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Detrimental Effect of Cancer Preventive Phytochemicals Silymarin, Genistein and Epigallocatechin 3-gallate on Epigenetic Events in Human Prostate Carcinoma DU145 Cells

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BACKGROUND. Targeting epigenetic events associated with autonomous growth of advanced prostate cancer (PCA) is a practical approach for its control, prevention, and treatment. Recently we showed that treatment of prostate carcinoma DU145 cells with cancer preventive flavonoid silymarin at 100–200 μM doses inhibits erbB1-Shc mitogenic signaling and modulates cell cycle regulators leading to a G_1 arrest and inhibition of cell growth and anchorage-independent colony formation. Here, we asked the question whether these important findings could be extended to other cancer preventive flavonoids and isoflavones such as epigallocatechin 3-gallate (EGCG) and genistein.

METHODS. DU145 cells were treated with similar doses (100–200 μM) of silymarin, genistein or EGCG, cell lysates prepared, and levels of activated signaling molecules (erbB1-Shc-ERK1/2) and cell cycle regulators (CDKIs, CDKs, and cyclins) analyzed employing immunoprecipitation and/or immunoblotting techniques. Cell growth studies were done by cell counting during 5 days of treatment with these agents, and cell death was determined by Trypan blue staining.

RESULTS. Treatment of cells with silymarin, genistein or EGCG at 100–200 μM resulted in a complete inhibition of TGFα-caused activation of erbB1 followed by a moderate to strong inhibition (10–90%) of Shc activation without an alteration in their protein levels. Silymarin and genistein, but not EGCG, also inhibited (10% to complete) ERK1/2 activation suggesting that these agents impair erbB1-Shc-ERK1/2 signaling in DU145 cells. In other studies, silymarin, genistein or EGCG caused a strong induction of Cip1/p21 (up to 2.4-fold) and Kip1/p27 (up to 150-fold), and a strong decrease in CDK4 (40–90%) but had moderate effect on CDK2, and cyclins D1 and E. An enhanced level of CDKIs also led to an increase in their binding to CDK4 and CDK2. Treatment of cells with silymarin, genistein or EGCG also resulted in 50–80% cell growth inhibition at lower doses, and complete inhibition at higher doses. In contrast to silymarin, higher doses of genistein showed cytotoxic effect causing 30–40% cell death. A more profound cytotoxic effect was observed with EGCG accounting for 50% cell death at lower doses and complete loss of viability at higher doses.

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CONCLUSION. These results suggest that similar to silymarin, genistein and EGCG also inhibit mitogenic signaling pathway(s) and alter cell cycle regulators, albeit at different levels, leading to growth inhibition and death of advanced and androgen-independent prostate carcinoma cells. More studies are, therefore, needed with these agents to explore their anti-carcinogenic potential against human prostate cancer. *Prostate 46:98–107, 2001.*

KEY WORDS: **EGFR; MAPK; cell cycle; silymarin; genistein; EGCG**

INTRODUCTION

Prostate cancer (PCA) is the most common invasive malignancy and second (to lung cancer) leading cause of cancer mortality in American men [1]. Progression of PCA depends on both genetic and epigenetic factors. The multi-step process of PCA progression leads from transformation over an androgen-dependent non-metastatic phenotype to a more malignant metastatic androgen-independent phenotype [2]. Receptor tyrosine kinases (RTKs) are the major components of signal transduction pathways that play an essential role in cell growth, proliferation, and differentiation [3]. However, enhanced tyrosine kinase activity due to overexpression of RTKs or non-RTKs, leads to persistent mitogenic signaling, and has been established as a major contributor to carcinogenesis [3]. In human PCA, RTKs and associated mitogenic and anti-apoptotic signaling have been shown to be both genetic and epigenetic causes for disease progression [4–10]. For example, an enhanced expression of epidermal growth factor receptor (EGFR or erbB) family members (erbB1/EGFR, erbB2, erbB3) and associated ligands has been shown to be a causal genetic event in advanced and androgen-independent PCA growth and metastasis [4–7]. These genetic alterations lead to an epigenetic feedback event where ligand (e.g., transforming growth factor α (TGFα)/epidermal growth factor (EGF)) and receptor (e.g. erbB1) make a functional autocrine loop, thus facilitating hormone-independent uncontrolled growth of PCA [8–10].

