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

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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...
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-β1-integrin mAb (clone 6S6) was purchased from Milipore (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 (St. 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. Louis, MO, USA), the upper part of Transwells (8 μm pore, Millipore) were coated with 100 μg/cm² Matrigel for 15 min at 37°C and then for 10 min at room temperature. Cells (2 × 10³) 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 × 10⁵ cells/ml, after a 3 h pretreatment period with DA. The two compartments were separated by a polycvinylpyrrolidone-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 × 10⁶/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’-AATCTACGGACACGCGCT3’ (forward) and 5’-CCAAACTGATGACGATGTC3’ (reverse) for MMP-9, 5’-ATCAGTGACCAGAGAC3’ (forward) and 5’TAGC- TGTACAAAAGTCG3’ (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 × 10⁵) 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 × 10⁶ 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 × 10⁴/100 μl/well) were incubated with DA for 24 h and 10 μl of CCK-8 solution was added into each well. After a 4 h incubation at 37°C in 5% CO₂, 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 × 10⁵ 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 × 10⁵) 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.
In order to investigate DA’s molecular mechanism of action, various events associated with cancer cell invasion were tested, including cell signaling, cell adhesion and the expression of matrix degrading enzymes. Activation of phosphatidylinositol 3-kinase (PI3K)/AKT is associated with migration and invasion of various types of cells through modulation of cytoskeletal movement [18–20]. As shown in Fig. 2, LY294002 (a specific inhibitor of PI3K) dose dependently blocked the invasion of the HT1080 cells. This confirmed that PI3K/AKT activity was involved in the invasion of HT1080 cells. The effect of decursin on PI3K activity was then assessed. As shown in Fig. 2B, DA dose dependently inhibited TNF-α-induced phosphorylation of AKT (Fig. 2B). The activity of ERK was not involved in migration since the presence of U0126 (a specific inhibitor of ERK) did not affect cellular migration, even though U0126 blocked ERK phosphorylation in cells stimulated with TNF-α (Fig. 2). These results indicate that the suppression of PI3K/AKT activity can be a mechanism responsible for DA-mediated inhibition of invasion and migration of cancer cells.

3.2. DA inhibits cancer cell adhesion through blocking the expression levels of β1-integrin

Inflammatory activation results in the production of pro-inflammatory mediators such as adhesion molecules, cytokines and matrix degrading enzymes. In order to determine whether DA affects cancer cell adhesion, the expression patterns of various adhesion molecules were assessed. High level expressions of β1-integrin was detected in HT1080 cells, while the expression of other adhesion molecules, such as ICAM-1 and CD11c was not detected (data not shown). When HT1080 cells were incubated in the presence of different DA doses, the expression levels of β1-integrin decreased in a dose dependent manner (Fig. 3A). Western blot analysis further confirmed that DA treatment results in reduction of β1-integrin expression levels (Fig. 3B). Accordingly, adhesion of HT1080 cells to fibronectin coated plates was inhibited by DA (Fig. 3C). The involve-
ment of $\beta_1$-integrin in cell adhesion toward fibronectin was demonstrated by blockage of the adhesion process with anti-$\beta_1$-integrin mAb but not with isotype matching mouse IgG (Fig. 3B). DA-induced down-regulation of $\beta_1$-integrin expression levels and subsequent inhibition of cellular adhesion to the ECM can be another mechanism that explains inhibition of cell migration and invasion by DA.

### 3.3. DA inhibits production of pro-inflammatory mediators of cancer cells

Migration of cancer cells through the ECM requires the action of matrix degrading enzymes. MMP-9 is a representative matrix degrading enzyme and is highly induced during the inflammatory processes. Stimulation of HT1080 cells with TNF-$\alpha$ induced MMP-9 expression and it was inhibited by DA (Fig. 4A). PMA-induced production of MMP-9 in MDA-MB-231 cells was also inhibited by DA (Fig. 4C). Suppression of MMP-9 expression in cancer cells by DA is expected to have contributed to DA-mediated inhibition of cellular invasion and migration.

Suppression of cellular invasion or migration and MMP-9 expression by DA may have been caused by its growth suppressive effects or cytotoxicity. DA and/or decursin have already been reported to have suppressive effects on cell cycle progression and induced apoptosis in certain prostate and breast cancer cell lines [3–6]. When cell viability was tested in HT1080 and MDA-MB-231 cells after treatment with DA, the viability of these cell lines was not affected by DA during the time span that has been used in this experiment (Fig. 4B and D). In order to find out whether it was a general phenomenon, various cancer cell lines were compared for the cytotoxic and anti-inflammatory effects. DA exerted a cytotoxic effect on MCF-7 and HeLa S3 cells, while other cell lines such as SW480 and A549 cells were not affected (data Supplement #1). Even though cell viability was not affected in SW480 and A549 cells, DA still suppressed TNF-$\alpha$-induced expression of MMP-9. It was of interest that two breast cancer cell lines responded to DA differently. DA was not cytotoxic to MDA-MB-231 (an estrogen-independent breast cancer cell line) while MCF-7 cells (an estrogen-dependent breast cancer cell line) were greatly affected. This observation is in agreement with a previous report which showed that the growth suppressive effect of DA on MDA-MB-231 cells requires a much higher concentration than that required for MCF-7 cells [4]. These data indicate that sensitivity of cancer cells toward DA's cytotoxicity or cell growth was different between different cell types, while DA's anti-inflammatory activity persisted even when it was not toxic to these cells.
The effects of DA on pro-inflammatory cytokines production were then tested. As shown in Fig. 4E and F, DA inhibited IL-8 production in HT1080 cells after stimulation with TNF-α in a dose dependent manner. In MDA-MB-231 cells, basal and/or PMA-induced expressions of both IL-6 and IL-8 were blocked by DA (Fig. 4G and H).

