A Flavonoid Antioxidant, Silymarin, Inhibits Activation of erbB1 Signaling and Induces Cyclin-dependent Kinase Inhibitors, G1 Arrest, and Anticarcinogenic Effects in Human Prostate Carcinoma DU145 Cells

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ABSTRACT

Prostate cancer (PCA) is the most common nonskin malignancy and the second leading cause of cancer deaths in United States males. One practical and translational approach to control PCA is to define a mechanism-based anticarcinogenic agent(s). Recently, we showed that silymarin, a flavonoid antioxidant isolated from milk thistle, possesses exceptionally high to complete protective effects against experimentally induced tumorigenesis. Because the epidermal growth factor receptor (erbB1) and other members of the erbB family have been shown to play important roles in human PCA, efforts should be directed to identify inhibitors of this pathway for PCA intervention. In this study, we assessed whether silymarin inhibits erbB1 activation and associated downstream events and modulates cell cycle regulatory proteins and progression, leading to growth inhibition of human prostate carcinoma DU145 cells. Treatment of serum-starved cells with silymarin resulted in a significant inhibition of transforming growth factor α-mediated activation of erbB1 but no change in its protein levels. Silymarin treatment of cells also resulted in a significant decrease in tyrosine phosphorylation of an immediate downstream target of erbB1, the adapter protein SHC, together with a decrease in its binding to erbB1. In the studies analyzing cell cycle regulatory molecules, silymarin treatment of cells also resulted in a significant induction of cyclin-dependent kinase inhibitors (CDKIs) Cip1/p21 and Kip1/p27, concomitant with a significant decrease in CDK4 expression, but no change in the levels of CDK2 and CDK6 and their associated cyclins E and D1, respectively. Cells treated with silymarin also showed an increased binding of CDKIs with CDKs, together with a marked decrease in the kinase activity of CDKs and associated cyclins. In additional studies, treatment of cells grown in 10% serum with anti-epidermal growth factor receptor monoclonal antibody clone 225 or different doses of silymarin also resulted in additional pathways independent of inhibition of erbB1 activation are involved in the effect of silymarin on an increase in CDK1 protein levels is mediated via inhibition of erbB1 activation only in the case of Kip1/p27; however, additional pathways independent of inhibition of erbB1 activation are possibly responsible for the silymarin-caused increase in Cip1/p21 in DU145 cells. In other studies, silymarin treatment also induced a G1 arrest in the cell cycle progression of DU145 cells and resulted in a highly significant to complete inhibition of both anchorage-dependent and anchorage-independent growth of DU145 cells in a dose- and time-dependent manner. Taken together, these results suggest that silymarin may exert a strong anticarcinogenic effect against PCA and that this effect is likely to involve impairment of erbB1-SHC-mediated signaling pathway, induction of CDKIs, and a resultant G1 arrest.

INTRODUCTION

PCA is the second most common malignancy (after nonmelanoma skin cancers) in American men and is the second leading cause of cancer death (after lung cancer) in women. Statistical predictions for 1997 show that 334,500 new PCA cases will have been diagnosed, and an estimated 41,800 deaths due to this disease will have occurred in the United States alone (3). The induction of human PCA has been viewed as a multistage process, involving progression from small, latent carcinomas of low histological grade to large, metastatic carcinomas of higher grade (2). It is becoming clear that in the genesis of PCA, a variety of pathogenic pathways exist. Among the widely accepted risk factors for PCA are age, race, ethnicity, dietary habits, and androgen secretion and metabolism (4). African Americans have the highest PCA rate in the world, followed by Caucasian Americans and then Southeast Asians (1–3). Both epidemiology and laboratory studies have suggested that diet and androgen can alter PCA risk via a common etiological pathway (4–7).

To design and conduct mechanism-based early phase preventive intervention clinical trials, a major goal of PCA research in recent years has been to focus on the biology of normal prostate and elucidate the molecular mechanism(s) of PCA induction. Indeed, several genetic alterations have been identified that lead to the induction and/or development of human PCA (Ref. 8 and references therein). It has been shown that the early components of signal transduction pathways, specifically those of tyrosine kinases, are of utmost significance for control of cell growth and differentiation (Ref. 9 and references therein). Ironically, a single genetic alteration in any of the cell signaling components can result in a continuous signaling, leading to an uncontrolled cell growth and proliferation. RTKs participate in transmembrane signaling, whereas non-RTKs take part in intracellular signal transduction, including signaling to the nucleus (9). Enhanced tyrosine kinase activity due to overexpression of RTKs and/or non-RTKs can lead to persistent autocrine stimulation of cells by secreted growth factors, which in turn can lead to a disease (9). Enhanced activity of tyrosine kinases has been implicated in a wide variety of human malignancies; several studies have shown increased expression of erbB family of RTKs in human malignancies, suggesting their role in the causation of this disease (8–10). With regard to human PCA, the aberrant expression of the erbB family of RTKs, such as EGFR (also known as erbB1), erbB2, and erbB3, has been demonstrated with strikingly high frequency in prostatic intraepithelial neoplasia and invasive PCA, both primary and metastatic (8, 10–14).

