blocks transmigration and inflammatory activation of cancer cells through inhibition of PI3K, ERK and NF-κB activation

Won-Jung Kim [a], Min-Young Lee [a], Jung-Hee Kim [a], Kyoungho Suk [b], Won-Ha Lee [a,*]

[a] School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea
[b] Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

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ABSTRACT

Inflammation is known to be closely associated with the development of cancer. Decursinol angelate (DA), a coumarin compound isolated from *Angelica gigas* and related compounds have been shown to possess potent anti-inflammatory activities. However, little is known about their effects on the inflammatory processes associated with cancer. In this study, the anti-inflammatory effect of DA was evaluated in cancer cell lines with respect to cellular invasion through the extracellular matrix (ECM) and the expression of pro-inflammatory mediators such as cytokine, cell adhesion molecules and matrix metalloproteinase (MMP)-9. DA inhibited the invasion of fibrosarcoma cell line, HT1080 and breast cancer cell line, MDA-MB-231 in the Matrigel invasion assay. DA-mediated suppression of cancer cell invasion was accomplished by suppression of PI3K activity known to be associated with cytoskeletal rearrangement related to cellular migration. DA also suppressed the adhesion of cancer cells to ECM mediated by down-regulation of β_1-integrin expression levels. Furthermore, DA inhibited the expression of pro-inflammatory cytokines and MMP-9 through suppression of PI3K, ERK and NF-κB activation. These results demonstrate that DA suppresses invasion and inflammatory activation of cancer cells through modulation of PI3K/AKT, ERK and NF-κB. These anti-inflammatory activities of DA may contribute to its anti-cancer activity.

1. Introduction

Decursinol angelate (DA) is a coumarin compound shown to have strong anti-cancer activity along with its structural isomer decursin [1,2]. The underlying molecular mechanism of their anti-cancer activity has been the subject of intense investigations. Recent reports demonstrated that DA and decursin induce G1 arrest and apoptosis in prostate and breast cancer cells [3,4]. In addition, these agents suppress androgen-induced cellular proliferation [5] and androgen-independent cellular proliferation by promoting degradation of β-catenin [6] in prostate cancer cell lines. Furthermore, DA and decursin suppress angiogenesis through inhibition of VEGF-induced proliferation, migration and tube formation of human umbilical vein endothelial cells [7–9].

DA and related compounds have been demonstrated to have anti-inflammatory activities in macrophage activation and in an animal model of asthma [10,11]. Since inflammation is now well known to have a close relationship with the onset and the development of cancer [12,13], it is expected that the anti-inflammatory activity of these compounds will in turn contribute to their anti-cancer activity. In order to test this hypothesis, the influence of DA on cancer-associated inflammatory processes was evaluated in various cancer cell lines. DA has been found to inhibit invasion and migration of cancer cells through ECM as well as production of inflammatory mediators. The molecular mechanisms of these activities were then investigated with respect to activation of signaling molecules and transcription factors. The possible contribution of

* Corresponding author. Tel.: +82 53 950 5388; fax: +82 53 943 6925.
E-mail address: whl@knu.ac.kr (W.-H. Lee).

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these anti-inflammatory and anti-migration activities toward DA's anti-cancer activity is also discussed.

2. Materials and methods

2.1. Cell culture and reagents

DA was isolated from the root of *Angelicae gigas* as a structural isomer of decursin as described previously [10]. U0126, monoclonal antibody (mAb) against phospho-IκB (clone 5A5), polyclonal antibodies against IκB, phospho-ERK, ERK, phospho-AKT and AKT were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-β_1-integrin mAb (clone 6S6) was purchased from Millipore (Billerica, MA, USA). Recombinant TNF-α was purchased from R&D systems (Minneapolis, MN, USA). Phorbol 12-myristyl 13-acetate (PMA) and sulfasalazine were obtained from Sigma (Sr. Louis, MO, USA). Cell lines were purchased from ATCC (Rockville, MD, USA). LY294002 was purchased from Calbiochem International Inc. (La Jolla, CA, USA),

