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Silymarin induces cyclin D1 proteasomal degradation via its phosphorylation of threonine-286 in human colorectal cancer cells

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ABSTRACT

Silymarin from milk thistle (*Silybum marianum*) plant has been reported to show anti-cancer, anti-inflammatory, antioxidant and hepatoprotective effects. For anti-cancer activity, silymarin is known to regulate cell cycle progression through cyclin D1 downregulation. However, the mechanism of silymarin-mediated cyclin D1 downregulation still remains unanswered. The current study was performed to elucidate the molecular mechanism of cyclin D1 downregulation by silymarin in human colorectal cancer cells. The treatment of silymarin suppressed the cell proliferation in HCT116 and SW480 cells and decreased cellular accumulation of exogenously-induced cyclin D1 protein. However, silymarin did not change the level of cyclin D1 mRNA. Inhibition of proteasomal degradation by MG132 attenuated silymarin-mediated cyclin D1 downregulation and the half-life of cyclin D1 was decreased in the cells treated with silymarin. In addition, silymarin increased phosphorylation of cyclin D1 at threonine-286 and a point mutation of threonine-286 to alanine attenuated silymarin-mediated cyclin D1 downregulation. Inhibition of NF-κB by a selective inhibitor, BAY 11-7082 suppressed cyclin D1 phosphorylation and downregulation by silymarin. From these results, we suggest that silymarin-mediated cyclin D1 downregulation may result from proteasomal degradation through its threonine-286 phosphorylation via NF-κB activation. The current study provides new mechanistic link between silymarin, cyclin D1 downregulation and cell growth in human colorectal cancer cells.

1. Introduction

In worldwide, colorectal cancer is the third common malignancy [1,2]. Colorectal cancer is developed by a multistep process accompanied by adenomatous polyps, acquiring a series of somatic mutation, and aberrant gene expression [3,4]. Cyclin D1 forms cyclin dependent kinases (CDK) 4 and 6, and regulates cell cycle transition from G1 to S phase, which results in an increase of cell proliferation [5–8]. Cyclin D1 has been reported to be overexpressed in 68.3% of human colorectal cancer indicating that the deregulation of cyclin D1 is associated with colorectal tumorigenesis [9,10]. Therefore, it has been accepted that the control of cyclin D1 level may provide a promising chemopreventive and therapeutic way for human colorectal cancer.

Both the surgery and adjuvant therapy have been regarded as the most effective treatment for human colorectal cancer. However, the complementary and alternative medicine is considered because of ineffectiveness of these therapeutic approaches. Thus, chemoprevention using phytochemicals, widely distributed vegetables, fruits and medicinal plants has received attention as an attractive and promising strategy for human cancer [11]. It has been reported that there is a strong inverse relationship between the consumption of vegetables and human colorectal cancer [12], which indicates that a constant intake of phytochemical-containing plants is beneficial for the prevention of the human colorectal cancer.

Silymarin is a complex of three flavonolignans (silybin, silydianin and silychristin) and two flavonoids (tamoxifen and quercetin) contained in the seeds of the milk thistle (*Silybum marianum*) [13,14] and has been clinically used for a long time to treat liver diseases due to its hepatoprotective effects [15–17]. In addition, silymarin has been reported to exert anti-inflammatory and anticarcinogenic properties [18–20]. In anti-cancer activity, silymarin has a number of the different molecular targets such as anti-inflammation, cell cycle regulation, apoptosis induction, inhibition of angiogenesis, inhibition of invasion & metastasis, and growth inhibition [21]. Especially, silymarin treatment induces binding of Cip1/p21 with CDK2 and CDK6 paralleled a significant decrease in CDK2-, CDK6-, cyclin D1-, and cyclin

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E-associated kinase activities, along with a decrease in cyclin D1 and E, which results in the inhibition of the proliferation of cells by inhibiting cell cycle progression at different stages of the cell cycle [21].

However, more detailed mechanism for silymarin-mediated decrease of cyclin D1 level still remains unanswered. Here, we propose a novel anti-cancer mechanism of silymarin. Silymarin induces cyclin D1 proteasomal degradation through NF-κB activation in human colorectal cancer cells.

