Anticarcinogenic Effect of a Flavonoid Antioxidant, Silymarin, in Human Breast Cancer Cells MDA-MB 468: Induction of $G_1$ Arrest through an Increase in Cip1/p21 Concomitant with a Decrease in Kinase Activity of Cyclin-dependent Kinases and Associated Cyclins

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ABSTRACT

There is an increasing interest in identifying potent cancer preventive and therapeutic agents against breast cancer. Silymarin, a flavonoid antioxidant isolated from milk thistle, exerts exceptionally high to complete anticarcinogenic effects in tumorigenesis models of epithelial origin. In this study, we investigated the anticarcinogenic effect of silymarin and associated molecular mechanisms, using human breast carcinoma cells MDA-MB 468. Silymarin treatment resulted in a significantly high to complete inhibition of both anchorage-dependent and anchorage-independent cell growth in a dose- and time-dependent manner. The inhibitory effects of silymarin on cell growth and proliferation were associated with a $G_1$ arrest in cell cycle progression concomitant with an induction of up to 19-fold in the protein expression of cyclin-dependent kinase (CDK) inhibitor Cip1/p21. Following silymarin treatment of cells, an incremental binding of Cip1/p21 with CDK2 and CDK6 paralleled a significant decrease in CDK2-, CDK6-, cyclin D1-, and cyclin E-associated kinase activity with no change in CDK2 and CDK6 expression but a decrease in $G_1$ cyclins D1 and E. Taken together, these results suggest that silymarin may exert a strong anticarcinogenic effect against breast cancer and that this effect possibly involves an induction of Cip1/p21 by silymarin, which inhibits the threshold kinase activities of CDKs and associated cyclins, leading to a $G_1$ arrest in cell cycle progression.

INTRODUCTION

Breast cancer is the most common nonskin malignancy and the second leading cause of cancer deaths in women in the United States (1). One approach to controlling breast cancer is chemopreventive intervention, a means of cancer control in which the disease is prevented, slowed, or reversed by the administration of one or a combination of naturally occurring or synthetic compounds. Several studies have shown that microchemicals present in the diet, several herbs, and plants are the most desirable class of agents for the prevention and/or intervention of various cancers (Refs. 2–8 and references therein). Among these, polyphenolic antioxidants are receiving increasing attention in recent years (5–8). One such naturally occurring polyphenolic flavonoid antioxidant is silymarin, isolated from milk thistle (Silybum marianum (L.) Gaertn; Ref. 9).

Silymarin is accepted for use in humans because it is used clinically in Europe and Asia for the treatment of alcoholic liver diseases (10). As a therapeutic agent, it is well tolerated and largely free from adverse effects, it is nontoxic in acute, subchronic, and chronic tests even at large doses, and there is no known LD$_{50}$ for this agent (Ref. 11 and references therein). Silymarin protects against hepatotoxicity and lipid peroxidation induced by several xenobiotic agents (9–13) and has been shown to be a strong antioxidant capable of scavenging free radicals (Refs. 14–16 and references therein). Limited short-term studies have suggested that silymarin may be a potent anticarcinogenic agent (17, 18). In our recent studies, we showed that silymarin exerts exceptionally high to complete protective effects against carcinogenesis in different mouse tumorigenicity models of epithelial origin (19, 20).

Defects in the regulation of cell cycle progression are thought to be one of the commonest features of transformed cells (21). Eukaryotic cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of CDKs at different phases (22, 23). The activities of CDKs are positively regulated by cyclins (24); there are five major types of cyclins, namely A, B, C, D, and E, which act at different checkpoints of cell cycle (21). Cyclins D and E are required for progression through $G_1$ (25). As cells enter $G_1$, the CDK4- and/or CDK6-cyclin D complex appears to be necessary for transition through early $G_1$ (23), whereas CDK2-cyclin E com-

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3 The abbreviations used are: CDK, cyclin-dependent kinase; CDK1, CDK inhibitor; RB, retinoblastoma; FACS, fluorescence-activated cell sorting.
plex is involved in transition from late G1 into S phase (26, 27). Whereas CDK activity is negatively regulated by CDKIs (24, 28), CDKI proteins are often absent or inactive in cancerous cells (Refs. 29 and 30 and references therein).

