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DIF-1 inhibits the Wnt/β-catenin signaling pathway by inhibiting TCF7L2 expression in colon cancer cell lines

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ABSTRACT

We previously reported that differentiation-inducing factor-1 (DIF-1), a morphogen in *Dictyostelium discoideum*, inhibits the proliferation of human cancer cell lines by inducing β-catenin degradation and suppressing the Wnt/β-catenin signaling pathway. To determine whether β-catenin degradation is essential for the effect of DIF-1, we examined the effect of DIF-1 on human colon cancer cell lines (HCT-116, SW-620 and DLD-1), in which the Wnt/β-catenin signaling pathway is constitutively active. DIF-1 strongly inhibited cell proliferation and arrested the cell cycle in the G_0/G_1 phase via the suppression of cyclin D1 expression at mRNA and protein levels without reducing β-catenin protein. TCF-dependent transcriptional activity and cyclin D1 promoter activity were revealed to be inhibited via suppression of transcription factor 7-like 2 (TCF7L2) expression. Luciferase reporter assays and EMSAs using the TCF7L2 promoter fragments indicated that the binding site for the transcription factor early growth response-1 (Egr-1), which is located in the −609 to −601 bp region relative to the start codon in the TCF7L2 promoter, was involved in DIF-1 activity. Moreover, RNAi-mediated depletion of endogenous TCF7L2 resulted in reduced cyclin D1 promoter activity and protein expression, and the overexpression of TCF7L2 overrode the inhibition of the TCF-dependent transcriptional activity and cyclin D1 promoter activity induced by DIF-1. Therefore, DIF-1 seemed to inhibit the Wnt/β-catenin signaling pathway by suppressing TCF7L2 expression via reduced Egr-1-dependent transcriptional activity in these colon cancer cell lines. Our results provide a novel insight into the mechanisms by which DIF-1 inhibits the Wnt/β-catenin signaling pathway.

1. Introduction

The Wnt/β-catenin signaling pathway plays a number of key roles in embryonic development and maintenance of homeostasis in matured tissues. It is well known that the constant activation of Wnt/β-catenin signaling can lead to cancer development [1–4]. Notably, there are numerous reports on the involvement of the Wnt/β-catenin signaling pathway in colorectal cancers. Most colorectal cancers contain somatic mutations in adenomatous polyposis coli (APC) or β-catenin, which are members of the Wnt/β-catenin signaling pathway, resulting in constitutive activation of target gene transcription by an accumulation of nuclear β-catenin [5–8]. Numerous target genes of Wnt/β-catenin signaling are proto-oncogenes that have been directly implicated in cancer development [9–11]. Among them, TCF7L2 has been shown to be involved in tumor formation, and the β-catenin/TCF7L2 complex is described as a master switch that controls the proliferation and differentiation of intestinal epithelial cells [12–14]. Therefore, anti-cancer drugs that suppress transcriptional activity dependent on the β-catenin/TCF7L2 complex may be of significant therapeutic value for colon cancer therapy.

Differentiation-inducing factors (DIFs) were identified in *Dictyostelium discoideum* as morphogens required for stalk cell differentiation [15,16]. In the DIF family, DIF-1 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone) was the first to be identified. DIF activity is not limited to *Dictyostelium* and has been shown to strongly inhibit the proliferation of human cells [17–19]. Previously, we reported that DIFs inhibit the Wnt/β-catenin signaling pathway via glycogen synthase kinase-3β (GSK-3β)

Abbreviations: DIF, differentiation-inducing factor; GSK-3β, glycogen synthase kinase-3β; TCF7L2, transcription factor 7-like 2; Egr-1, early growth response-1; APC, adenomatous polyposis coli; RT-PCR, reverse transcription-polymerase chain reaction; RNAi, RNA interference; EMSA, electrophoretic mobility shift assay.

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activation followed by β-catenin degradation, leading to cell cycle arrest at the G_0/G_1 phase via the suppression of cyclin D1 expression in various human cells [20–27]. However, the effect of DIFs on human colon cancer cells, in which the Wnt/β-catenin signaling pathway is constitutively activated by inhibition of β-catenin destruction mechanisms, has not been elucidated.

In this study, we examine the effect of DIF-1 on human colon cancer cell lines that contain mutations in β-catenin (HCT-116) and APC (DLD-1 and SW-620).

2. Materials and methods

2.1. Chemicals and antibodies

DIF-1 was synthesized as described elsewhere [15,16]. MG132 was purchased from the Peptide Institute, Osaka, Japan. SB216763 was purchased from BIOMOL international (Farmingdale, NY). TOPflash (TCF reporter plasmid) and FOPflash (negative control for TOPflash) were purchased from Upstate Biotechnology (Lake Placid, NY). Wild-type and mutant cyclin D1 pGL3 basic luciferase reporter constructs were a generous gift from Drs. O. Tetsu and F. McCormick, University of California, San Francisco. The polyclonal anti-cyclin D1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-Egr-1 antibody was purchased from Cell Signaling Technology (Danvers, MA). The monoclonal anti-β-catenin antibody was purchased from BD Biosciences (San Jose, CA). The monoclonal anti-α-tubulin antibody was purchased from Calbiochem (Darmstadt, Germany). The monoclonal anti-GAPDH antibody was purchased from Abcam (Cambridge, UK). The monoclonal anti-TCF7L2 antibody was purchased from MILLIPORE (Temecula, CA).