In addition, EGF, TGF-α, and erbB1 have been shown to be...
associated with the regulation of prostatic cell mitogenesis (15). For example, hormone-independent prostate carcinoma cells commonly express high levels of erbB1 and TGF-α, thus making a functional autocrine loop for the hormone-independent growth of PCA (16, 17). Using hormone-independent prostate carcinoma cell lines PC-3 and DU145, it has been shown that high-affinity, ligand-blocking monoclonal antibodies to erbB1 prevent its activation and also result in the growth inhibition of these cells (16–18). Together, these studies implicate that the erbB family of RTK-mediated signaling pathways may be contributory mechanisms for human PCA (14, 19), and therefore, one practical and translational approach for the intervention of PCA could be to identify the inhibitors of erbB family of RTK-mediated signaling pathway(s).

Several epidemiological studies, supported by long-term animal tumor experiments or vice versa, have suggested that microchemicals present in our diet, as well as several herbs and plants with diversified pharmacological properties, could be the most desirable agents for the prevention and/or intervention of human cancer incidence and mortality due to stomach, colon, breast, esophagus, lung, bladder, and even PCAs (20–25). Measuring the effects of these agents in cancer chemopreventive intervention studies in human populations has now become one important objective of experimental cancer research. The potential for inhibiting tumor development in both targeted high-risk and general population has increased significantly in recent years (20–25). Accordingly, many new classes of chemical compounds are being evaluated in clinical trials as cancer preventive and/or therapeutic agents for several malignancies (20–25); fewer efforts, however, have been made with regard to PCA (Refs. 26–29 and references therein). At present, about 30 classes of chemicals with such effects have been described, which may have practical implications in reducing human cancer incidence (22–28). Among these, polyphenolic antioxidants are receiving increased attention (22–25, 30).

Silymarin, a polyphenolic flavonoid antioxidant isolated from milk thistle (Silybum marianum (L.) Gaertn; Ref. 31), is being used clinically in Europe and Asia for the treatment of alcoholic liver diseases (32, 33). As a therapeutic agent, silymarin is well tolerated and largely free of adverse effects (Refs. 34 and 35 and references therein), so much so that it is also being marketed recently in the United States and Europe as nutritional supplement by Pure Encapsulations (Sudbury, MA). Studies on mice, rats, rabbits, and dogs, using different modes of administration, showed that silymarin is nontoxic in acute tests even at large doses (36). Similarly, it is nontoxic in subchronic and chronic tests and does not show any side effects (36); there is no known LD₅₀ for silymarin in laboratory animals (35–38). Several studies have shown that silymarin affords protection against lipid peroxidation induced by xenobiotic agents (39, 40) and that it is a strong antioxidant capable of scavenging free radicals (Refs. 41–44 and references therein). In studies using the mouse skin models of carcinogenesis, we have shown that silymarin inhibits skin tumor promoter-caused induction of epidermal ornithine decarboxylase activity and mRNA expression (45) and that it possesses exceptionally high to complete protective effects against experimental tumorigenesis (46, 47). Likewise, experiments involving a mammary gland culture initiation-promotion protocol also demonstrated the ability of silymarin to inhibit tumor promotion (48). Taken together, these findings suggested a possibility that silymarin could also be a useful anticarcinogenic agent for PCA. In this study, we demonstrate the inhibitory effect of silymarin on activation of erbB1 signaling pathway in hormone-independent human prostate carcinoma DU145 cells. Furthermore, we found that inhibition of erbB1 activation by silymarin was associated with alterations in the levels of cell cycle regulatory molecules, cessation of cell cycle progression, and inhibition of both anchorage-dependent and anchorage-independent growth of DU145 cells.

**MATERIALS AND METHODS**

**Materials.** Human prostate carcinoma cell line DU145 was from American Type Culture Collection (Bethesda, MD). RPMI 1640, human recombinant TGF-α, and all other culture materials were from Life Technologies, Inc. (Gaithersburg, MD). Silymarin was from Aldrich Chemical Co. (Milwaukee, WI). Anti-ErbB-1, anti-SH2, and anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Cip1/p21 antibody was from Calbiochem (Cambridge, MA). Anti-Kip1/p27 and anti-CDK4 antibodies and mAb 225 were from Neomarkers, Inc. (Fremont, CA). Antibodies to cyclin D1, cyclin E, CDK2, and CDK6; rabbit antiumo-globulin- and goat antirabbit immunoglobulin- horseradish peroxidase-conjugated secondary antibodies; and RB-GST 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 Culture Conditions and Silymarin Treatment.** DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions. For studies assessing the effect of silymarin on activation of erbB1 signaling, 70–80% confluent cultures were washed twice with PBS, and then starved in serum-free medium for 36 h. After 20 h of starvation, the medium was replaced with fresh serum-free medium to remove any autocrinely secreted TGF-α ligand. During the last 2 h of starvation, the cultures were treated with either ethanol alone or varying concentrations of silymarin (25–150 μM of medium) 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. At the end of these treatments, cultures were added with either PBS alone or TGF-α (50 ng/ml of medium) and incubated for 15 min at 37°C. Thereafter, medium was aspirated, and monolayers were quickly washed twice with cold PBS and added with 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 0.2 units/ml aprotinin) per 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 an additional 15 min. For studies assessing the effect of silymarin on constitutive activation of erbB1 signaling, 70–80% confluent cultures grown in 10% serum were treated with either solvent alone, mAb 225 (6 μg/ml, equivalent to 40 nm), or varying concentrations of silymarin (50–100 μg/ml) for 16 h, and the cell lysates were prepared as detailed above. The lysates were cleared by centrifugation for 5 min in a tabletop centrifuge at 4°C, the supernatants were collected, and protein concentration was determined. For all other studies, DU145 cells were cultured as detailed above, and 70–80% confluent cultures (without serum starvation) were treated with either ethanol alone, mAb 225 (6 μg/ml), or varying concentrations of silymarin (25–75 μg/ml of medium) in ethanol. Sixteen h after these treatments, the medium was aspirated, monolayers were quickly washed twice with cold PBS, and cell lysates were prepared as detailed above. For those studies assessing the time-dependent effect of silymarin, cultures were treated with silymarin at a concentration of 75 μg/ml of medium; cells were harvested 8, 24, and 48 h later; and lysates were prepared.