At least seven CDKIs have been identified in mammalian cells, and on the basis of homology, they fall into the p21 and p16 families, each of which comprises several members (24, 28). The p21 family includes p21 (also known as Cip1, WAF1, and so forth; Ref. 31), Kip1/p27 (32, 33), and Kip2/p57 (34, 35). These CDKIs preferentially bind to and inhibit most CDK-cyclin complexes (21, 28, 30). Out of several CDKIs, Cip1/p21 is an important mediator of cell cycle arrest imposed by p53 in response to DNA damage (31, 36, 37). However, Cip1/p21 expression can also be induced by other factors in a p53-independent fashion (38–40). Several in vitro studies have demonstrated that relative levels of Cip1/p21 may be critical in determining the threshold kinase activity of various CDK-cyclin complexes (41, 42), suggesting that appropriate levels of Cip1/p21 may be critical in cell growth regulation.

When these data are taken together, it can be appreciated that the agents that overcome the loss of tumor suppressor genes and/or the presence of aberrant endogenous cell cycle regulation that contributes to the tumorigenic process should be useful for the preventive intervention of cancer. Using MDA-MB 468 cells, which are estrogen receptor-negative breast carcinoma cells that overexpress a transcriptionally active mutant p53 protein (43, 44), in this study, we demonstrate the anticarcinogenic effect of silymarin and suggest the involvement of G1 phase of the cell cycle and regulatory proteins associated with it as a possible molecular mechanism of the effect of silymarin.

### MATERIALS AND METHODS

#### Cell Line and Other Reagents

The MDA-MB 468 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in improved minimum essential Eagle’s medium with 2 mM glutamine (Biofluids, Rockville, MD), 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO2 atmosphere. Silymarin was purchased from Aldrich Chemical Co. (Milwaukee, WI) and dissolved in ethanol. The final concentration of ethanol in culture medium during silymarin treatment did not exceed 0.5% (v/v), and therefore, the same concentration of ethanol was present in control dishes. Anti-Cip1/p21 antibody was from Calbiochem (Cambridge, MA). Anti-Kip1/p27 and anti-CDK4 antibodies were from Neomarkers, Inc. (Fremont, CA). Antibodies to cyclin D1, cyclin E, CDK2, and CDK6; rabbit antismouse immunoglobulin-horseradish peroxidase-conjugated and goat antirabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibodies; and RB-glutathione S-transferase fusion protein were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Histone H1 was from Boehringer Mannheim Corp. (Indianapolis, IN). [γ-32P]ATP (specific activity, 3000 Ci/mmol) was from New England Nuclear (Boston, MA). ECL detection system was from Amersham Corp. (Arlington Heights, IL).

#### Cell Growth Assay

For the anchorage-dependent cell growth assay, MDA-MB 468 cells were plated at 0.5 × 10^5 cells/60-mm plate under the culture conditions detailed above. On day 2, cells were fed with fresh medium and either left untreated or treated with ethanol alone or silymarin at a dosage of 10, 25, 50, or 75 μg/ml of medium dissolved in ethanol. The cultures were fed with fresh medium with or without same concentrations of silymarin every alternate day up to the end of the experiment; each treatment and time point had four plates. At days 1–5 of these treatments, cells were trypsinized and collected in counting vials. Each plate was washed thoroughly with isotonic buffer containing 0.1% formalin, and washings were collected in the original vials with trypsinized cells. Each vial was counted in a Coulter counter to determine the total number of cells. In a parallel experiment, at various experimental intervals, the number of viable cells was also determined by the trypan blue dye exclusion test.

#### Soft Agar Colony Formation Assay

MDA-MB 468 cells were cultured under the conditions detailed above. For anchorage-independent cell growth, a soft agar colony formation assay was performed using 6-well plates. Each well contained 2 ml of 0.5% agar in medium as the bottom layer, 1 ml of 0.38% agar in medium and 2000 cells as the feeder layer, and 1 ml of 0.38% agar in medium either with vehicle ethanol or with different doses of silymarin in ethanol as the top layer. Each treatment had three wells. Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a group of more than 10 cells was counted as a colony. The rate of colony growth and the optimum time for scoring colonies was assessed by counting colonies at 5, 7, 10, 15, 20, and 30 days and was found to be optimal at day 10. The wells were also examined on day 1 to eliminate possible artifacts caused by any clumps of cells.

