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Molecules and Cells

Dieckol from *Ecklonia cava* Suppresses the Migration and Invasion of HT1080 Cells by Inhibiting the Focal Adhesion Kinase Pathway Downstream of Rac1-ROS Signaling

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We have previously isolated dieckol, a nutrient polyphenol compound, from the brown alga, *Ecklonia cava* (Lee et al., 2010a). Dieckol shows both antitumor and antioxidant activity and thus is of special interest for the development of chemopreventive and chemotherapeutic agents against cancer. However, the mechanism by which dieckol exerts its antitumor activity is poorly understood. Here, we show that dieckol, derived from *E. cava*, inhibits migration and invasion of HT1080 cells by scavenging intracellular reactive oxygen species (ROS). H_2O_2 or integrin signal-mediated ROS generation increases migration and invasion of HT1080 cells, which correlates with Rac1 activation and increased expression and phosphorylation of focal adhesion kinase (FAK). Rac1 activation is required for ROS generation. Depletion of FAK by siRNA suppresses Rac1-ROS-induced cell migration and invasion. Dieckol treatment attenuated intracellular ROS levels and activation of Rac1 as well as expression and phosphorylation of FAK. Dieckol treatment also decreases complex formation of FAK-Src-p130Cas and expression of MMP2, 9, and 13. These results suggest that the Rac1-ROS-linked cascade enhances migration and invasion of HT1080 cells by inducing expression of MMPs through activation of the FAK signaling pathway, whereas dieckol downregulates FAK signaling through scavenging intracellular ROS. This finding provides new insights into the mechanisms by which dieckol is able to suppress human cancer progresssion and metastasis. Therefore, we suggest that dieckol is a potential therapeutic agent for cancer treatment.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are generally considered toxic by-products of respiration. However, a wealth of recent evidence suggests that the production of these ROS might be an integral component of intracellular signaling (Aslan and Ozben, 2003; Poli et al., 2004). Particularly, aberrant ROS generation has been found in many types of cancer cells (Wu, 2006) and thus the involvement of ROS signaling in tumor progression and metastasis has been the focus of much research.

Tumor metastasis is a complicated pathological process including epithelial-mesenchymal transition (EMT), migration, and invasion of the tumor cells. Tumor cells isolated from metastases are highly migratory and invasive. Mounting evidence indicates that integrin-focal adhesion kinase (FAK) signaling is closely associated with tumor metastasis (Mitra and Schlaepfer, 2006). Integrin engagement with the extracellular matrix (ECM) facilitates focal adhesion, which entails the recruitment of FAK to cell-substratum interaction sites and increases phosphorylation responsible for activation of FAK. The activated FAK then binds to Src family kinases and other intracellular signaling molecules such as p130Cas and Grb2 to trigger multiple downstream pathways to regulate focal adhesion dynamics and cell migration. Overexpression and increased phosphorylation of FAK have been found in a variety of human cancer cells (Gabarra-Niecko et al., 2003) and these changes are correlated with the acquisition of an invasive migration phenotype and enhanced metastasis (Cance et al., 2000; Kornberg, 1998). FAK -/- fibroblasts exhibit cell migration defects, while overexpression of FAK in a number of cell lines, including FAK -/- cells, promotes migration on fibronectin (Cary et al., 1996; Owen et al., 1999; Sieg et al., 1999). Furthermore, viral Src (v-Src) transformation reverses the integrin-stimulated motility defects of FAK -/- fibroblasts as does FAK re-expression. However, FAK -/- v-Src cells are not invasive and FAK re-expression, as well as Y397 phosphorylation and FAK kinase activity, were required for the generation of an invasive cell phenotype (Hsia et al., 2003). Various FAK downstream pathways have also been found to regulate cell migration and invasion (Hsia et al., 2003). Therefore, it is clear that FAK contributes to the regulation of cell migration and invasion during tumor progression and metastasis.

Interestingly, several recent studies have shown that FAK phosphorylation and activation, associated with tumor cell mi-

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gration and invasion, are increased by integrin-Rac1-mediated ROS generation. Cell adhesion on fibronectin generates a transient ROS increase dependent on the small GTPase Rac1. The induced ROS promote oxidative inhibition of a FAK tyrosine phosphatase, thereby preventing dephosphorylation and inactivation of FAK (Chiarugi et al., 2003). In addition, NADPH oxidase, that produces ROS, is an effecter protein of Rac1 and both Rac1 and NADPH oxidase enhance FAK phosphorylation. This phosphorylation subsequently leads to EGF-induced ERK activation in focal adhesions, which is necessary for focal adhesion turnover and membrane protrusion for cell migration (Flinder et al., 2010). Therefore, FAK is an attractive therapeutic target responsible for metastatic functions in cancer cells related to migration and invasion. Thus, the screening and validation FAK signaling inhibitors promises to elucidate new anticancer strategies.

We recently isolated dieckol from the brown alga, *Ecklonia cava* (Heo et al., 2009; Lee et al., 2010a). This alga is produced in abundance on Jeju Island in Korea. It is popular in Korea and Japan as a food ingredient, supplement of animal feed and fertilizers, and a therapeutic. Dieckol is one of the major polyphenolic compounds in *E. cava*. Although dieckol has been reported to effectively protect cells against various cellular injuries, including cancer, aging, and inflammation induced by oxidative stress, the molecular description of this compound has been limited with respect to its role in apoptosis induction and suppression of aberrant proliferation by oxidative stress. As such, the mechanistic evidence illustrating dieckol's anti-cancer properties remain unclear. Therefore, we investigated the effects of natural dieckol, isolated from *E. cava*, on intracellular ROS generation and tumor cell migration and invasion. We found that dieckol inhibits migration and invasion of HT1080 human fibrosarcoma cells by suppressing the FAK signaling pathway downstream of Rac1/ROS. These results provide a novel insight into the possible mechanisms that underlie the anti-cancer activity of dieckol.