**Immunoprecipitation and Western Blotting.** For erbB1 signaling studies, 400 μg of protein lysate per sample were diluted to 1 ml with lysis buffer and added with 2 μg of anti-EGRF or anti-SH-2 antibody followed by rotating this mixture at 4°C for 4 h. Thereafter, 25 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 erbB1 or SHC was denatured with 30 μl of 1X SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on an 8% gel, and separated proteins were transferred on to nitrocellulose membrane by Western blotting. As needed, membranes were probed with antiphosphotyrosine, anti-EGRF, and anti-SH2 antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. For cell cycle regulatory molecules, 40–100 μg of protein lysate per
sample were denatured with 2 × SDS-PAGE sample buffer, samples were subjected to SDS-PAGE on 12% gel, and separated proteins were transferred on to membrane by Western blotting. 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 CDKIs with CDKs, 50 µg of protein lysate per sample were mixed with 0.5 µg of anti-Cip1/p21 or anti-Kip1/p27 antibody and 5 µl of protein G-agarose beads for immunoprecipitations as described above. The immunoprecipitated proteins were denatured with sample buffer and subjected to 12% SDS-PAGE followed by Western blotting. The levels of CDKs bound to CDKIs were determined by specific primary antibodies to CDK2, CDK4, and CDK6 followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system.

Kinase Assays. CDK2- and cyclin E-associated H1 histone kinase activity was determined as described by Wu et al. (49). Briefly, using anti-CDK2 or anti-cyclin E antibody (2 µg) and protein A-agarose beads (20 µl), CDK2 and cyclin E, respectively, were immunoprecipitated from 200 µg of protein lysate per sample as detailed above. Beads were washed three times with lysis buffer and then once with kinase assay buffer (50 mM Tris-HCl, 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 0.1 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, CDK4-, CDK6-, and cyclin D1-associated RB kinase activity was determined as described by Wu et al. (49) and detailed above with some modifications. Briefly, vehicle- or silymarin-treated DU145 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 µM β-mercaptoethanol, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin and aprotinin), and using anti-CDK4, anti-cyclin D6, and anti-cyclin D1 antibody (2 µg) and protein G-agarose or protein A-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-mercaptoethanol, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM MgCl2, and 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-GST fusion protein, 0.5 µl of [γ-32P]ATP, 0.5 µl of 0.1 mM ATP, and 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.

FACS Analysis and Cell Growth Assay. DU145 cells at 70-80% confluency were treated with either ethanol alone or silymarin at a dose of 75 µg/ml of medium in ethanol. Twenty-four and 48 h at these treatments, the medium was aspirated, monolayers were quickly washed twice with cold PBS, cells were trypsinized, and cell pellets were collected. The cell pellets were washed twice with PBS and the cells were fixed in cold methanol and rewarmed 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.

For cell growth assays, DU145 cells were plated at a density of 0.5 × 105 cells per 60-mm plate. On day 2, cells were fed with fresh medium and left untreated, or they were treated with ethanol alone or silymarin at doses of 5, 10, 25, 50, 75, and 100 µg/ml of medium dissolved in ethanol. In other studies, DU145 cells were plated at a density of 0.5 × 105 cells per 60-mm plate. The next day, cells were fed with fresh medium and treated with ethanol alone, mAb 225 at doses of 5 and 12 µg/ml of medium, or silymarin at doses of 50, 75, and 100 µg/ml of medium dissolved in ethanol. The cultures were fed with fresh medium with or without same concentrations of mAb 225 or silymarin every other day until the end of the experiment. Each treatment and time point had four plates. At days 1–6 after these treatments, cells were trypsinized and collected in counting vials. Each plate was washed thoroughly with isotonic buffer with 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 cell number.

Soft Agar Colony Formation Assay. DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin (complete medium) as detailed above. Soft agar colony formation assay was performed using 6-well plates. 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 1000 cells as the feeder layer, and 1 ml of 0.38% agar in complete medium with either vehicle ethanol or a different dose 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 were 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.