The effects of decursin or decursinol were then tested on pro-inflammatory mediators production and cytotoxicity (data Supplement #2). Decursin blocked TNF-α-induced production of MMP-9 and IL-8 without any cytotoxicity at a concentration range similar to DA (40–80 μM). Decursinol also blocked the production of pro-inflammatory mediators in a dose dependent manner, but higher concentrations (up to 400 μM) were required. Data indicate that decursinol exhibits both anti-inflammatory (data Supplement #2) and anti-invasion/migration activities (Fig. 1E and F). These activities of decursinol are expected to contribute to the anti-cancer activity of DA (or decursin), although metabolic conversion of DA (or decursin) into decursinol may affect the efficiency of its anti-inflammatory activity.

3.4. DA exerts its anti-inflammatory activity through inhibition of PI3K, ERK and NF-κB activation

In order to determine the molecular mechanism responsible for DA’s suppression of the inflammatory processes, RT-PCR analysis of MMP-9 was performed on HT1080 cells. As shown in Fig. 5, MMP-9 gene expres-

![Fig. 4.](image-url)
The expression of MMP-9 and/or cytokine requires the activation of various signaling molecules, including PI3K [21,22], ERK [23,24] and NF-κB [25–27] in HT1080 cells. In order to confirm the involvement of these signaling molecules in TNF-α-induced activation of HT1080 cells, specific inhibitors of PI3K, ERK and NF-κB were used. The expression of both MMP-9 and IL-8 was inhibited by these agents in a dose dependent manner (Fig. 6). The activation status of these signaling molecules was then analyzed in the presence or absence of DA. As shown in Fig. 2B, AKT and ERK phosphorylation was blocked by DA. These data indicated that PI3K and ERK were the targets of DA with respect to the TNF-α-induced activation of MMP-9 and IL-8. Inhibitors for p38 and JNK MAPK were not able to inhibit the expression of MMP-9 (data not shown).

Nuclear translocation of NF-κB, which is normally detained in cytosol through its association with IκB, requires activation-induced phosphorylation and subsequent degradation of IκB. A specific inhibitor of NF-κB blocked the expression of IL-8 and MMP-9 (Fig. 6), demonstrating the involvement of it in the production of inflammatory agents. The status of IκB was tested in HT1080 cells after stimulation with TNF-α in the presence or absence of DA. As shown in Fig. 7A, DA inhibited the phosphorylation and subsequent degradation of IκB in a dose dependent manner. Accordingly, immunofluorescence analysis of NF-κB p65 subunit showed that DA blocked nuclear translocation of NF-κB (Fig. 7B and data Supplement #3). Western blot analysis nuclear extracts further confirmed that DA blocked nuclear translocation of NF-κB (Fig. 7A).

In conclusion, DA exerted its anti-inflammatory effects through inhibition of PI3K/AKT, ERK and NF-κB activation. DA also suppressed cancer cell migration and invasion through the inhibition of PI3K activation, cellular adhesion to ECM through β1-integrin and the production of matrix degrading enzyme. The adhesion of cancer cells to ECM in the cancer microenvironment affects its survival as well as metastatic potential [28]. DA-mediated reduction of β1-integrin expression levels, and the subsequent reduction in its interaction with ECM proteins, is expected to be a contributory factor for the reduction in cell migration, and the anti-cancer activity of DA.

Inflammation that accompanies chronic diseases and infection, has been shown to be closely associated with increased incidence of various cancers [12,13]. Tumor-infiltrating macrophages are known as the key cell type which links cancer and inflammation through promotion of

**Fig. 5.** DA inhibits the transcription of MMP-9 gene. A, HT1080 cells were treated with TNF-α. Total cellular RNAs were collected at various time points after stimulation and RT-PCR analysis was then performed using specific primers for MMP-9 and GAPDH. PCR products were electrophoresed in 1% agarose gel and stained with ethidium bromide. B, HT1080 Cells were treated with 50 ng/ml TNF-α and/or 80 μM of DA for 24 h and RT-PCR analysis was performed as in A.

**Fig. 6.** TNF-α induces MMP-9 expression through PI3K, ERK and NF-κB activation in HT1080 cells. HT1080 cells were stimulated with TNF-α in the presence of the indicated amounts of LY294002, U0126, or sulfasalazine (SSZ). SSZ was added as a specific inhibitor of NF-κB. Culture supernatants were collected 24 h after activation for the measurement of IL-8 concentration using ELISA or MMP-9 activity using gelatin zymogram. MMP-9 levels were measured through densitometer and normalized with corresponding MMP-2 band intensities. Levels of IL-8 or MMP-9 were then compared with that in cells treated with TNF-α only. The experiments were performed more than three times with essentially the same results.
Conflict of interest

None of the authors of this study has a conflict of interest to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2010.03.012.

References