MATERIALS AND METHODS

Cell culture and reagents

HT1080 human fibrosarcoma cells from the American Type Culture Collection (ATCC) were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin, in 5% CO_2 in air at 37°C. For experiments, cells were passaged at least 3 times and detached with Trypsin-EDTA. Matrigel was a product from BD Biosciences (USA). Antibodies against FAK, Y397 FAK, Rac, and actin were obtained from Santa Cruz Biotechnology (USA), BD Biosciences (USA), Cell Signaling Technology (USA), and Sigma (USA), respectively. DCF-DA was from Molecular Probes. Chemicals and reagents were purchased form Sigma if not differently stated. pEF-Myc-Bos construct (Myc-Rac1G12V and Myc-Rac1t17N) were described previously (Miki et al., 1998).

Cell viability (MTT) assay

HT1080 cells were seeded in 96-well plates at a density of 1×10^3 cells/well in DMEM containing 10% fetal bovine serum. Twenty-four hours after seeding, the medium was changed to serum-free DMEM and the cells were incubated with 100 μM H_2O_2 for 48 h. The cells were then incubated with or without 25 μg/ml dieckol for 24 h. Thereafter, the medium was carefully removed and 100 μl of MTT [3-(4, 5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (1 mg/ml final concentration) solution was added to each well prior to incubation for another 3 h at 37°C in 5% CO_2. The absorbance was then measured on a microplate reader (iMark Bio-Rad) at 540 nm.

Cell migration and invasion assay

Cell migration was determined using the wound-healing scratch assay as previously described (Meng et al., 2006). Cells were seeded on a 3.5 cm dish and grown overnight. After serum starvation for 24 h, cells were preincubated with 100 μM H_2O_2 for 48 h and then incubated with or without 25 μg/ml dieckol for 24 h. A sterile 200-μl pipette tip was used to scratch the cells to form a wound. Migration of the cells to the wound was visualized with an inverted Olympus phase-contrast microscope. The representative fields were photographed. The healing rate was quantified using measurements of the gap size after the culture. Ten different areas in each assay were chosen to measure the distance of migrating cells to the origin of the wound.

For invasion assay, the undersurface of the porous membranes in the Matrigel Invasion Chambers (BD Biosciences, USA) were coated with fibronectin (25 μg/ml) at room temperature for 1 h and washed 3 times in DMEM containing 0.1% bovine serum albumin (DMEM-BSA). DMEM-BSA was added to the lower compartment of the chamber. Cells were starved in DMEM-BSA overnight and treated with H_2O_2 and/or dieckol as described above, trypsinized and collected, and then 200 μl of each cell suspension (2×10^5 cells/well in DMEM-BSA) was added to the upper compartment of the chamber and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. Cells on the upper surface of the membrane were removed, and cells that had migrated to the under surface of the membrane were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS), stained with crystal violet (0.4% dissolved in 10% ethanol) for 15 min, washed twice with PBS, and counted under a phase-contrast microscope with a 10× objective lens. The numbers of cells in 9 randomly selected fields from triplicate chambers were counted in each experiment.

Cell adhesion assay

HT1080 cells were incubated in the presence or absence of dieckol (25 μg/ml) for 24 h. The cells were harvested and resuspended in culture medium. The cells were then transferred to a 24-well plate that was precoated with fibronectin (25 μg/ml). After incubation for 1 h at 37°C, the medium was discarded and washed with PBS to remove the non-adherent cells. Attached cells were photographed and quantified.

Measurement of ROS

Dichlorofluorescein diacetate (DCF-DA) was used to evaluate the generation of ROS by oxidative stress. Cells (4×10^4 cells/well) in 24-well plates were first incubated with H_2O_2 for 48 h and then incubated with or without dieckol for 24 h. The cells were then washed with PBS and incubated with 10 μM DCF-DA for 30 min at room temperature. Fluorescence was measured using a fluorescence plate reader.

Transient transfection of RNAi

Two FAK small interfering RNAs (siRNAs) and a non-specific siRNA were ordered from Invitrogen. The siRNA sequences are siRNA1: 5′-GGAUUUCUAAACCAGUUUATT-3′ (sense), 5′-UAAACUGGUUUAGAAAUCCTT-3′ (antisense); siRNA2: 5′-GAAGGAAUCAGUUACCUAATT-3′ (sense), 5′-UUAGGUA ACUGAUUCCUUCTT-3′ (antisense). Transient transfection of siRNA was accomplished using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Small GTPase Rac1 activity assay

GST-PAK-CRIB fusion protein was expressed as described (Miki et al., 2000) and immobilized on glutathione-sepharose beads (Amershan Biosciences, USA). Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1% NP-40, 10% glycerol, 200 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM DTT, and 1 mM PMSF. A measure of 5-10 μl of cell lysate was subjected to Western blotting for loading control. A total 1 ml of cell lysate was mixed with 50 μl (bed volume) of GST-PAK CRIB beads and rotated at 4°C for 40 min. The beads were washed 3 times with cold wash buffer containing 25 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 40 mM NaCl, 1% NP-40, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF and 20 μl of SDS sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 6% 2-mercaptoethanol, 10% glycerol, and 0.5 mg/ml bromophenol blue was added. Samples were separated by electrophoresis, and Rac1-GTP was detected by Western blotting.