RESULTS

Effect of Silymarin on the Activation of erbB1 in DU145 Cells. It has been shown that androgen-independent prostate carcinoma cell lines DU145 and PC3 overexpress erbB1 as well as synthesize and secrete TGF-α, which interacts with erbB1 for autonomous growth through an autocrine feedback loop (16-18). These cell lines, therefore, provide a valuable system to explore the agents that can inhibit erbB1-mediated signaling pathways in PCA. In the present study, we used DU145 cells to assess the inhibitory effects of silymarin on erbB1 activation and its associated downstream events. As shown in Fig. 1A, 36 h of serum starvation of DU145 cells resulted in a complete reduction of activated erbB1, as evidenced by no reactivity of immunoprecipitated erbB1 with antiphosphotyrosine antibody in Western blotting (Lane 1). On the other hand, treatment of starved cultures with 50 ng/ml TGF-α for 15 min resulted in a highly significant activation of erbB1 as evident by a very strong reactivity with antiphosphotyrosine antibody (Fig. 1A, Lane 2). Pretreatment of cultures with silymarin during the last 2 h of starvation at the concentrations of 75, 100, and 150 µg/ml followed by treatment with TGF-α at the same dose resulted in a highly significant inhibition of TGF-α-mediated activation of erbB1 in DU145 cells (Fig. 1A, Lanes 4–6); the concentration of silymarin lower than these did not show such inhibition (Fig. 1A, Lane 3). To determine whether the decrease in TGF-α-mediated tyrosine phosphorylation of erbB1 by silymarin was due to a decrease in erbB1 expression, the membrane was also probed with anti-EGFR antibody. As shown in Fig. 1B, all of the lanes showed equal reactivity of immunoprecipitated erbB1 with anti-EGFR antibody in Western blotting, indicating no change in erbB1 protein levels. Treatment of starved cultures with silymarin alone did not show any effect on tyrosine phosphorylation and protein levels of erbB1 (data not shown).

Effect of Silymarin on the Activation of SHC and the Binding of SHC to erbB1 in DU145 Cells. Activation of erbB1 results in the activation of several different signaling molecules recruited by erbB1 (Refs. 9 and 50–53 and references therein). One such immediate recruitment following erbB1 activation is the adaptor protein SHC (9, 50–53). SHC proteins contain a src homology-2 domain that binds to phosphotyrosine-containing sequences, including those of tyrosine kinase receptors (erbB1 in the present study) upon ligand activation (Refs. 9, 51, 54, and 55 and references therein). We therefore performed the studies to assess whether inhibition of erbB1 activation by silymarin in DU145 cells also impaired the activation of SHC proteins and their binding to erbB1. As shown in Fig. 1C, the results obtained for tyrosine phosphorylation of SHC paralleled those for erbB1 acti-
cells were as follows: Lane 1, vehicle control; Lane 2, treated with TGF-α for 15 min; conjugated appropriate secondary antibody as detailed in "Materials and Methods." Cell lysates were prepared. erbB1 or SHC was immunoprecipitated using anti-EGFR or treated either with vehicle alone or with varying concentrations of silymarin and, at the end of these treatments, with PBS or TGF-α (50 ng/ml of medium) for 15 min at 37°C. Confluency, they were serum starved for 36 h. During the last 2 h of starvation, they were

46-kDa SHC proteins (Fig. 1C, Lanes 3-6). When the membrane was probed with antiphosphotyrosine, anti-EGFR, or anti-SHC antibody and then peroxidase-conjugated appropriate secondary antibody as detailed in "Materials and Methods." Visualization of proteins was done using the ECL detection system. A, tyrosine phosphorylation of erbB1; B, protein levels of erbB1; C, tyrosine phosphorylation of SHC; D, protein levels of SHC; E, binding of SHC to erbB1. Serum-starved cultures of DU145 cells were as follows: Lane 1, vehicle control; Lane 2, treated with TGF-α for 15 min. Lanes 3–6, treated with 50, 75, 100, and 150 μg/ml of silymarin, respectively, and after 2 h with TGF-α for 15 min. IP, immunoprecipitation; IB, Western immunoblot.

The starvation of DU145 cells for 36 h resulted in no reactivity of immunoprecipitated SHC to antiphosphotyrosine in Western blot (Fig. 1C, Lane 1). Treatment of starved cells with TGF-α showed activation of both 52- and 46-kDa SHC proteins, as evidenced by strong reactivity of immunoprecipitated SHC to antiphosphotyrosine (Fig. 1C, Lane 2). However, treatment of cultures with different doses of silymarin for 2 h prior to the addition of TGF-α showed a highly significant decrease in tyrosine phosphorylation of both 52- and 46-kDa SHC proteins (Fig. 1C, Lanes 3–6). When the membrane was probed with anti-SHC antibody, there was no change in SHC protein levels (Fig. 1D), suggesting that the decrease in SHC activation was not due to a decrease in protein content. To assess the binding of SHC to erbB1, samples were immunoprecipitated with anti-EGFR and immunoblotted with anti-SHC antibody. As shown in Fig. 1E, compared to a strong binding in TGF-α alone treated sample (Lane 2), silymarin treatment at various doses also resulted in a highly significant decrease in the binding of SHC to erbB1 (Lanes 3–6).