#### Cell Cycle Analysis

MDA-MB 468 cells at 80–90% confluency were either untreated or treated with ethanol alone or varying concentrations of silymarin (25–75 μg/ml of medium) in ethanol. Twenty-four, 48, and 72 h after these treatments, medium was aspirated, monolayers were quickly washed twice with cold PBS, cells were trypsinized, and cell pellets were collected. The cells were washed twice with PBS, fixed in cold methanol, and rewarshed with PBS to remove methanol. After being suspended in 500 μl of PBS, cells were digested with 20 μg/ml RNase at 37°C for 30 min and chilled on ice for 10 min, and then cellular DNA was stained with propidium iodide (50 μg/ml) by incubation for 1 h at room temperature in the dark. Cell cycle distribution was analyzed by flow cytometry using the Becton Dickinson FACS system.

#### Silymarin Treatment of Cells for Cell Cycle Regulatory Molecules

MDA-MB 468 cells were grown as detailed above, and 80–90% confluent cultures were treated with either ethanol alone or varying concentrations of silymarin (10–75 μg/ml of medium) in ethanol. Sixteen h after these treatments, medium was aspirated, cells were quickly washed twice with cold PBS, and 0.5 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP40, 1 μg/ml pepstatin, 0.2 units/ml aprotinin) was added to each plate. After 15 min in lysis buffer at 4°C, the cell lysate was scraped from the plate, collected in microcentrifuge tubes and left on ice for additional 15 min. The lysates were cleared by centrifugation for 5 min in a table-top centrifuge at 4°C, the
clear supernatants were collected, and protein concentration was determined. For the studies assessing the time-dependent effect of silymarin, cultures were treated with either ethanol or silymarin at a concentration of 75 μg/ml of medium, cells were harvested 4, 8, 16, 24, and 48 h later, and cell lysates prepared as described above.

**Western Immunoblotting and Immunoprecipitation.** For Western immunoblotting, 40–100 μg of protein lysate per sample were denatured with 2 × SDS sample buffer, samples were subjected to 12% SDS-PAGE, and separated proteins were transferred on to membrane. The levels of Cip1/p21, Kip1/p27, CDK2, CDK4, CDK6, cyclin D1, and cyclin E were determined using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. For studies evaluating the binding of CDKs with cyclins, 50 μg of protein lysate was mixed with 0.5 μg of anti-CDK2 or anti-CDK6 antibody, and then this mixture was rotated at 4°C for 4 h. Thereafter, 5 μl of protein A-agarose beads were added, and this mixture was incubated overnight at 4°C. The next day, beads were collected by centrifugation and washed four times with lysis buffer, and the immunoprecipitated CDK2 or CDK6 was denatured with 30 μl of 1 × SDS sample buffer. The proteins were subjected to SDS-PAGE on 12% gel, and the separated proteins were transferred on membrane by Western blotting. The membrane was probed with anti-cyclin E antibody in the case of CDK2 or anti-cyclin D1 antibody in the case of CDK6 followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. Similarly, the levels of CDK2 and CDK6 bound to Cip1/p21 were determined by immunoprecipitating Cip1/p21 using anti-Cip1/p21 antibody and 5 μl of protein G-agarose beads, and following SDS-PAGE and Western blotting, the membranes were probed with specific primary antibody to CDK2 or CDK6 followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system.

**Kinase Activity Assays.** CDK2- and cyclin E-associated H1 histone kinase activity was determined as described by Wu et al. (45). Briefly, using anti-CDK2 or anti-cyclin E antibody (2 μg) and protein A-agarose beads (20 μl), CDK2 and cyclin E were immunoprecipitated from 200 μg of protein lysate per sample as described above. Beads were washed three times with lysis buffer and then once with kinase assay buffer (50 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 40 μl of “hot” kinase solution [0.25 μl (2.5 μg) of histone H1, 0.5 μl of [γ-32P]ATP, 0.5 μl of 1 mM ATP, and 38.75 μl of kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography. Similarly, CDK6- and cyclin D1-associated RB kinase activity was determined as described by Wu et al. (45) and detailed above with some modifications. Briefly, vehicle- or silymarin-treated cells were lysed in RB lysis buffer (50 mM HEPES-KOH, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 80 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin and aprotinin), and using anti-CDK6 or anti-cyclin D1 antibody (2 μg) and protein G-agarose beads (20 μl), specific proteins were immunoprecipitated from 200 μg of protein lysate per sample as detailed above. Beads were washed three times with RB lysis buffer and then once with RB kinase assay buffer (50 mM HEPES-KOH, pH 7.5, containing 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM MgCl₂, 1 mM DTT). Phosphorylation of RB was measured by incubating the beads with 40 μl of hot RB kinase solution [0.25 μl (2 μg) of RB-glutathione S-transferase fusion protein, 0.5 μl of [γ-32P]ATP, 0.5 μl of 0.1 mM ATP, 38.75 μl of RB kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