RESULTS

Dieckol suppresses adhesion, migration, and invasion of human fibrosarcoma HT1080 cells

Previously, we isolated dieckol from *E. cava* and showed that it possesses antioxidant activity (Heo et al., 2009; Lee et al., 2010a). Although there are many reports showing polyphenolic compounds, including dieckol, exert antitumor activity, many details regarding their molecular mechanisms remain unclear.

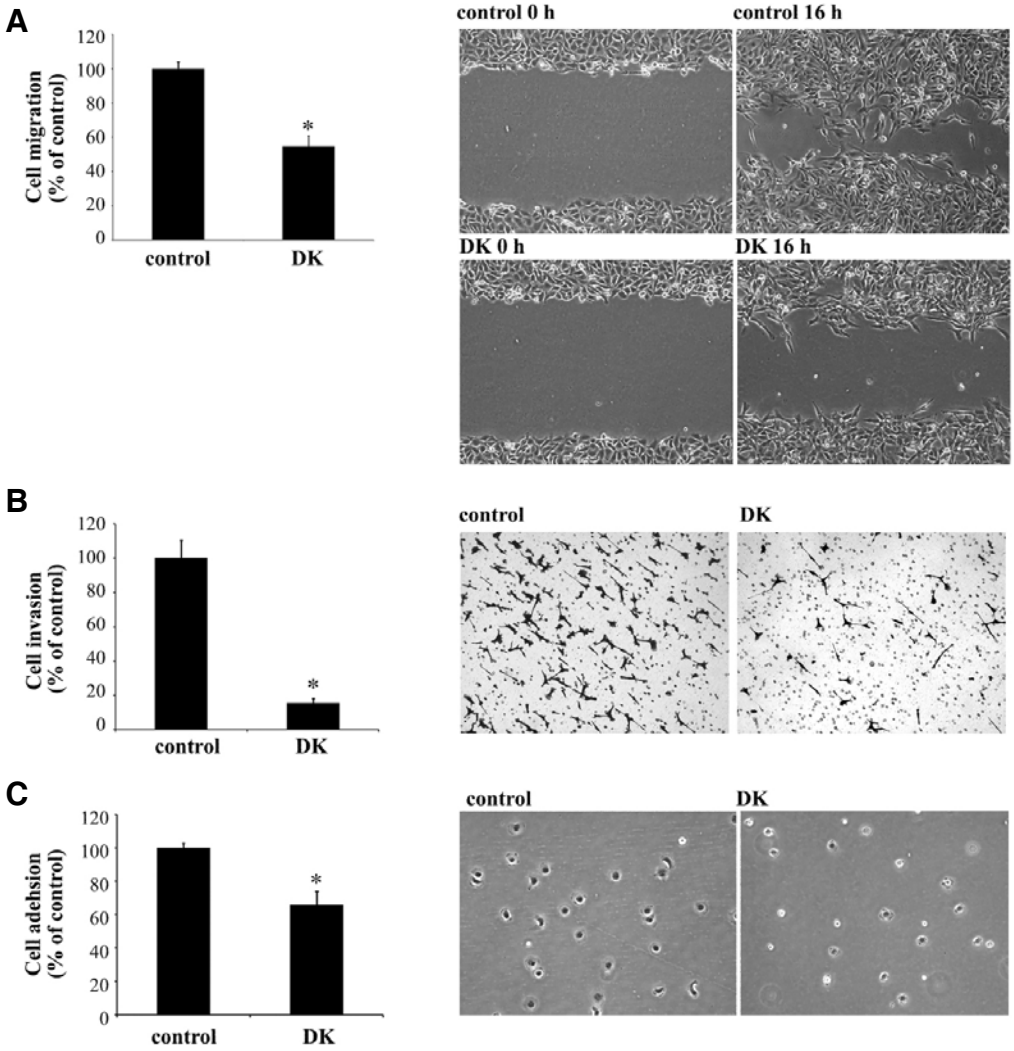

Fig. 1. Dieckol inhibits migration, invasion, and adhesion of HT1080 cells. (A) Inhibitory effect of dieckol on the migration of HT1080 cells. Wound-healing scratch assays were performed with HT1080 cells plated onto fibronectin-coated dishes. After serum starvation, cells were incubated in the absence or presence of 25 μg/ml dieckol for 24 h. A sterile 200-μl pipette tip was used to scratch the cells to form a wound. Cell migration was quantified with measurements of the gap size of 4 different images at 0 and 16 h; representative images are shown. Results of 3 independent experiments were averaged. PBS-treated cells were used as a control. *P < 0.05 compared with control. (B) Dieckol inhibits invasion of HT1080 cells. Matrigel invasion assays were performed with HT1080 cells incubated with 25 μg/ml dieckol. *P < 0.05 compared with control. (C) Dieckol inhibited the early adhesion of HT1080 cells. Cells were treated as in (A). Cell adhesion was determined on a fibronectin-coated dish. The adhered cells were quantified by cell counting as described in the "Materials and Methods". Values are means ± standard deviation from 3 independent experiments. *P < 0.05 compared with control.