2.2. Measurement of cellular invasion and migration

For the measurement of cell invasion through Matrigel (Sigma, St. Luis, MO, USA), the upper part of Transwells (8 μm pore, Millipore) were coated with 100 μg/cm^2 Matrigel for 15 min at 37 °C and then for 10 min at room temperature. Cells (2×10^5) were pretreated with DA for 3 h and added into the upper well; the lower wells were filled with a 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 were stained with Hematoxylin. Pictures (100×) of the membrane were taken in five random fields and the cell numbers were counted. Migration of cells was assessed in a 48-well Boyden chamber (Probe Inc., Gaithersburg, MD, USA). Briefly, the lower wells were filled with 27 μl RPMI (supplemented with 10% serum) and the upper wells were filled with 50 μl of cells at a concentration of 4×10^6 cells/ml, after a 3 h pretreatment period with DA. The two compartments were separated by a polyvinylpyrrolidone-free filter (Neuro Probe Inc.) with 8-μm pores. After incubation for 24 h at 37 °C, the number of cells that had migrated into the lower wells was counted, pictures were taken and cells were counted as described above. The experiments were performed in triplicate samples.

2.3. Gelatin zymogram, western blot, and ELISA

Cancer cells (1×10^4/well) were incubated in 96-well plates in the presence or absence of 20–80 μM of DA and/or 1–20 ng/ml of TNF-α. The culture supernatants were collected 24 h after activation, and gelatin zymogram analyses were performed as described previously [14,15]. Cell lysates were obtained at various time points after activation, and Western blot analysis was performed as described previously [16,17]. For the measurement of cytokine concentrations, culture supernatants were collected 24 h after activation and cytokines were measured by sandwich ELISA (Endogen Inc., Woburn, MA, USA); detection limits were <10 pg/ml.

2.4. RT-PCR

Five microgram of total RNAs isolated from cells were treated with RNase free DNase (BD-Pharmingen), and then used to generate first-strand cDNAs using a Revert-Aid™ first-strand cDNA synthesis kit with 500 ng oligo $(dT)_{12-18}$ primers. PCR primers were designed with ABI PRISM Primer Express 2.0 (Applied Biosystems) and made by Geno Tech Corp (Daejeun, Korea). Primer sequences are 5′AATCTCACCGACAGGCAGCT3′ (forward) and 5′CCAAACTGGATGACGATGTC3′ (reverse) for MMP-9, 5′ATCACTGCCACCCAGAAGAC3′ (forward) and 5′TGAGCTTGACAAAGTGGTCG3′ (reverse) for GAPDH. PCR products were run on 2% agarose gel following PCR reaction to confirm the size and purity of the products.

2.5. Flow cytometry

Flow cytometry analysis was performed using FACS-calibur (Becton–Dickinson, Mountain View, CA). For flow cytometric analysis of cell surface antigens, cells (5×10^5) were pelleted and incubated with 0.3 μg of fluorescence-labeled primary or secondary antibodies in 30 μl of FACS solution (a PBS containing 0.5% BSA and 0.1% Sodium Azide) for 20 min on ice. For background fluorescence, the cells were stained with an isotype-matching control antibody. The fluorescence profiles of 2×10^4 cells were collected and analyzed.

2.6. Cell viability assay and cell adhesion assay

For the measurement of cell viability, Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) was used. Briefly, cells in 96-well plates (1×10^4/100 μl/well) were incubated with DA for 24 h and 10 μl of CCK-8 solution was added into each wells. After a 4 h incubation at 37 °C in 5% CO_2, absorbance at 450 nm was measured using a microplate reader. For the measurement of cell adhesion, culture plates (96-well plates) were coated with 10 μg/ml fibronectin overnight. HT1080 cells pretreated with or without DA for 3 h were added into each well (4×10^5 cells/well). After 30 min, unattached cells were removed by PBS washing and the CCK-8 assay was performed to measure the amount of attached cells.

2.7. Immunofluorescence assay

For subcellular localization of NF-κB, HT1080 cells (2×10^4) were grown on a cover glass, washed in PBS and fixed with 4% formaldehyde in distilled water. The cells were then permeabilized with 1% Triton in PBS for 10 min, incubated with anti-p65 mAb (clone F-6, Santa Cruz, CA, USA) (10 μg/ml in PBS containing 3% BSA) at 37 °C for 45 min, washed, and then incubated with the Alexa Fluor 488-labeled goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA) (10 μg/ml) at 37 °C for 45 min, washed again, counterstained with DAPI