Effect of Silymarin on Cell Cycle Regulatory Molecules in DU145 Cells. Defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells (56). Eukaryotic cell cycle progression is regulated by a series of CDKs; their activity is positively regulated by cyclins and negatively regulated by CDKIs (57). The significance of growth factors and the signaling pathway(s) mediated by them to regulate the progression of cell cycle in eukaryotes has been identified as an important component of their function (18, 58, 59). We therefore assessed whether inhibition of erbB1 and SHC activation by silymarin was associated with the alterations in cell cycle regulatory molecules in DU145 cells. As shown in Fig. 2A, compared to ethanol-treated controls, treatment of DU145 cells in culture with silymarin resulted in a significant up-regulation of CDKI Cipl/p21 protein levels. The effect of silymarin was both dose- and time-dependent. Maximum up-regulation was observed at 24 h after treatment, because no significant increase was evident after this time (Fig. 2A). Similar to Cipl/p21, treatment of DU145 cells with silymarin also resulted in a significant up-regulation in the protein levels of another CDKI, Kipl/p27, which was also dependent on the dose of silymarin as well as the time of treatment (Fig. 2B). When the effect of silymarin on the expression of CDKs was assessed, as shown in Fig. 2D, silymarin treatment of DU145 cells resulted in a dose- and time-dependent decrease in the protein levels of CDK4. However, no change in the levels of CDK2 (Fig. 2C) and CDK6 (Fig. 2E) was observed following silymarin treatment up to a concentration of 75 μg/ml for 48 h. Next, we assessed the effect of silymarin on the protein levels of cyclins associated with CDK2 (cyclin E) and with CDK4 and CDK6 (cyclin D1). As shown in Fig. 2, F and G, treatment of DU145 cells with silymarin did not result in any alterations in the expression of cyclin D1 and cyclin E, respectively, up to 48 h of exposure at a dose of 75 μg/ml.
Because we observed, following silymarin treatment of DU145 cells, a significant increase in the expression of CDKIs Cipl/p21 and Kipl/p27, which bind to and inactivate CDKs (57), we next assessed whether an up-regulation of CDKIs exerted any effect on the kinase activity of CDKs and associated cyclins in silymarin-treated DU145 cells. CDK2 and cyclin E were immunoprecipitated from control and silymarin-treated sample lysates, and kinase activity was determined using histone H1 as a substrate. As shown in Fig. 3A, compared to vehicle-treated control, silymarin treatment for 16 h resulted in a dose-dependent decrease in CDK2 kinase activity. No change, however, was observed in cyclin E-associated kinase activity (data not shown). For CDK4, CDK6, and cyclin D1-associated kinase activity, these cell cycle regulatory proteins were immunoprecipitated from control and varying doses of silymarin-treated sample lysates, and kinase activity was determined using histone H1 as a substrate. Similar to CDK2, as shown in Fig. 3A, compared to ethanol-treated control, silymarin treatment for 16 h resulted in a significant decrease in CDK4, CDK6, and cyclin D1-associated kinase activity in a dose-dependent manner. To further explore whether the observed decrease in kinase activity of CDKs and cyclin D1 was due to an increase in the binding of CDKs with the induced levels of Cipl/p21 and Kipl/p27 following silymarin treatment, we immunoprecipitated Cipl/p21 and Kipl/p27 from vehicle- and silymarin-treated sample lysates and immunoblotted with anti-CDK2, anti-CDK4, and anti-CDK6 antibodies. As shown in Fig. 3B, treatment of DU145 cells with a 75 µg/ml dose of silymarin for 16 h indeed resulted in a significant increase in the binding of all three CDKs (CDK2, CDK4, and CDK6) to Cipl/p21. However, in the case of Kipl/p27, a marked increase in binding was observed only between CDK4 and this CDKI following silymarin treatment (Fig. 3C). Together, the data shown in Fig. 3 clearly demonstrate that silymarin treatment of DU145 cells results in a significant decrease in the kinase activity of CDKs and cyclin D1 and that this effect is due to an increased binding of CDKs with the induced levels of CDKIs.