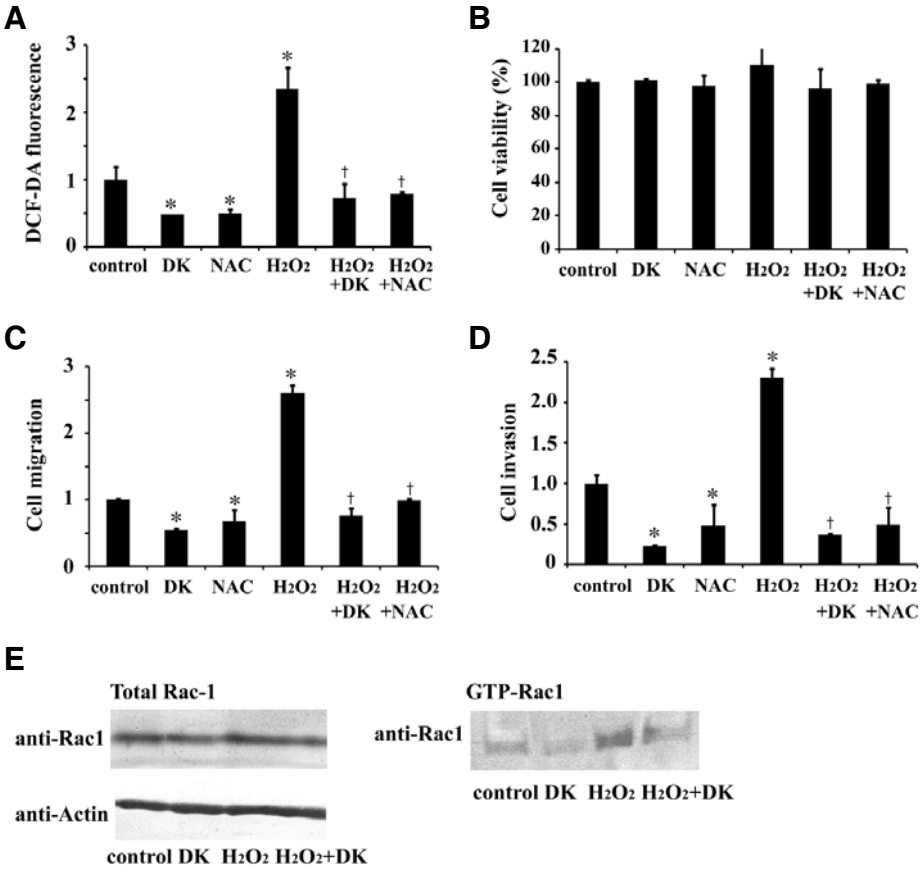

Fig. 2. Dieckol suppresses migration and invasion of HT1080 cells by reducing intracellular ROS. (A) Dieckol attenuates intracellular ROS. Cells (4×10^4 cells/well) were incubated in the presence or absence of 100 μM H_2O_2 for 48 h followed by incubation with or without 25 μg/ml dieckol for 24 h. Cellular ROS levels were assessed by DCF-DA. All data are mean ± S.D. *P < 0.05 compared with control; †P < 0.05 compared with H_2O_2. (B) Effects of diekol and/or H_2O_2 on cell viability. Cells were treated with H_2O_2 (100 μM), Diekol (25 μg/ml), and/or Nac (10 mM) for 48 h and assessed by MTT. Results of 3 independent experiments were averaged. (C, D) Cells were treated as in (A). Wound-healing scratch assays and Matrigel invasion assays were performed. All data are mean ± S.D. *P < 0.05 compared with control; †P < 0.05 compared with H_2O_2. (E) The purified GST-PAK-PBD fusion protein was incubated with lysates of HT1080 cells treated as in (A). Bound proteins were collected and GTP-bound Rac1 was detected via western blot with anti-Rac1 antibody. Representative of 3 independent experiments.

In this study, we investigated whether dieckol has an effect on metastatic phenotypes of cancer cells, such as dynamic migration and invasion. Dieckol did not affect cell viability below the concentrations of 50 μg/ml (Supplementary Fig. 1). As shown in Fig. 1A and Supplementary Fig. 1, treatment of HT1080 cells with non-cytotoxic dieckol (25 μg/ml) effectively decreased their motility in wound healing assays. Invasion of dieckol-treated HT1080 cells into Matrigel was also reduced to approximately 19% that of control cells which received no dieckol treatment (Fig. 1B; Supplementary Fig. 1). In addition, the adhesion assay on the fibronectin-coated plate showed that dieckol also decreased cell adhesion (Fig. 1C; Supplementary Fig. 1). The dieckol-treated cells became more round and poorly spread. Therefore, these results suggest that dieckol regulates an intracellular signaling cascade involved in adhesion, migration, and invasion of HT1080 cells.

Dieckol downregulates adhesion, migration, and invasion of HT1080 cells by scavenging intracellular reactive oxygen species (ROS)

Dieckol's antioxidant properties have been well documented with recent studies showing ROS contribute to cell migration and invasion. We therefore, examined whether dieckol decreases ROS in HT1080 cells. Dieckol treatment attenuated intracellular ROS levels to approximately 50% that of the control, as shown in Fig. 2A. This was consistent with the result using *N*-acetyl cysteine (NAC), a ROS-quenching agent, which suggests that reduced intracellular ROS following dieckol treatment plays a role in the decreased migration and invasion of HT1080 cells. To address this, we treated HT1080 with H_2O_2 and then incubated with dieckol and evaluated cell migration and invasion. A relatively low concentration of H_2O_2 (100 μM) was used to increase intracellular ROS levels. Cellular exposure to H_2O_2 over 2 days did not affect cell viability (Fig. 2B). Elevated ROS levels, due to H_2O_2 treatment, increased migration and invasion of HT1080 cells. H_2O_2 treatment increased ROS level to approximately 2.3-fold in comparison to level of control. Migration and invasion level were increased 2.6- and 2.3-fold in comparison to level of control, respectively. Importantly, dieckol significantly inhibited these H_2O_2-induced cell migration and invasion effects (Figs. 2C and 2D), indicating that intracellular ROS mediates migration and invasion of HT1080 cells and dieckol downregulates cell migration and invasion by reducing the ROS levels.

