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Synthetic triterpenoids inhibit GSK3β activity and localization and affect focal adhesions and cell migration

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ABSTRACT

Synthetic triterpenoids are a class of anti-cancer compounds that target many cellular functions, including apoptosis and cell growth in both cell culture and animal models. We have shown that triterpenoids inhibit cell migration in part by interfering with Arp2/3-dependent branched actin polymerization in lamellipodia (To et al., 2010). Our current studies reveal that Glycogen Synthase Kinase 3 beta (GSK3β), a kinase that regulates many cellular processes, including cell adhesion dynamics, is a triterpenoid-binding protein. Furthermore, triterpenoids were observed to inhibit GSK3β activity and increase cellular focal adhesion size. To further examine whether these effects on focal adhesions in triterpenoid-treated cells were GSK3β-dependent, GSK3β inhibitors (lithium chloride and SB216763) were used to examine cell adhesion and morphology as well as cell migration. Our results showed that GSK3β inhibitors also altered cell adhesion sizes. Moreover, these inhibitors blocked cell migration and displaced proteins at the leading edge of migrating cells, consistent with what was observed in triterpenoid-treated cells. Therefore, the triterpenoids may affect cell migration *via* a mechanism that targets and alters the activity and localization of GSK3β.

1. Introduction

The triterpenoids are a class of compounds biosynthesized in plants by the cyclization of squalene. Oleanolic acid is one of the 2000 triterpenoids found in nature, and is widely used in Asia for its weak anti-inflammatory and anti-tumorigenic properties [26]. The modification of oleanolic acid to the synthetic oleanolic triterpenoid, 2-cyano-3,12-dioxooleana-1,9 (11)-dien-28-oic acid (CDDO) as well as its methyl ester (CDDO-Me) and imidazolide (CDDO-Im) derivatives increases the biological activities of these compounds [29]. CDDO and its derivatives are effective in inducing cytoprotection, apoptosis, and in reducing tumor proliferation, as assessed using animal models and cancer cell culture studies [19,20,25]. However, the effects of triterpenoids on cell adhesion and cell migration, both of which are important players in tumor metastasis, are still poorly understood.

Cell migration is a highly orchestrated process involving different cellular components that respond to stimuli such as chemo-attractants in the biological system [36]. This process mainly encompasses of the reorganization of the actin and microtubule cytoskeletons, and cellular proteins that establish cell polarity, and the formation of a definitive leading edge [27,36]. As a cell extends its protrusions to explore its surrounding environment in preparation for cell movement, these protrusions are stabilized by adhesion structures used as traction points that allow cells to advance towards or away from stimuli [24]. These adhesion structures are composed of complexes with adhesion proteins known as focal complexes, focal adhesions or fibrillar adhesions depending on their size, morphology and dynamic [40]. Focal complexes are small nascent adhesions that are often observed in rapidly migrating cells and most of them turn over within minutes as the cell continues its migratory movement [23]. However, some of these nascent adhesions mature and evolve into focal adhesions that attach to the ends of stress fibers to maintain the structure of the cell [43].

Abbreviations: CDDO, 2-cyano 3,12-dioxooleana-1,9 (11)-dien-28-oic acid; Im, imidazolide; Me, methyl ester; FAK, focal adhesion kinase; GSK3β, Glycogen Synthase Kinase 3 Beta; APC, adenomatous polyosis coli; DMSO, dimethyl sulfoxide; NaCl, sodium chloride; LiCl, lithium chloride; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H–pyrrole-2,5-dione; ANOVA, analysis of variance; Rac1, Ras-related C3 botulinum toxin substrate 1; IQGAP1, IQ motif containing GTPase activating protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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Most of these focal adhesions eventually disassemble, albeit at a slower rate, in order for the cell to maintain its flexibility as it moves forward. The adhesions that mature are known as fibrillar adhesions and they are involved in the remodeling of the extracellular matrix [9]. As such, a delicate balance between the disassembly and the maturation of adhesions is critical in order to regulate cell migration. In fact, the reduction of adhesion turnover has been shown to increase the size of focal adhesions and reduce cell motility [28,34].

Within these multi-protein adhesion complexes, different scaffolding and signaling molecules are recruited to regulate the dynamics of the adhesion structures. For instance, paxillin and Focal Adhesion Kinase (FAK) are important proteins that are commonly found at focal adhesion sites. Although it possesses no kinase activity itself, paxillin is an important scaffolding protein that initiates the recruitment of other signaling proteins, including FAK, to the complex. In fact, mice deficient in paxillin die during embryogenesis due to defects in cell migration [10] and $Pxn^{-/-}$ embryonic stem cells show defects in cell spreading [37]. FAK plays an essential role in the regulation of important intracellular signaling pathways, which in turn, governs the turnover of cell contacts with the extracellular matrix and the promotion of cell migration [33]. The loss of FAK can lead to embryonic lethality [8] and $Fak^{-/-}$ fibroblasts have enlarged focal adhesions and reduced cell motility [12].

Glycogen Synthase Kinase 3 Beta (GSK3β), originally identified for its role in glycogen metabolism and Wnt-mediated cell proliferation, has been reported to regulate cell migration and adhesion dynamics [14,30]. GSK3β is found constitutively active in cells and can phosphorylate microtubule-associated proteins as well as interact with microtubule motor proteins and regulate microtubule organization and microtubule-dependent vesicle transport [22,38]. In addition, GSK3β regulates several Rho small GTPases including Rac1 and Arf6, which in turn, control membrane ruffling, cell spreading and lamellipodia formation [7,16,35]. Interestingly, a small pool of inactive GSK3β localizes at the leading edge of migrating cells and enables APC to localize to the plus end of microtubules thereby stimulating microtubule growth and stability [44]. However, global inactivity of GSK3β can inhibit cell migration [13].