**Densitometric and Statistical Analysis.** Autoradiograms of the Western immunoblots were scanned with a Microtek MSF-300GS image scanner (Microtek, Torrance, CA) that was linked to a Macintosh IIsi computer. The image generated by the Microtek gray-scale scanner was captured by Image Studio and then analyzed for intensity of gray relative to background by Scan Analysis. The quantitation of image intensities on film was carried out at less than maximal densities. Radiolabeled bands for kinase activities were quantitated with a PhosphorImager and analyzed with Image-Quant software (Molecular Dynamics). As needed, the two-tailed Student’s t test was used to assess statistical significance of the difference between vehicle- and silymarin-treated samples. Unless specified otherwise, the results shown in each case are representative of three independent experiments with similar findings.

**RESULTS**

**Silymarin Exerts both Antiproliferative and Anticarcinogenic Effects in MDA-MB 468 Cells.** Using an anchorage-dependent growth assay, we first assessed whether silymarin affords an antiproliferative effect in human breast carcinoma cells MDA-MB 468. As shown in Fig. 1, silymarin treatment significantly inhibited the cell proliferation in a dose- and time-dependent manner. Whereas lowest concentration of silymarin tested (10 μg/ml) showed almost no inhibition, a concentration of 25 μg of silymarin/ml showed a complete inhibition of cell proliferation, with almost 50% inhibition (P < 0.001) at day 5 (Fig. 1). Treatment of cells with silymarin at higher doses of 50 and 75 μg/ml showed a complete inhibition (P < 0.0001) of anchorage-dependent cell growth beginning at day 2 and onward at all time points studied, up to 5 days of treatment (Fig. 1). A 75 μg/ml dose of silymarin also showed a reduction in initial cell number.

Inhibition of soft agar colony formation potential of malignant cells is used extensively as a short-term in vitro assay to assess the anticarcinogenic effects of agents undergoing testing. Using this assay, we next assessed the anticarcinogenic effect of silymarin in breast carcinoma cells MDA-MB 468. First, we determined the optimal time period and number of cells per plate, as well as number of cells per colony. In these studies, we found that 2000 cells grown in soft agar gave rise to optimum colonies of more than 10 cells per colony after 10 days of seeding (data not shown). As many as 97 ± 7 (mean ± SE of...
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Fig. 1 Silymarin treatment inhibited proliferation of breast carcinoma cells MDA-MB 468. Cells were plated at 0.5 × 10⁵ cells/60-mm plate. On day 2 after plating, cultures were treated with ethanol or silymarin at a concentration of 10–75 μg/ml of medium, and total number of cells were counted for varying time periods (1–5 days) as described in “Materials and Methods.” The cell growth data shown are mean ± SE of four independent plates; each sample was counted in duplicate.

Fig. 2 Silymarin treatment inhibited anchorage-independent growth of breast carcinoma cells MDA-MB 468. Cells were cultured as described in “Materials and Methods,” and soft agar colony formation assay was performed using 6-well plates as detailed in “Materials and Methods.” Each well contained 2 ml of 0.5% agar in complete medium as the bottom layer, 1 ml of 0.38% agar in complete medium and 2000 cells as the feeder layer, and 1 ml of 0.38% agar in complete medium either with vehicle ethanol or a different dose of silymarin in ethanol as the top layer. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a group of more than 10 cells was counted as a colony. The data shown are means ± SE of three independent wells at optimum time of 10 days from the start of cell seeding; the experiment was repeated once with similar results.

Further increase in G₁ accumulation of cells was observed when the dose of silymarin was increased from 50 to 75 μg/ml (data not shown).

