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

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A B S T R A C T

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 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.

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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
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-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and BAY 11-7082 were purchased from Sigma-Aldrich (St. Louis, MO, USA). pNF-κB-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% fetal 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% CO2. 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′-aactacttgacgcgtctc-3′ and reverse 5′-ccacttagctggcaacca-3′ and GAPDH: forward 5′-accagagactctgctgg-3′ and reverse 5′-ttctagacggccagttc-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 × 105 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 13%, 28% and 47% in 24 h and 24%, 39% and 59% in 48 h, respectively (Fig. 1B). The results indicate that 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
cells and 3 h in SW480 cells after silymarin treatment. To determine if the downregulation of cyclin D1 is responsible for transcriptional down-regulation, we tested mRNA level and promoter activity of cyclin D1 in HCT116 and SW480 cells treated with the different concentrations of silymarin. As shown in Fig. 2D, mRNA level was not affected by treatment of silymarin. In addition, silymarin did not affect cyclin D1 promoter activity (Fig. 2E). These results indicate that silymarin may decrease protein stability of cyclin D1.

3.3. Silymarin-mediated proteasomal degradation of cyclin D1 via threonine-286 phosphorylation

To confirm that silymarin affects proteasomal degradation of cyclin D1, the cells were pretreated with the proteasome inhibitor (MG132) and then co-treated with silymarin. As a result, pre-treatment of MG132 blocked silymarin-induced downregulation of cyclin D1 in HCT116 and SW480 cells (Fig. 3A and B). To verify these results, the...
cells were pre-treated with DMSO or silymarin and then exposed to cy-

cloheximide for indicated times. As shown in Fig. 3C, silymarin treat-

ment decreased half-life of cyclin D1 protein in HCT116 cells.

There is growing evidence that cyclin D1 phosphorylation on

threonine-286 is associated with its proteasomal degradation via the

ubiquitin–proteasome pathway [22]. Thus, we evaluated the effect of

silymarin on the threonine-286 phosphorylation of cyclin D1. As

shown in Fig. 3D, cyclin D1 phosphorylation (threonine-286) began to

increase at 1 h in silymarin-treated HCT116 cell. To verify that cyclin

D1 phosphorylation (threonine-286) by silymarin results in cyclin D1

proteasomal degradation, HCT116 cells were transfected with wild type

HA-tagged cyclin D1 for 24 h and then treated with 200 μM of silymarin for the indicated times. (E) HCT116 cells were transfected with wild type

HA-tagged cyclin D1 or HA-tagged T286A cyclin D1 and then treated with 200 μM of silymarin for 10 h. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against cyclin D1, phospho-

cyclin D1 (Thr286) or HA-tag. Actin was used as internal control.

Fig. 3. Proteasomal degradation of cyclin D1 by silymarin in HCT116 and SW480 cells. (A, B) HCT116 and SW480 cells were plated overnight. The cells were pretreated with MG132 for 2 h and then co-treated with silymarin for the indicated times. (C) HCT116 cells were pretreated with DMSO for 200 μM of silymarin for 3 h and then co-treated with 10 μg/ml of cycloheximide (CHX) for the indicated times. (D) HCT116 cells were plated overnight and then treated with 200 μM of silymarin for the indicated times. (E) HCT116 cells were transfected with wild type

HA-tagged cyclin D1 for 24 h and then treated with 200 μM of silymarin for the indicated times. (F) HCT116 cells were transfected with wild type HA-tagged cyclin D1 or HA-tagged T286A cyclin D1 and then treated with 200 μM of silymarin for 10 h. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against cyclin D1, phospho-

cyclin D1 (Thr286) or HA-tag. Actin was used as internal control.
Overall, these data proposed that the downregulation of cyclin D1 by silymarin depends on proteolytic proteasomal degradation via threonine-286 phosphorylation.