**Effect of Silymarin on Constitutive Activation of erbB1 and Its Association with CDKI Up-Regulation in DU145 Cells.** On the basis of the findings that silymarin inhibits TGF-α-mediated activation of erbB1 and its downstream target SHC in serum-starved DU145 cells (Fig. 1) and that silymarin also up-regulates the protein levels of CDKIs in DU145 cells grown in 10% serum (Fig. 2), we performed additional studies to identify whether silymarin also inhibits constitutive activation of erbB1 signaling, of which an increase in CDKIs is a plausible downstream result. For these studies, DU145 cells were cultured under normal growth conditions in the presence of 10% serum, and at 70–80% confluence, they were treated either with solvent, different concentrations of silymarin, or mAb 225. The selection of mAb 225 and its dose level in this experiment were based on previous studies showing that treatment of DU145 cells with a 40 nM concentration of mAb 225, a high-affinity ligand blocking anti-EGFR monoclonal antibody, inhibits erbB1 receptor activation and cell growth via an induction of Kipl/p27 and G1 arrest (16–18). Therefore, mAb 225 is an appropriate and specific erbB1 blocking reagent to be used to compare the effect of and delineate the mechanism associated with silymarin-induced inhibition of erbB1 activation, followed by an increase in the protein levels of CDKIs in DU145 cells. As evidenced by a very strong reactivity with antiphosphotyrosine antibody, erbB1 was found to be highly activated in DU145 cells when they were cultured under normal growth conditions in the presence of 10% serum (Fig. 4A, Lane 1). However, parallel to the inhibition of TGF-α–caused activation of erbB1 (Fig. 1A), treatment of cultures grown in 10% serum with a 50–100 µg/ml dose of silymarin resulted in a significant inhibition of constitutive activation of erbB1 (Fig. 4A, Lanes 3–5). Whereas observed inhibition at 50 and 75 µg/ml doses of silymarin was comparable to that when mAb 225 was used (Fig. 4A, Lanes 3 and 4 versus Lane 2), much stronger inhibition of erbB1 activation was evident at a 100 µg/ml dose of silymarin (Fig. 4A). As shown in Fig. 4B, the observed inhibition in erbB1 activation was not due to a decrease in erbB1 protein levels. Results comparable to those of erbB1 activation were also found for the activation of SHC. As shown in Fig. 4C, cells grown in 10% serum showed high levels of constitutive SHC activation, which was inhibited significantly by both mAb 225 and the different doses of silymarin used. There was no change in total SHC protein levels following these treatments (Fig. 4D).

When the inhibitory effects of silymarin and mAb 225 on constitutive erbB1 signaling in DU145 cells were analyzed in terms of their association with the up-regulation of CDKI protein levels, striking
of SHC: and D, protein levels of SHC. In other studies, total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in "Materials and Methods," and tyrosine phosphorylation of erbB 1; B. protein levels of erbB 1; C, tyrosine phosphorylation antibody and then peroxidase-conjugated appropriate secondary antibody followed by precipitated using anti-EGFR or anti-SHC antibody, and following SDS-PAGE and Western blotting, membranes were probed with antiphosphotyrosine, anti-EGFR, or anti-SHC antibody and then peroxidase-conjugated appropriate secondary antibody followed by visualization by the ECL detection system as detailed in "Materials and Methods." A, tyrosine phosphorylation of erbB1; B, protein levels of erbB1; C, tyrosine phosphorylation of SHC; and D, protein levels of SHC. In other studies, total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in "Materials and Methods," and the membrane was probed with anti-Cipl/p21 (E) or anti-Kipl/p27 (F) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Cultures of DU145 cells were as follows: Lane 1, vehicle control; Lane 2, treated with 6 fig/ml mAb 225; Lanes 3-5, treated with 50, 75, and 100 fxg/ml of silymarin, respectively, for 16 h. IP, immunoprecipitation; IB, Western immunoblot.

**Fig. 4.** Effect of silymarin on constitutive activation of erbB1 and its association with CDK1 up-regulation in DU145 cells. Cells were cultured as described in "Materials and Methods," and at 70–80% confluency (without serum starvation), they were treated with vehicle alone, mAb 225, or varying concentrations of silymarin for 16 h as described in "Materials and Methods." Cell lysates were prepared, erbB1 or SHC was immunoprecipitated using anti-EGFR or anti-SHC antibody, and following SDS-PAGE and Western blotting, membranes were probed with antiphosphotyrosine, anti-EGFR, or anti-SHC antibody and then peroxidase-conjugated appropriate secondary antibody followed by visualization by the ECL detection system as detailed in "Materials and Methods." A, tyrosine phosphorylation of erbB1; B, protein levels of erbB1; C, tyrosine phosphorylation of SHC; and D, protein levels of SHC. In other studies, total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in "Materials and Methods," and the membrane was probed with anti-Cipl/p21 (E) or anti-Kipl/p27 (F) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Cultures of DU145 cells were as follows: Lane 1, vehicle control; Lane 2, treated with 6 fig/ml mAb 225; Lanes 3-5, treated with 50, 75, and 100 fxg/ml of silymarin, respectively, for 16 h. IP, immunoprecipitation; IB, Western immunoblot.