2. Materials and methods

2.1. Reagents

Cell culture media, Dulbecco's modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). The 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and BAY 11-7082 were purchased from Sigma-Aldrich (St. Louis, MO, USA). pNFκB-Luc cis-Reporter plasmid was purchased from Agilent Technologies (Santa Clara, CA, USA). Cyclin D1 promoter was provided from Addgene (Cambridge, MA, USA). Antibodies against cyclin D1, phospho-cyclin D1 (Thr286), HA-tag and β-actin were purchased from Cell Signaling (Beverly, MA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

2.2. Cell culture and treatment

Human colon cancer cell lines, HCT116 and SW480 were purchased from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM/F-12 supplemented with 10% fatal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37 °C under a humidified atmosphere of 5% CO_2. Silymarin was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

2.3. Cell proliferation assay

Cell growth was measured using MTT assay system. Briefly, cells were plated onto 96-well plates and grown overnight. The cells were treated with 0, 50, 100 and 200 μg/ml of silymarin for 24 and 48 h. Then, the cells were incubated with 50 μl of MTT solution (1 mg/ml) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 nm.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 μg) was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was carried out using PCR Master Mix Kit (Promega, Madison, WI, USA) with human primers for cyclin D1 and GAPDH as follows: cyclin D1: forward 5′-aactacctggaccgcttcct-3′ and reverse 5′-ccacttgagcttgttcacca-3′ and GAPDH: forward 5′-acccagaagactgtggatgg-3′ and reverse 5′-ttctagacggcaggtcaggt-3′.

2.5. SDS-PAGE and Western blot

After silymarin treatment, cells were washed with 1× phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich), and centrifuged at 15,000 ×g for 10 min at 4 °C. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with specific primary antibodies in 5% non-fat dry milk at 4 °C overnight. After three washes with TBS-T, the blots were incubated with horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized in Polaroid film.

2.6. Expression vectors

Wild type HA-tagged cyclin D1 and point mutation of T286A of HA-tagged cyclin D1 were provided from Addgene (Cambridge, MA, USA). Transient transfection of the vectors was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturer's instruction.

2.7. Transient transfection and luciferase activity

Transient transfection was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories) according to the manufacturer's instruction. Cells were plated in 12-well plates at a concentration of 2×10^5 cells/well. After growth overnight, plasmid mixtures containing 0.5 μg of NF-κB-Luc-plasmid or cyclin D1 promoter, and 0.05 μg of pRL-null vector were transfected for 24 h. The transfected cells were treated with silymarin for 24 h. The cells were then harvested in 1× luciferase lysis buffer, and luciferase activity was normalized to the pRL-null luciferase activity using a dual-luciferase assay kit (Promega).

2.8. Statistical analysis

All the data are shown as mean ± SEM (standard error of mean). Statistical analysis was performed with one-way ANOVA followed by Dunnett's test. Differences with $^*P < 0.05$ were considered statistically significant.

3. Results

3.1. Inhibitory effect of silymarin on the proliferation of HCT116 and SW480 cells

To investigate if silymarin affects the proliferation of human colon cancer cells, HCT116 (APC wild type) and SW480 (APC mutant) were treated with the different concentrations of silymarin for 0, 24 and 48 h and the cell proliferation was measured. As shown in Fig. 1A, HCT116 cells treated with 50, 100, and 200 μM of silymarin reduced the cell growth by 11%, 22% and 48% in 24 h and 16%, 36% and 54% in 48 h, respectively. And SW480 cells treated with 50, 100, and 200 μM of silymarin reduced the cell growth by 13%, 28% and 47% in 24 h and 24%, 39% and 59% in 48 h, respectively (Fig. 1B). The results indicate that silymarin suppressed cell growth of human colorectal cancer cells in dose- and time-dependent manner, and anti-proliferative activity of silymarin is APC-independent.

3.2. Inhibitory effect of silymarin on cyclin D1 expression in HCT116 and SW480 cells

To test if silymarin affects cyclin D1 expression, we treated HCT116 and SW480 cells with 50, 100 and 200 μM of silymarin for 24 h. As shown in Fig. 2A and B, silymarin treatment (100 and 200 μM) down-regulated cyclin D1 expression. Next, we observed the effects of silymarin on cyclin D1 expression at different time points. As shown in Fig. 2C, cyclin D1 expression begins to decrease at 6 h in HCT116