Together, it can be appreciated that possibly other than gene therapy, it is difficult to fix genetic alterations for the control of advanced PCA. Therefore, targeting epigenetic events such as impairment of tyrosine kinase activity and associated mitogenic signaling pathway(s) could be a practical and translational approach to control advanced and androgen-independent PCA, and to prevent and treat the disease from further progression. Fruits, vegetables, common beverages, and several herbs and plants with diversified pharmacological properties have been shown to be rich sources of micro-chemicals with cancer preventive effects in humans [11,12]. Among these, naturally occurring flavonoids and isoflavones have been receiving increasing attention in recent years [13–15]. Accordingly, the major objective of present study was to assess the effect of cancer preventive phytochemicals silymarin (flavanone), genistein (isoflavone), and epigallocatechin 3-gallate (EGCG) (flavanol) on epigenetic events involved in uncontrolled growth of advanced and androgen-independent PCA.

Silymarin is a dietary supplement present in milk thistle (*Silybum marianum*) seeds, and used clinically in Europe, Asia, and the United States for the treatment of liver disease [16]. In recent years, several studies by us have shown the cancer preventive effects of silymarin in skin tumorigenesis models and its anti-carcinogenic activity in human prostate, breast, and cervical carcinoma cells [16–19]. Genistein is a dietary agent present in soybeans, and has received much attention as a potential anti-carcinogenic agent due to its effect on a number of cellular processes [20,21]. Several epidemiological and animal tumor studies have shown the preventive effects of genistein against various cancers [15,22]. With regard to PCA, the anti-carcinogenic and cancer preventive effects of genistein are well studied using cell and organ cultures, and animal models [23–25]. The efficacy of genistein is also being evaluated in PCA patients [26]. Tea (*Camellia sinensis*) is one of the most common beverages all over the world. Several studies from our group and by others have shown the cancer preventive and anti-carcinogenic effects of tea polyphenols on various cancers including skin, lung, esophagus, stomach, liver, intestine, pancreas, breast, and prostate [14,27]. As a major component, EGCG constitutes ~50% (w/w) of the total green tea extract, and is attributed for both cancer preventive and anti-carcinogenic effects of green tea [14,27–30].

Based on the above findings, in the present study, our major goal was to assess the effect of silymarin, genistein, and EGCG on erbB1-Shc-ERK1/2-mediated mitogenic signaling and modulation of cell cycle regulators in androgen-independent human prostate carcinoma DU145 cells. The rationale for these studies was also based on our recent findings where we showed that treatment of DU145 cells with silymarin at

100–200 μM doses inhibits erbB1-Shc mitogenic signaling and modulates cell cycle regulators leading to a G_1 arrest and inhibition of cell growth and anchorage-independent colony formation [18]. Accordingly, our other goal was to determine whether these important findings with silymarin could be extended to other cancer preventive flavonoids and isoflavones such as EGCG and genistein.

MATERIALS AND METHODS

Materials

DU145 cells were from American Type Culture Collection (Manassas, VA); and RPMI-1640 medium, fetal bovine serum, penicillin–streptomycin, TGFα, and all other cell culture reagents were from Life Technologies (Gaithersburg, MD). Silymarin, genistein, and EGCG were from Sigma-Aldrich (St. Louis, MO). Antibodies to EGFR, Shc, and phosphotyrosine were from Upstate Biotechnology (Waltham, MA), and antibodies against CDK2, Cyclin D1, Cyclin E, rabbit anti-mouse IgG, and goat-anti-rabbit IgG-horse radish peroxidase conjugated secondary antibodies and protein A/G agarose beads were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Cip1/p21 was from Calbiochem (Cambridge, MA), and anti-Kip1/p27 and anti-CDK4 antibodies were from Neomarkers Inc. (Fremont, CA). Phospho- (and regular) MAPK/ERK1/2 antibodies were from New England Biolabs (Beverly, MA). ECL detection system was from Amersham Corp. (Arlington Heights, IL).

Cell Culture and Treatments

DU145 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in an atmosphere of 95% air and 5% CO_2 under 90–95% humidity. For erbB1-mediated signaling studies, cells were grown to 70–80% confluency, and then serum-starved for 48 hr. During the final 2 hr of starvation, cells were treated with ethanol or 100–200 μM doses of silymarin, genistein or EGCG in ethanol. The selection of these doses was based on our recent study with silymarin at identical doses [18]. The final concentration of ethanol in the culture medium during the treatment with agents was 0.5% (v/v) and, therefore, the same concentration of ethanol was present in control dishes. Cells were then treated with PBS or TGFα at a concentration of 100 ng/ml of medium, and incubated for 10 min at 37°C. Thereafter, the medium was removed, cells washed with PBS two times, and cell lysates prepared in non-denaturing 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 PMSF, 0.5% NP-40, and 0.2 U/ml aprotinin) as detailed recently [18]. The lysates were cleared by centrifugation at 10,000 rpm for 15 min in tabletop centrifuge at 4°C, the supernatants were collected, and protein concentration determined [18]. To assess the protein levels of cell cycle regulatory molecules, ~80% confluent cells (without serum starvation) were treated with either ethanol or 100–200 μM doses of silymarin, genistein or EGCG in ethanol for 20 hr and cell lysates prepared [18].