The changes in cell migration and invasion under oxidative stress also suggest involvement of the small GTPase Rac1, which is an upstream regulator critical for actin reorganization and adhesive and invasive cell migration. Generation of ROS in a variety of physiological conditions is associated with Rac1 activation (Nimnual et al., 2003; Werner and Werb, 2002). To test endogenous Rac1 activation, the GST-PAK binding assay was performed with cell lysates. We found that H_2O_2 treatment induces activation of Rac1, whereas dieckol treatment decreases H_2O_2-induced Rac1 activation (Fig. 2E). This data suggests that the Rac1-ROS cascade is involved in cell migration and invasion of these HT1080 cells.

Cell migration and invasion are closely associated with cell-ECM interaction. Figure 1C showed that dieckol inhibits adhesion of HT1080 cells on fibronectin. Thus, we examined whether the Rac1-ROS signaling is also involved in integrin-mediated

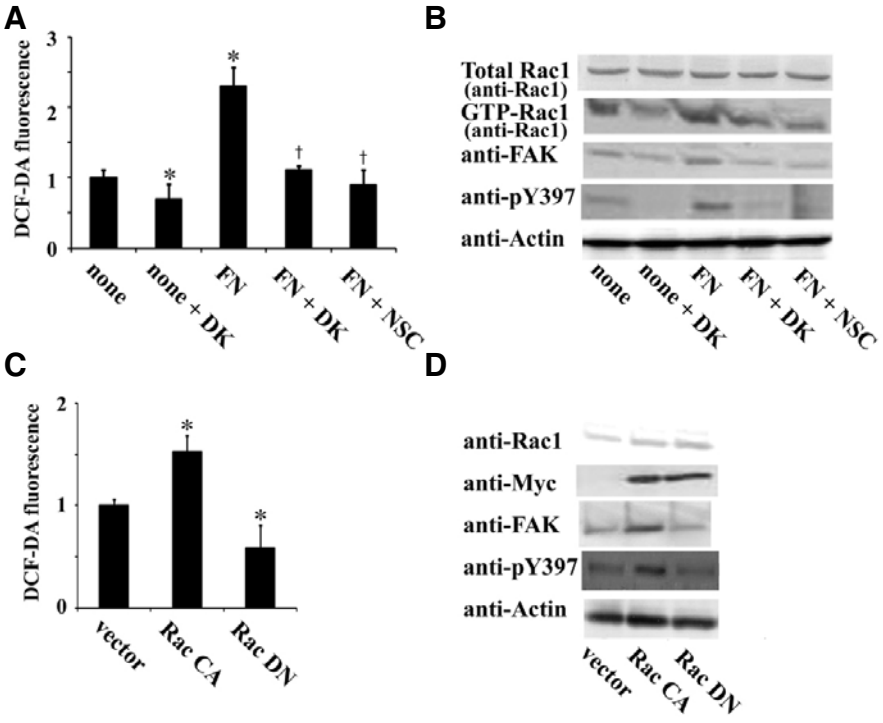

Fig. 3. Dieckol inhibits Rac1-ROS-mediated adhesion of HT1080 cells. (A) Serum starved cells were detached and suspended for 30 min in the presence or absence of 25 μg/ml dieckol or a Rac inhibitor NSC (1 mM) and then seeded onto fibronectin (FN)-coated or uncoated (none) culture dishes for 60 min under same medium condition. ROS was evaluated with DCF-DA. All data are mean ± S.D. *P < 0.05 compared with none; †P < 0.05 compared with FN. (B) Rac1 activation was examined during cell adhesion to fibronectin. Cells were treated as in (A). Total Rac and GTP-bound Rac1 were detected via Western blot with anti-Rac1 antibody. Cell lysates were also analyzed for FAK, pFAK (Y397), and β-actin. Representative of 3 independent experiments. (C, D) Expression of myc-tagged Rac CA or Rac DN mutants in HT1080 cells. Empty vector or Rac-expressing cells were serum starved before detaching and maintained in suspension for 30 min. The cells were seeded onto fibronectin-coated dishes for 60 min. ROS was evaluated with DCF-DA. Cell lysates were also analyzed for Rac, Myc, FAK, pFAK (Y397), and β-actin.

HT1080 cell adhesion and whether dieckol inhibits the signal. Serum starved HT1080 cells were presuspended for 30 min in the presence or absence of dieckol and then seeded onto fibronectin-coated or uncoated culture dishes for 60 min under same medium condition. Cell adhesion on fibronectin induced a dramatic increase in ROS production and Rac1 activation, whereas dieckol treatment inhibited adhesion-induced ROS increase and Rac1 activation (Figs. 3A and 3B). In addition, treatment with NSC, a Rac inhibitor, inhibited the effects on ROS production as well as Rac1 activation during cell adhesion (Figs. 3A and 3B). These findings indicate that integrin-mediated cell adhesion and Rac1 activation are associated with increased generation of intracellular ROS. A causal link between Rac1 activation and adhesion-induced ROS generation was further determined by overexpressing constitutively active Rac1 mutant (Rac CA) or the corresponding dominant-negative form (Rac DN) (Fig. 3C). Rac1 CA-overexpressing cells plated on fibronectin produced approximately 150% increased ROS in comparison to level in empty vector-expressing control cells, whereas Rac DN-expressing cells showed approximately 58% reduced ROS production in adherent cells. Therefore, these data indicate that Rac1 lies downstream of adhesion molecules for the oxidative signals trigged by cell-ECM interaction and Rac1 activation is required for intracellular ROS generation in response to adhesion signals. Dieckol inhibits adhesion-mediated Rac1-dependent ROS generation signal.