Silymarin Induces Cip1/p21 Protein Expression. CDK1 Cip1/p21 plays a major role in the induction of G₁ cell cycle arrest by an elevation in its mRNA and protein levels through both p53-dependent and p53-independent mechanisms (38, 40, 48–51). On the basis of our results showing a dose- and time-dependent inhibition of MDA-MB 468 cell proliferation caused by silymarin concomitant with a significant increase in G₁ population of the cells, we next examined the effect of silymarin treatment on the protein levels of CDKIs Cip1/p21 and Kip1/p27. Exposure of cells to silymarin resulted in a highly significant induction of Cip1/p21 protein levels in both dose-dependent (Fig. 4A) and time-dependent (Fig. 4B) manners. As quantitated by densitometric analysis of the immunobLOTS, treatment of cells with silymarin at 10 μg/ml dose for 16 h resulted in a small induction (1.8-fold) of Cip1/p21; however, 25, 50, and 75 μg/ml doses of silymarin showed 6-, 18-, and 19.5-fold increase, respectively, in Cip1/p21 protein levels as compared to vehicle-treated control (Fig. 4A). In another study, we also followed the time kinetics of Cip1/p21 protein induction caused by silymarin. As shown in Fig. 4B, silymarin treatment of cells at 75 μg/ml dose showed an induction of Cip1/p21 as early as 8 h. A longer treatment time with the same silymarin dose resulted in a progressive increase in Cip1/p21 protein levels (Fig. 4B). Kip1/p27 was not detectable in MDA-MB 468 cells without or with silymarin treatment (data not shown).
Silymarin Inhibits the Levels of G1 Cyclins and Their Binding to CDKs but not the Levels of CDKs. Eukaryotic cell cycle progression is regulated by a series of CDKs and cyclins at different phases; the transition from G1 to S is thought to be regulated by CDK4 and/or CDK6 complexed with cyclin D and CDK2 complexed with cyclin E (23, 26). Therefore, we analyzed the expression and binding of these cell cycle-regulatory proteins in MDA-MB 468 cells treated either with ethanol (control) or with silymarin in ethanol. As compared to the control, Western blot analysis of the samples obtained from 75 μg/ml silymarin treatment for different time periods showed no change in the protein levels of CDK2 (Fig. 5A) and CDK6 (Fig. 5B). Under the experimental conditions used, CDK4 was not detectable in any of the control or silymarin-treated samples (data not shown), which is consistent with earlier studies in MDA-MB 468 breast carcinoma cells (52). With regard to G1 cyclins, treatment of cells with same dose of silymarin for varying time resulted in a time-dependent decrease in both cyclin D1 (Fig. 5C) and cyclin E (Fig. 5D) protein expression. Although in the case of cyclin D1, the observed decrease was more significant at 24 and 48 h after silymarin treatment, it was also significant at early time points for cyclin E (Fig. 5, C and D). The decrease in protein level of cyclin E was also associated with a gradual decrease in its binding to CDK2 (Fig. 5E) with increasing silymarin treatment time. However, in the case of cyclin D1 binding to CDK6, silymarin treatment did not show a considerable change at all time points studied. Comparing these data with a decrease in cyclin D1 protein levels caused by silymarin, it can be suggested that the observed decrease in cyclin D1 is possibly that associated with newly synthesized protein that has not yet bound to CDK6. More detailed studies, however, are needed to further explore this possibility.

Silymarin Results in a Decrease in CDK- and Cyclin-associated Kinase Activity via an Increased Binding of Cip1/p21 to CDKs. CDKs complexed with their catalytic subunit cyclins regulate the cell cycle progression in different phases; progression is also controlled by a threshold functional levels of CDKIs (21, 24). Therefore, a decrease in the levels of CDKs and/or cyclins associated with a specific phase of cell cycle is supposed to result in an arrest in that phase of progression. The other possibility is an induction in CDKI expression leading to an increase in its binding to and subsequent inactivation of...
CDK-cyclin complex followed by an arrest in cell cycle progression. The results obtained in our study showing that silymarin treatment of MDA-MB 468 cells does not result in a decrease in CDKs expression but reduces G1 cyclins D1 and E suggest that silymarin exerts its effect by (a) decreasing the levels of the catalytic subunit cyclins (Fig. 5, C and D) and thereby causing a decrease in their binding to associated CDKs, and (b) inducing Cip1/p21 expression (Cip1/p21 binds to CDKs...
and inactivates their kinase activity). To support the second suggestion, we assessed the effect of silymarin on kinase activity of CDKs and associated cyclins and the binding of CDKs to CDK1 Cip1/p21.