**Fig. 5.** Effect of silymarin on cell cycle progression of DU145 cells. Cells were cultured as described in "Materials and Methods," and at 70–80% confluency (without serum starvation), they were treated with either vehicle alone or 75 fxg/ml concentration of silymarin as described in "Materials and Methods." Twenty-four and 48 h later, cells were trypsinized, and cell pellets were collected as described in "Materials and Methods." The cells were fixed in cold methanol and digested with RNase, and then cellular DNA was stained with propidium iodide as described in "Materials and Methods." Cell cycle distribution was then determined by FACS analysis.
of more than 10 cells per colony after 10 days of seeding (data not shown). As many as 40.67 ± 4.2 (mean ± SE of three independent experiments) colonies/1000 cells (per plate) were counted in ethanol-treated controls (data not shown). However, treatment of cells with silymarin resulted in a highly significant inhibition in soft agar colony formation of DU145 cells (Fig. 7). In qualitative analysis, compared to ethanol control (Fig. 7A), silymarin treatment at doses of 25 (Fig. 7B) and 75 (Fig. 7C) μg/ml resulted in a significant decrease in both colony number and size. In quantitative analysis, as shown in Fig. 7D, compared to ethanol control, treatment with silymarin at lower doses of 1, 5, 25, and 50 μg/ml resulted in 21% (P < 0.1), 38% (P < 0.01), 65% (P < 0.001), and 80% (P < 0.001) inhibition, respectively, in number of colonies per plate. It is important to mention here that these results are significantly different from those observed for anchorage-dependent growth studies, in which low doses of silymarin were not effective in inhibiting cell growth. Results comparable to those of anchorage-dependent growth inhibition by silymarin at higher doses, however, were also observed in soft agar colony formation assay, in which silymarin treatment resulted in 96% (P < 0.0001; Fig. 7D) and complete inhibition (data not shown) in number of colonies at doses of 75 and 100 μg/ml, respectively.

**DISCUSSION**

Alarming statistics about human PCA clearly indicate that it is an invasive cancer; behind lung cancer, it is the second leading cause of cancer related deaths among males in the United States (1–3). Geographic variations in the incidence of clinical cancer worldwide are considerable, with an incidence over 100-fold higher in the United States than in China (61, 62). Several studies have suggested that various factors, specifically androgen (testosterone), play a major role in the pathogenesis as well as in the promotion of PCA (2, 4–7). The significance of androgen in prostate carcinogenesis can be attributed by the findings that PCA rarely occurs in eunuchs or men with a deficiency in 5α-reductase, the enzyme responsible for converting testosterone to its active metabolite dihydrotestosterone (Refs. 63–65 and references therein). Because the growth and development of PCA is initially androgen-dependent, androgen deprivation has been extensively explored as a strategy for PCA prevention and therapy (Ref. 65 and references therein). Although PCA patients treated with androgen deprivation therapy often have remission of their PCA, within a few years, tumor regrowth occurs that is largely due to progression of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation (Ref. 63 and references therein). When the data are taken together, it can be appreciated that although androgen deprivation is a useful strategy for the prevention and/or therapy of androgen-dependent PCA, additional approaches are needed for advanced and androgen-independent PCA.

In addition to the loss of androgen dependence due to lack of androgen receptor (66), functional autocrine and paracrine growth factor/growth factor receptor interactions are believed to be contributors to the multifactorial mechanisms of androgen independence in PCA cell proliferation (16–18). For example, human prostate carcinoma cell lines PC-3 and DU145, which are derived from androgen-independent tumors and lack androgen receptor (66), express high levels of erbB1 (16–18). In addition, coexpression of erbB1 and TGF-α has been demonstrated in advanced and metastatic PCA (8, 10–14, 67). These studies suggest that a functional autocrine loop may contribute to hormone-independent cancer growth and successful proliferation of PCA at metastatic sites (18) and that agents that could inhibit the activation of RTKs, such as those of erbB family, may be useful for the intervention of PCA. In earlier studies, it has been shown that anti-EGFR monoclonal antibodies 225 and 528 bind to
 erbB1 with high affinity, blocking the binding of the ligands EGF and TGF-α to their receptor. This leads to inhibition of erbB1 activation, as well as reduced growth of nontransformed prostatic epithelial cells and human PCA cell lines PC-3 and DU145 (16–18). Comparing the inhibitory effects of (a) silymarin on ligand-induced erbB1 activation, and (b) both silymarin and mAb 225 on constitutive activation of erbB1 in DU145 cells observed in the present study to those reported with anti-EGFR antibody (18), there is a possibility that silymarin also directly affects the erbB1, leading to an inhibition in the binding of its ligand TGF-α. More detailed studies, however, are needed to address this possibility.

Stimulation of erbB1 kinase activity is known to result in the activation of several different signaling molecules, as well as the recruitment of adaptor proteins (9, 50–53). One such recruitment following erbB1 activation is SHC, which, following tyrosine phosphorylation, acts as an adaptor for other src homology-2-containing proteins in the signal transduction pathway(s) (9, 50–55). Being one of the immediate downstream signaling molecules recruited by erbB1, inhibition of ligand-induced, as well as endogenous, activation of erbB1 by silymarin also resulted in a significant decrease in SHC tyrosine phosphorylation, concomitant with a decrease in its binding to erbB1, suggesting that SHC is also targeted by silymarin via erbB1. To the best of our knowledge, ours is the first study showing the involvement of SHC in erbB1 signaling and its inhibitory modulation by an anticarcinogenic agent in human prostate carcinoma cell line DU145. More detailed studies, however, are needed with other agents to explore the modulation of erbB1-SHC downstream events in human PCA.