Dieckol attenuates ROS-induced increase of Focal adhesion kinase (FAK) expression and phosphorylation

Cell adhesion, migration, and invasion are fundamental components of tumor cell metastasis and FAK has been found to be responsible for cell adhesion, migration, and invasion during tumorigenesis in response to oxidative stresses (Chiarugi et al., 2003; Flinder et al., 2010). ROS-induced increase of FAK expression and tyrosine phosphorylation has been connected with elevated tumorigenesis. As shown in Fig. 4A, dieckol treatment of HT1080 cells decreased expression and Y397 phosphorylation of FAK. In addition, we found that the treatment of cells with H_2O_2 increased FAK expression and Y397 phosphorylation, whereas dieckol inhibited these H_2O_2-induced effects on FAK. This suggests that the increased cell migration and invasion induced by H_2O_2 might be dependent on the role of FAK. To determine the relationship between the activation of FAK and H_2O_2-induced cell migration and invasion, HT1080 cells were transfected with FAK siRNA and then treated with H_2O_2 for 2 days. Two FAK siRNAis were designed to inhibit FAK expression and both of them attenuated expression of FAK (Fig. 4B). We observed these FAK siRNAs attenuated cell migration and invasion of HT1080 cells. The inhibitory effects were more significant in H_2O_2-induced cells than in the control cells, indicating an essential role of FAK in H_2O_2-induced cell migration and invasion of HT1080 cells (Fig. 4B). These data suggest that dieckol treatment in the presence or absence of H_2O_2 treatment suppresses expression and phosphorylation of FAK, which results in decreased cell migration and invasion.

We further determined that a critical role of ROS-induced FAK activation in cells adhered on fibronectin. Cell adhesion on fibronectin induced increase of FAK expression and tyrosine phosphorylation, whereas dieckol or NSC treatment inhibited the effects on FAK expression and phosphorylation as well as Rac1 activation (Fig. 3B). In addition, Rac CA-overexpressing increases FAK activation, whereas Rac DN-overexpressing decreases FAK activation (Fig. 3D). Increased invasive migration activity of Rac CA-expressing cells was affected by FAK levels. RNAi of FAK inhibited Rac CA-induced HT1080 cell invasion to approximately 53% that of control (Fig. 4C). Taken together, these data indicate that cell adhesion-induced Rac1 activation and ROS generation subsequently induces FAK