As shown in Fig. 6A, compared to vehicle-treated control, treatment of cells with silymarin at 75 μg/ml dose for varying time periods resulted in a highly significant reduction in CDK2 kinase activity in a time-dependent manner. At longer treatment times of 24 and 48 h, decreases of as much as 40 and 73%, respectively, in CDK2 kinase activity were observed (Fig. 6A). Similarly, silymarin treatment also showed a 40 and 75% reduction in cyclin E-associated histone H1 kinase activity at 24 and 48 h of treatment (Fig. 6B), suggesting that presumably this decrease was associated with CDK2 bound to cyclin E. We next assessed whether the observed decrease in CDK2 kinase activity was due to an increase in its binding to the induced levels of Cip1/p21 following silymarin treatment. As shown in Fig. 6C, indeed, treatment of cells with silymarin at 75 μg/ml dose for varying time periods resulted in a significant increase in the binding of CDK2 with Cip1/p21. A more marked increase in binding was evident at 24 and 48 h of silymarin treatment (Fig. 6C) than at earlier times (data not shown). Together, the results shown in Fig. 6 demonstrate that silymarin treatment of cells results in a significant decrease in CDK2- and cyclin E-associated kinase activity and that this effect is due to an increased binding of CDK2 with Cip1/p21.

As with CDK2 kinase activity, silymarin treatment at 75 μg/ml dose resulted in a significant decrease in CDK6 kinase activity; a more profound effect was observed at 24 (43% reduction) and 48 (70% reduction) h of treatment than at earlier time points of 8 and 16 h (Fig. 7A). Corroborative to the experiments involving CDK6, cyclin D1-associated kinase activity was also found to be decreased significantly in a time-dependent manner following silymarin treatment of cells (Fig. 7B). Immunoprecipitation of Cip1/p21 followed by immunoblotting with CDK6 antibody clearly demonstrated a time-dependent increase in the binding of CDK6 with Cip1/p21, specifically at 24 and 48 h of silymarin treatment (Fig. 7C), supporting a reduction in CDK6- and cyclin D1-associated kinase activity.

**DISCUSSION**

In the present study, we demonstrated strong anticarcinogenic effect of silymarin in human breast carcinoma cells MDA-MB 468 through their accumulation in G1 phase of the cell cycle and suggested the modulation of cell cycle-regulatory proteins and associated kinase activity as a possible molecular mechanism of the effect of silymarin. The results obtained provide convincing evidence that silymarin exerts its effect on cell cycle progression of MDA-MB 468 cells by two pathways. First, by a significant increase in Cip1/p21 expression that leads to its increased binding with CDK2 and CDK6, resulting in a marked decrease in their kinase activity, and second, by a decrease in G1 cyclins D1 and E. When these cell cycle-regulatory protein results are compared with those obtained for cell cycle phase distribution and proliferation following silymarin treatment of MDA-MB 468 cells, it can be suggested that a significant increase in Cip1/p21 could be the major cause of the...
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Confluency were treated with silymarin at a dosage of 75 μg/ml of CDK6 in breast carcinoma cells MDA-MB 468. Cultures at 80-90% confluency were treated with silymarin for 8, 16, 24, and 48 h as described in “Materials and Methods.”

Fig. 7 Silymarin treatment resulted in a decrease in CDK6 and cyclin D1-associated kinase activity via an increased binding of Cipl/p21 to CDK6 in breast carcinoma cells MDA-MB 468. Cultures at 80-90% confluency were treated with silymarin at a dosage of 75 μg/ml of medium for 8, 16, 24, and 48 h as described in “Materials and Methods.” Cell lysates were prepared, and CDK6 (A) and cyclin D1 (B) kinase activity was determined by in bead RB kinase assay using immunoprecipitated CDK6 and cyclin D1 from total cell lysates using specific antibodies as described in “Materials and Methods.” After the assay, labeled substrate was subjected to SDS-PAGE, and the gel was dried and exposed to X-ray film. The PhosphorImager quantification data of the radioactive bands (insets) are plotted in bar graphs (main figure) and represent arbitrary units. For the binding study (C), Cipl/p21 was immunoprecipitated and subjected to SDS-PAGE followed by Western blotting. Membrane was probed with anti-CDK6 antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. IP: immunoprecipitation; IB: Western immunoblot.

effect of silymarin on G1 arrest and on antiproliferative and anticarcinogenic responses. More detailed mechanistic studies, however, will be needed in the future to describe whether the alterations observed in cell cycle regulatory molecules at protein levels caused by silymarin are due to changes in their gene expression. In addition, it will also be important to identify the upstream signaling target that is modulated by silymarin, resulting in a significant increase in Cipl/p21 level and other events.