Since the triterpenoids have previously been shown to inhibit cell migration [31,32], we sought to examine whether the underlying mechanism of action involves targeting kinases such as FAK and GSK3β, both of which are important regulators of focal adhesion dynamics.

2. Materials and methods

2.1. Cell culture and antibodies

Rat2 fibroblasts were cultured using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in a 37 °C incubator with 5% CO_2. Anti-FAK (BD610087), anti-Rac1 (610650), and anti-paxillin (BD610051) antibodies were purchased from BD Transductions Laboratories (Mississauga, Canada). Anti-phospho-FAK Y397 (#3283), anti-phospho-FAK Y576/577 (#3281), anti-phospho-FAK Y925 (#3284), anti-GSK3β (#9315), anti-phospho-GSK3β serine 9 (#9336) and anti-β-catenin (#8480) antibodies were purchased from Cell Signaling Technologies (Pickering, Canada). Anti-phospho-FAK serine 722 (sc-16,662-R), anti-IQGAP1 (sc-10,792) and anti-GAPDH (sc-25,778) antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). Alexa Fluor 555-conjugated phalloidin (A34055) was purchased from Invitrogen Molecular Probes (Oregon, USA). Biotinylated CDDO-Me (compound 6) was a generous gift from Dr. M.B. Sporn (Hanover, NH) and has been previously characterized [42].

2.2. Scratch assays and immunofluorescence microscopy

Rat2 fibroblasts were cultured to confluency before a scratch was made with a pipette tip. Cells were then processed for either cell migration assay studies or immunofluorescence microscopy. For cell migration studies, cells were treated with media containing 0.1% DMSO or 50 mM NaCl (as vehicle controls), 1 μM CDDO-Im, 1 μM CDDO-Me, 50 μM SB216763, or 50 mM lithium chloride and brightfield images were taken over 16 h. For immunofluorescence microscopy, cells were allowed to establish polarity and a leading edge for 4 h prior to the 2-h drug treatment. The cells were then fixed, permeabilized and incubated with AlexaFluor 555-Phalloidin, anti-Rac1, anti-IQGAP1, anti-FAK, anti-paxillin or anti-GSK3β antibodies. All immunofluorescence images were taken using an Olympus IX81 microscope controlled by QED *In vivo* software or the Olympus Fluoroview Confocal microscope controlled by Fluoroview software (Olympus, Canada).

2.3. Affinity pull-down assay using biotinylated CDDO-me

To examine the co-localization of GSK3β and CDDO-Me, Rat2 fibroblasts were grown to confluency, scratched with a pipette tip and allowed to polarize for 6 h. Cells were then fixed, permeabilized and incubated with anti-Rac1 antibodies followed by treatment with either 0.1% DMSO, 10 μM biotin, 10 μM CDDO-Me or 10 μM biotinylated CDDO-Me. Streptavidin-Cy3 was used to visualize biotinylated CDDO-Me. Samples were stained with DAPI to visualize the nuclei. To confirm that FAK and GSK3β were triterpenoid-binding targets, cell lysates were incubated with 0.1% DMSO, 10 μM biotin, 10 μM CDDO-Me or 10 μM biotinylated CDDO-Me for two hours, followed by streptavidin beads. Samples were processed by SDS-PAGE and probed with anti-FAK and anti-GSK3β antibodies.

2.4. Western blotting

Rat2 fibroblasts were incubated with 0.1% DMSO or 50 mM NaCl (as controls), 1 μM CDDO-Im, 1 μM CDDO-Me, 50 mM Lithium Chloride or 50 μM SB216763 for 2 h before lysis. Samples were processed using SDS-PAGE and probed with anti-GSK3β, anti-phospho-GSK3β, anti-paxillin, anti-phospho-paxillin, anti-FAK, anti-phospho-FAK, anti-β-catenin or anti-GAPDH antibodies.

2.5. Statistical analyses

Results were provided as means ± SD. One-way ANOVA with a Tukey's post-hoc test was performed to assess the statistical differences between experimental groups. Statistical significance was considered at $p < 0.05$.

2.6. Focal adhesion quantitation studies

Rat2 fibroblasts were scratched and allowed to polarize for 4 h before they were treated with 0.1% DMSO or 50 mM NaCl (as controls), 1 μM CDDO-Im, 1 μM CDDO-Me, 50 mM Lithium Chloride or 50 μM SB216763 for 2 h prior to being subjected to the immunofluorescence microscopy protocol. Images were acquired using a Zeiss LSM 510 confocal system at 40 × magnification. All images were representative of at least three independent experiments in which 10 fields of cells were acquired for each condition. Focal adhesion size throughout the whole cell was analyzed and quantified using ImageJ. Data were subjected to statistical analysis using a one-way ANOVA. P values < 0.05 were considered statistically significant.

3. Results

We have previously shown that CDDO-Im and CDDO-Me inhibit branched actin polymerization, which has been associated with cell spreading and focal adhesion dynamics [32]. We therefore investigated whether triterpenoids alter the size of focal adhesions in migrating cells.