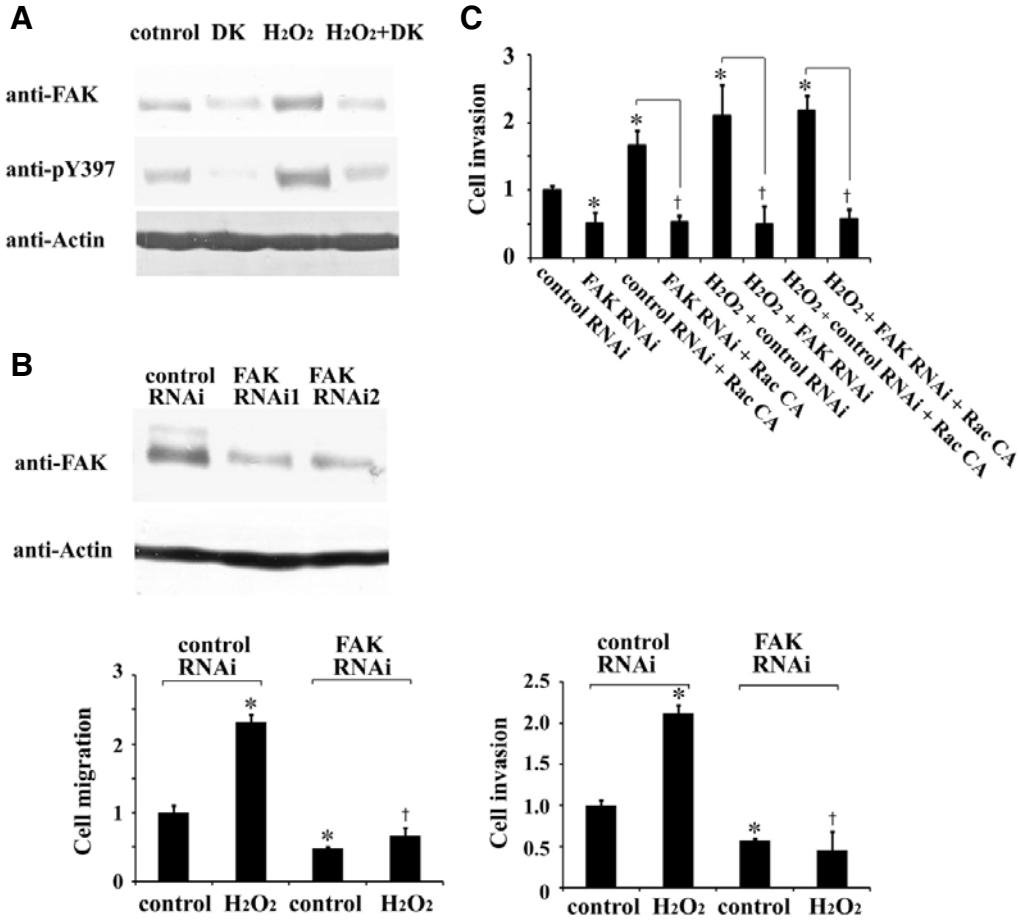

Fig. 4. Dieckol attenuates FAK expression and phosphorylation at Y397. (A) HT1080 cells were incubated with 100 μM H_2O_2 for 48 h and then incubated with or without 25 μg/ml dieckol for 24 h. Cell lysates were analyzed for FAK, pFAK (Y397), and β-actin. (B) HT1080 cells were transfected with FAK siRNA or the same amount of control siRNA. In 72 h, cell lysates were prepared and FAK expression was analyzed by western blot. After transfection with FAK siRNA, the cells were incubated for 48 h and then the cells were harvested for migration and invasion assays. All data are mean ± S.D. *P < 0.05 compared with control RNAi; †P < 0.05 compared with control of FAK RNAi. (C) Suppressive effects of decreased expression of FAK on Matrigel invasion were examined in Rac CA-induced invasion of HT1080 cells. *P < 0.05 compared with control RNAi; †P < 0.05 compared with control RNAi + Rac CA, H_2O_2 + control RNAi, or H_2O_2 + control RNAi + Rac CA.

activation, which is required for migration and invasion of HT1080 cells. Dieckol shows an inhibitory effect on FAK activation by suppressing the Rac-ROS signaling.

Dieckol decreases the Fak-Src-p130Cas complex formation and inhibits Matrix metalloproteinases (MMPs) secretion and expression

Activated FAK promotes assembly of focal complexes, including signaling proteins such as Src and p130Cas, and initiates activation of signaling cascades for cancer progression (Hsia et al., 2003). MMPs are highly susceptible to the catalytic activities of both FAK and Src. MMPs are responsible for invasive cell migration via their extracellular matrix-degrading activity. The Rac1-ROS pathway induces expression of MMPs in a FAK activation-dependent manner (Li et al., 2003; Zeng et al., 2006). Therefore, we first examined whether dieckol treatment affects assembly of FAK-containing focal complexes. HT1080 cells were stimulated with H_2O_2 for 2 days and then treated with dieckol for 24 h. As shown in Fig. 5A, H_2O_2 stimulation slightly increased levels of Src and p130Cas in anti-FAK immunoprecipitates, whereas the trimeric complex between FAK, Src, and p130Cas was reduced following dieckol treatment. In addition, we examined MMP expression by mRNA quantification via reverse transcription-PCR (Fig. 5B). H_2O_2 treatment increased MMP2, MMP9, and MMP13 expression levels in HT1080 cells. Dieckol treatment inhibited the H_2O_2-induced expression of these MMPs. MMP13 mRNA levels increased by > 5-fold after H_2O_2 treatment and were significantly inhibited by dieckol treatment. These findings suggest that dieckol suppresses HT1080 cell migration and invasion by inhibiting FAK pathway downstream signaling in response to intracellular ROS.

DISCUSSION

Because ROS play such a pivotal role in tumor progression and metastasis, chemical or enzymological antioxidants aimed to reduce oxidative stress are a promising anti-cancer strategy. In particular, dietary chemoprevention may be a promising, cost-effective, and safe approach in the prevention of ROS-triggered tumor progression. The marine brown alga *E. cava* has been