Cell cycle progression is regulated through several different CDK regulatory mechanisms (Ref. 24 and references therein). Two major mechanisms for CDK regulation are binding with its catalytic subunit cyclin followed by activation of CDK-cyclin complexes and binding with CDKIs followed by inactivation of CDK-cyclin complexes (33). An alteration in the formation of these complexes could lead to an increased cell growth and proliferation and a decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis (24, 39, 53–55). In mammalian cells, cyclins D and E have been implicated in the control of early G1 and late G1 to S transitions, respectively (56–60). Interesting and important, therefore, are the findings showing an increased expression of G1 cyclins, such as cyclin D1, in several types of human tumors, including breast, lung, esophagus, and so forth, and tumor-derived cell lines (61–64), which explains the uncontrolled growth and proliferation of cancer cells. Further support for the involvement of cyclin D in cancer growth came from the studies showing that overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control leading to a decrease in the duration of G1 phase, decreased cell size, and tumor induction in nude mice (61) and that antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells (65). On the basis of these and other findings, Weinstein (46) has suggested that cyclin D and related proteins might be useful markers and targets for cancer prevention. In our studies, therefore, a significant decrease in the protein levels of both cyclin D1 and cyclin E following silymarin treatment of MDA-MB 468 cells suggests that silymarin could be a useful agent for the preventive intervention of malignancies overexpressing G1 cyclins. In addition, the effect of silymarin on these cyclins observed in the present study also partially explains the accumulation of MDA-MB 468 cells in G1 phase of the cell cycle following silymarin treatment.

In addition to an increased expression of G1 cyclins in cancer cells, providing them an uncontrolled growth advantage, the irony is that most of the human tumors and derived cell lines either possess nonfunctional CDKI, lack CDKI (often), or have low expression of CDKI (Ref. 29 and references therein). For example, in breast carcinoma cells MDA-MB 468, used in the present study, the absence of p16 has been shown (66, 67). In addition, studies conducted by us did not find any detectable levels of Kip1/p27 in MDA-MB 468 cells (data not shown). Cipl/p21, therefore, appears to be the major CDKI associated with cell cycle progression in MDA-MB 468 cells. Cipl/p21 binds to CDK4-cyclin D1, CDK6-cyclin D1, and CDK2-cyclin E complexes during G1 (21, 24, 30). A highly significant increase in Cipl/p21 following silymarin treatment of MDA-MB 468 cells concomitantly also resulted in its significantly increased binding to both CDK2 and CDK6. These increased
bindings, by and large, also correlated with a significant decrease in the kinase activity of CDK2 and CDK6, as well as cyclin E and cyclin D1, which are associated with them, respectively. Because MDA-MB 468 cells lack CDK4 (52), consistent with the results obtained in the present study, CDK2 and CDK6 could be the major downstream targets for the effect of silymarin on accumulation of MDA-MB 468 cells in G1 phase of the cell cycle followed by inhibition of both anchorage-dependent and anchorage-independent cell growth. Because breast carcinoma cells MDA-MB 468 possess mutant p53 and have lost the gene encoding the RB protein (43, 44), the Cip1/p21 increase and G1 arrest produced by silymarin in these cells is not likely to be dependent on functional p53 or the presence of RB, and therefore, the p53-independent pathway may be predominant in the accumulation of cells in G1 phase caused by silymarin and achieved via an increase in Cip1/p21. It is also important to emphasize here that because mutations in p53 are frequent in most of the human cancers (43), the antiproliferative and anti-carcinogenic effects of silymarin in a p53-independent fashion could be widely useful for the preventive intervention of such human malignancies. Moreover, in a relatively novel approach for the intervention of cancer, development of silymarin as a potent inhibitor of CDK activity, and thereby cell cycle progression and cell growth, is warranted.

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