2.2. Cell culture

Human colon cancer cell lines HCT-116 (expressing wild-type APC and mutant β-catenin), DLD-1 and SW-620 (expressing mutant APC and wild-type β-catenin) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 0.1 μg/ml streptomycin.

2.3. Cell proliferation assay

HCT-116 cells (3×10^4 cells/well), DLD-1 cells (5×10^4 cells/well) and SW-620 cells (7.5×10^4 cells/well) were seeded in 24-well plates and treated with or without various concentrations of DIF-1 for specific periods. Cells were harvested by trypsin/EDTA treatment and counted using a Coulter Counter (Beckman Coulter, Indianapolis, IN).

2.4. Flow cytometric analysis

Cells were suspended in a hypotonic solution containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate and 0.1% Triton X-100. PI-stained samples (1×10^5 cells) were analyzed for fluorescence with a Becton-Dickinson FACSCalibur (Franklin Lakes, NJ).

2.5. Western blotting

Western blotting was performed as described elsewhere [21]. Samples were separated by 12% SDS–PAGE and then transferred to a polyvinylidene difluoride membrane using a semidry transfer system (1 h, 12 V). Immunoreactive proteins were visualized by treatment with a detection reagent (LumiGLO; Cell Signaling Technology). Densitometric analysis was performed using NIH Image J Software.

2.6. Purification of nuclear proteins

Nuclear proteins were isolated from cells cultured in 60-mm dishes using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) followed by Western blot and EMSA analyses.

2.7. RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). Using 1 μg RNA, the expression of cyclin D1, TCF7L2, Egr-1, GAPDH mRNA was analyzed by RT-PCR. The following primers were used: TCF7L2 (forward: 5′-ACG AGG GCG AAC AGG AGG AG-3′, reverse: 5′-TGG GCG AGA GCG ATC CGT TG-3′) and Egr-1 (forward: 5′-GGT CAG TGG CCT AGT GAG C-3′, reverse: 5′-TGC TGT CGT TGG ATG GCA C-3′). Other primer sets are described elsewhere [21].

2.8. RNAi

TCF7L2 Select Stealth™ RNAi was purchased from Invitrogen. Transfection of dsRNA (100 nM) was performed using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. A Stealth™ RNAi negative control, which is the GC-matched scrambled sequence, was also purchased from Invitrogen.

2.9. Construction of the TCF7L2 reporter plasmid

The 5′-flanking region of the human *TCF7L2* gene (−1306/−1 bp relative to the start codon) was PCR-amplified from human genomic DNA followed by cloning into pGL3-Basic firefly luciferase reporter vector. A series of deletion plasmids (−869/−1, −629/−1, −604/−1, −578/−1, −434/−1, −223/−1 bp) were generated by PCR using TCF7L2-Luc (−1306/−1 bp) as a template.

2.10. TCF7L2-overexpressing plasmid

Total cellular RNA was extracted from HCT-116 cells and TCF7L2 cDNA was obtained by RT-PCR. The cDNA (GenBank accession number FJ010167) was verified by DNA sequencing and subcloned into pcDNA3 (Invitrogen).

2.11. Luciferase reporter assay

Cells were transfected with luciferase reporter plasmids and pRL-SV40, a Renilla luciferase expression plasmid as a control for transfection efficiency, using Lipofectamine Plus reagent (Invitrogen). Then, cells were cultured for 24 h followed by stimulation with DIF-1 at the indicated periods. Luciferase activity was determined with a luminometer (Lumat LB 9507, Berthold Technologies, Barsinghausen, Germany) and normalized to Renilla luciferase activity.

2.12. EMSA

Complementary oligonucleotides corresponding to the −634/−605 and −618/−589 bp regions in the TCF7L2 promoter were synthesized (5′-GGC GCC CGA AAG GAT CAT TGT TAG CCG CCC-3′ and 5′-CAT TGT TAG CCG CCC CCG CCC CGC CCA CCC-3′) and labeled at the 3′-end with biotin using a Biotin 3′ End DNA Labeling Kit (Pierce). Nuclear protein extracts (5 μg) were incubated with biotin-3′-labeled oligonucleotides with or without a 100-fold molar excess of unlabelled oligonucleotide as a competitor. To identify DNA binding to protein, nuclear protein extracts were incubated with 500 ng anti-Egr-1 or anti-α-tubulin antibodies prior to labeled probe addition. The anti-α-tubulin antibody was