3.4. NF-κB activation contributes to silymarin-mediated cyclin D1 proteasomal degradation

It has been reported that many kinases including ERK1/2 [23], p38 [24], GSK3β [25] and NF-κB [26] are associated with cyclin D1 proteasomal degradation. To determine the upstream kinases associated with silymarin-mediated cyclin D1 proteasomal degradation, HCT116 cells were pretreated with BAY 11-7082 as a selective inhibitor of NF-κB and then co-treated with silymarin. As shown in Fig. 4A, DMSO-pretreated cells induced cyclin D proteasomal degradation by silymarin, while NF-κB inhibition by BAY 11-7082 attenuated silymarin-induced downregulation of cyclin D1. Then, we evaluated the effect of NF-κB inhibition on silymarin-induced phosphorylation of cyclin D1 and found that silymarin induced cyclin D1 phosphorylation by 2.8-fold in absence of BAY 11-7082, while NF-κB inhibition by BAY 11-7082 attenuated silymarin-induced downregulation of cyclin D1. In addition, silymarin increased NF-κB luciferase activity (Fig. 4C). However, other kinases such as ERK1/2, p38 and GSK3β did not affect cyclin D1 proteasomal degradation by silymarin (data not shown). These findings indicate that NF-κB activation by silymarin at least in part contributes silymarin-mediated cyclin D1 proteasomal degradation.

4. Discussion

Silymarin has been used as a natural medicine for treating hepatitis and cirrhosis and to protect liver from toxic substances for more than 2000 years [21]. In addition, silymarin has been reported to have anti-oxidative, anti-lipid peroxidative, anti-fibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory and liver regenerating, and anti-cancer effects [21]. Anti-cancer activity of silymarin has been observed in human breast cancer, skin cancer, androgen-dependent and -independent prostate cancer, cervical cancer, colorectal cancer, ovarian cancer, hepatocellular carcinoma, bladder cancer, and lung cancer cells [27–29]. For the regulation of cell cycle, silymarin downregulates cyclin D1 which results in the inhibition of the proliferation of cells by inhibiting cell cycle [21]. However, more detailed mechanism for silymarin-mediated decrease of cyclin D1 level still remains unanswered.

The level of cyclin D1 can be regulated by multiple mechanisms. One is through transcriptional regulation. In this study, our data showed that silymarin did not affect cyclin D1 mRNA expression, indicating that silymarin-mediated downregulation of cyclin D1 may be independent on transcription. Another mechanism to regulating cyclin D1 level is through the activation of proteasomal degradation, which is regarded as one of the important anti-cancer mechanisms, previously reported with curcumin [30], retinoic acid [31] and troglitazone [32]. In the presence of MG132 as a proteasome inhibitor, silymarin-mediated
downregulation of cyclin D1 was attenuated compared to silymarin-treated cells without MG132, which suggests that silymarin downregulates cyclin D1 through activating proteasomal degradation. Cyclin D1 proteasomal degradation is associated with its phosphorylation at threonine-286 [22]. Silymarin induced threonine-286 phosphorylation of cyclin D1 and the point mutation of threonine-286 to alanine ameliorated silymarin-mediated cyclin D1 degradation. These findings indicate that proteasomal degradation of cyclin D1 by silymarin may depend on threonine-286 phosphorylation.

Cyclin D1 proteasomal degradation has been shown to occur dependently of ERK1/2, p38, GSK3β, and NF-κB [23–26]. From our data that silymarin-mediated activation of NF-κB and suppression of silymarin-mediated cyclin D1 phosphorylation and subsequent degradation by selective inhibition of NF-κB by BAY 11-7082, NF-κB activation by silymarin at least in part contributes to silymarin-mediated cyclin D1 proteasomal degradation.

In conclusion, silymarin-induced proteasomal degradation of cyclin D1 might inhibit proliferation in human colorectal cancer cells. Although silymarin has been reported to regulate cell cycle [21], the current study provides information on molecular events of silymarin-regulated cell cycle.

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References