The significance of growth factors and the signaling pathway(s) initiated by them to regulate cell cycle progression in eukaryotes has been identified as an important component of their function (Refs. 56–60 and 68 and references therein). Several studies have shown that cell signaling pathways determine cell growth and inhibition through cell cycle regulation (56–60, 68). However, cancer cells often display abnormalities in genes that govern the responses of these cells to external growth factors; growth factors receptors; proteins involved in the pathways of signal transduction in the cytoplasm, the nucleus, or both; and nuclear transcription factors (68). In addition, defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells (56). Eukaryotic cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of CDKs at different phases (69–71). The activities of CDKs are regulated positively by cyclins and negatively by CDKIs (57). It is becoming increasingly clear that cyclin D1 and its associated CKD4 normally control cell cycle events in G1, and cyclin E associated with CDK2 mediates late G1 to early S (71–74). The cyclin D1 gene is turned on at G1 phase upon receipt of a cell cycle activation signal by growth factors (56, 60, 68). The cyclin D1 protein then binds with CDK4 (it is also known to bind with CDK2 and CDK6), and this complex hyperphosphorylates RB leading to its release from E2F (56, 59, 60, 68). The free transcription factor E2F then activates c-myc, resulting in cellular proliferation by progression through G1 (56, 59, 60, 68). The CDK activity, however, is negatively regulated by CDKIs (57, 75). In general, the relative abundance of CDKIs present at any point in the cell cycle sets thresholds for CDK-cyclin activation that must be overcome for the cell cycle to proceed (56, 57, 60, 68, 75). Impairment of a growth-stimulatory signaling pathway (such as erbB1, raf, or mitogen-activated protein kinase) has been shown to modulate the expression of CDKIs such as Cip1/p21 and Kip1/p27 (18, 49, 58, 59). An induced
CDK1 binds to and subsequently inhibits cyclin-CDK activity, which interferes with hyperphosphorylation of RB, keeping it in the hypophosphorylated form and bound to E2F, thereby blocking cell proliferation and inducing cell growth arrest (56, 60, 68). A decrease in the levels of cyclins and/or associated CDKs also leads to an arrest in cell cycle progression at a specific phase(s), depending on the particular regulatory molecule involved (56, 57, 60, 68).

In the present study, we observed that silymarin, which inhibited both ligand-induced and constitutive activation of erbB1 and its downstream signaling events, also up-regulated the levels of CDKIs Cip1/p21 and Kip1/p27 that and the increased CDKI levels were largely responsible for a marked decrease in the kinase activity of CDKs due to their increased binding with CDKs. In addition, silymarin treatment of DU145 cells also resulted in a significant decrease in the levels of CDK4. Consistent with the findings of alterations in cell cycle regulatory proteins, silymarin induced an arrest in G1 and inhibited both anchorage-dependent and anchorage-independent growth of human prostate carcinoma cell line DU145. However, when the results obtained with silymarin were compared with those when cells were treated with mAb 225, striking differences were observed in terms of CDKI up-regulation and inhibition of cell growth. Treatment of DU145 cells with mAb 225 resulted in the induction of Kip1/p27 only, not Cip1/p21. In addition, mAb 225 treatment resulted in almost 50% inhibition in cell growth, but silymarin showed complete inhibition. Whereas it would have been argued that the inhibitory effect of silymarin on cell growth is dose-dependent and higher doses show complete inhibition, doses of mAb 225 even two times higher did not result in any further inhibition of cell growth. Furthermore, the data obtained with mAb 225 in the present study were in accord with an earlier report showing that mAb 225 treatment results in G1 arrest and up-regulation of CDKI Kip1/p27 together with modulation of other cell cycle regulatory molecules and causes about 50% inhibition of cell growth in human prostate carcinoma cell line DU145 (18).

When the data are taken together, it can be argued that erbB1 receptor inactivation by blocking antibody leads to partial inhibition of cell growth as a biological response and that other mechanisms are still operational and responsible for DU145 cell growth independent of erbB1 signaling pathway. The results obtained in the present study, in conjunction with those reported earlier (18), clearly show that specific blocking of erbB1 receptor activation leads to an up-regulation of Kip1/p27 but not Cip1/p21, suggesting that silymarin possesses at least two independent growth inhibitory pathways. As shown by a schematic representation in Fig. 8, we suggest that, like mAb 225, silymarin inactivates erbB1-SHC signaling pathway leading to up-regulation of Kip1/p27 followed by its increased binding with CDK causing a decrease in CDK- and cyclin-associated kinase activity. This leads to a G1 arrest and resultant cell growth inhibition. In addition, by a mechanism not yet known at this point, silymarin also induces the expression of Cip1/p21 via an erbB1 inactivation-independent pathway that is presumably responsible for the remainder of the cell growth inhibitory potential of silymarin in DU145 cells. Further studies are needed to identify the targets associated with the up-regulation of Cip1/p21 by silymarin and to define whether the effect of silymarin observed in DU145 cells is a specific phenomenon or also occurs in other malignant cells. However, in summary, based on the findings reported here, it can be concluded that silymarin may exert a strong anticarcinogenic effect against human PCA.

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**REFERENCES**


A Flavonoid Antioxidant, Silymarin, Inhibits Activation of erbB1 Signaling and Induces Cyclin-dependent Kinase Inhibitors, G₁ Arrest, and Anticarcinogenic Effects in Human Prostate Carcinoma DU145 Cells


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