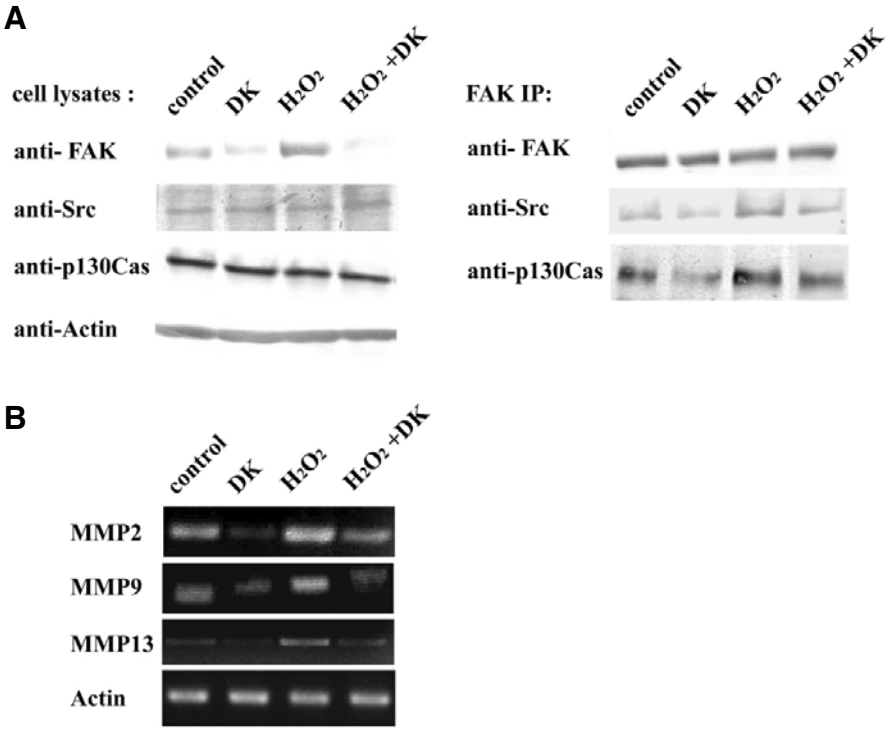

Fig. 5. Dieckol decreases FAK-Src-p130Cas complex formation and inhibits its expression of MMPs. (A) HT1080 cells were incubated with 100 μM H_2O_2 for 48 h and then incubated with or without 25 μg/ml dieckol for 24 h. The cell lysates were immunoprecipitated with anti-FAK antibody and bound proteins were assayed by specific antibodies of anti-FAK, Src, and p130Cas. (B) Cells were treated as described in (A). Expression of MMPs was determined by reverse transcription-PCR.

found to possess various phlorotannins, such as eckol, 6, 6′-bieckol, and dieckol. Most previous studies have shown that the phlorotannins from *E. cava* have strong antioxidant activity (Kang et al., 2004; Li et al., 2009). Here, we showed that ROS directly mediates migration and invasion of human sarcoma HT1080 cells and antioxidant dieckol from *E. cava* suppresses the FAK signaling responsible for migration and invasion of HT1080 cells through the reduction of Rac1-mediated ROS.

We used exogenous H_2O_2 directly as a source of ROS, as well as ROS generated endogenously through integrin-mediated cell adhesion. In both cases, increased ROS induced activation of FAK that contributes to cell migration and invasion. Integrin-Rac1 pathway normally induces increased levels of intracellular ROS. FAK plays a key role in not only integrin-mediated signaling pathways relevant to cell adhesion, migration, and invasion but also the positive feedback loop for integrin-Rac1-mediated ROS generation (Honore et al., 2003; Lee et al., 2010b; Werner and Werb, 2002). FAK directly potentiates the activity of Rac1 in matrix sites (Chang et al., 2007). Specific guanine exchange factors (GEFs) for Rac1 such as DOCK180 and ELMO are also activated by the integrin signal via FAK or ILK (McLean et al., 2004). On the other hand, ROS generated from Rac1-induced NADPH oxidase modifies the low molecular weight protein tyrosine phosphatase (LW-PTP) for FAK, causing elevated tyrosine phosphorylation and activation of FAK (Chiarugi et al., 2003). FAK-inactivating phosphatase, PTP-PEST, is also targeted by ROS (Gu et al., 1998; Richardson and Parsons, 1996). PTP-PEST is bound to paxillin, acts as a negative regulator of FAK, Src, p130Cas, and Rac1, and inhibits focal complex turnover and cell migration (Flinder et al., 2010; Wu et al., 2008). Therefore, ROS-mediated inhibition of PTP-PEST facilitates subsequent activation of FAK, Rac1, NADPH oxidase activation and local ROS production. Taken together, ROS generated at focal adhesion points is likely to provide cellular signaling pathway specific to extracellular stimuli through activating FAK. In addition, ROS and dieckol regulated expression level of FAK. Inhibition of FAK expression was associated with suppression of cell migration and invasion. Dieckol treatment caused decrease of FAK in both protein and messenger RNA level (Supplementary Fig. 2). Cell treatment with MG132, a proteasome inhibitor, did not affect FAK expression in the presence or absence of dieckol, suggesting that dieckol inhibits FAK expression at the transcriptional level. Furthermore, dieckol was found to reduce NF-kB p65 protein levels in both nuclear and cytosolic fraction (Supplementary Fig. 2). Therefore, NF-KB is likely to be an important transcription regulator controlling FAK expression under oxidative states. This finding provides important information to explore the role of dieckol suppressing tumor progression.

We also showed that the increase in FAK expression and phosphorylation mediated by ROS levels parallels the increased interaction between FAK, Src, and p130Cas. Activation of Src and p130Cas is deficient in focal complexes of cells lacking FAK, which is associated with loss of cell migration (Owen et al., 1999). Rac1-NADPH oxidase induces FAK phosphorylation at Y397, which is necessary for the recruitment and activation of Src and p130Cas (Mitra et al., 2005). Therefore, interaction of focal adhesion proteins is likely to be important in the cell migration processes. Furthermore, FAK signal activation induces MMP activation. Expression of dominant negative FAK inhibited MMP2 and MMP9 secretion from human carcinoma cells (Hauck et al., 2001). FAK re-expression in FAK-null cells increased the secretion of these MMPs (Sein et al., 2000). The role of ROS in MMP activation is also supported by several reports (Mori et al., 2004; Wenk et al., 1999; Ranganathan et al., 2001). Consistent with these results, we found that H_2O_2 treatment enhanced expression of MMP2, 9, and 13, whereas their expression was decreased when FAK levels were depleted by RNAi (data not shown). Therefore, it is reasonable that ROS enhances migration and invasion of HT1080 cells by inducing

MMP expression through activation of the FAK signaling pathway. Because not only gelatinase MMP2 and MMP9, but also collagenase MMP13 are remarkably upregulated by H_2O_2 treatment, these MMPs may also provide attractive targets for preventing invasive migration of HT1080 cells under oxidative stress.

Taken together, our findings suggest that Rac1-ROS activation directly affects migration and invasion of HT1080 cells through activation of FAK. Additionally, we found that dieckol suppresses the FAK signaling pathway downstream of Rac1/ROS by scavenging intracellular ROS. These data provide a basis for further investigations into dieckol-based anti-cancer treatment.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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