Immunoprecipitation and Western Blotting

Cell lysates (200–400 μg protein lysate per sample) were diluted to 1 ml with lysis buffer, and pre-cleared with 25–30 μl protein A/G agarose beads by gentle rotation at room temperature for 1 hr followed by the removal of beads using 5 min centrifugation at 2,000 rpm. The pre-cleared lysates were incubated overnight at 4°C with continuous rotation with 2 μg primary antibody against erbB1, Shc, Cip1/p21 or Kip1/p27, and 25 μl protein A/G agarose beads. Thereafter, immunocomplexes were collected by centrifugation at 2,000 rpm for 5 min, and washed four times with lysis buffer. For immunoblotting, immunocomplexes or cell lysates (20–80 μg protein) were denatured with sample buffer, the samples were subjected to SDS-PAGE (8/12% gel) and transferred on to nitrocellulose membranes. Membranes were blocked with blocking buffer at room temperature for 1 hr and, as desired, probed with primary antibody against phosphotyrosine, erbB1, Shc, phospho- (or regular) MAPK/ERK1/2, Cip1/p21, Kip1/p27, cyclin D1, cyclin E, CDK2 or CDK4 overnight followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system.

Cell Growth and Viability Assays

DU145 cells were plated at a density of 1×10^5 cells per 60 mm dish, and on the following day (Day 0), fed with fresh medium and treated with ethanol or 50–200 μM doses of silymarin, genistein or EGCG in ethanol. The cultures were fed with a fresh medium with or without the same concentrations of compounds every alternate day up to the end of the experiment. On days 1–5 after these treatments, cells were trypsinized, collected, and counted on a hemocytometer. Trypan blue dye exclusion was used to determine cell death.

Densitometric and Statistical Analysis

Autoradiograms of the Western immunoblots were scanned using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The blots were adjusted for brightness and contrast for minimum background,

and the mean density for each band was analyzed using Scanimage Program (National Institutes of Health, Bethesda, MD). The densitometric data (arbitrary numbers) are shown under the immunoblots at appropriate places, and are average of three independent experiments with less than ± 10% variation. For ERK1/2 phosphorylation studies, the densitometric analysis data for phospho-ERK1/2 blots were corrected for loading with the density of ERK1/2 blots. The two-tailed Student *t*-test was employed to assess statistical significance of difference between vehicle and agent treated samples. The results shown are representative of three independent experiments with similar findings, unless otherwise specified.

RESULTS

Inhibition of TGFα-Caused erbBI Activation

First we assessed the effect of silymarin, genistein, and EGCG on TGFα-caused erbB1 activation in DU145 cells. A 48-hour serum starvation of cells resulted in a complete disappearance of constitutively active erbB1 [18] as evident by a lack of reactivity of immunoprecipitated erbB1 with anti-phosphotyrosine antibody (Fig. 1A, lane 1). Treatment of serum-starved cells with TGFα, however, resulted in a strong activation of erbB1 (Fig. 1A, lane 2). Pretreatment of cultures with silymarin, genistein, and EGCG during the last 2 hr of serum starvation followed by TGFα stimulation resulted in a complete inhibition of ligand-caused activation of erbB1 (Fig. 1A, lanes 3–11). The densitometric quantification of the bands (Fig. 1A) showed that, compared to TGFα-treated positive control, silymarin treatment at 100 μM dose followed by TGFα stimulation resulted in no effect on erbB1 activation. However, 150 and 200 μM doses of silymarin and all three doses of genistein and EGCG showed complete inhibition ($P < 0.001$, $n = 3$) of ligand-caused erbB1 activation (Fig. 1A, lanes 6–11). The observed inhibitory effect of these agents was not due to a decrease in erbB1 protein levels in all but 100 μM genistein sample (Fig. 1B) suggesting that these agents inhibit TGFα-caused erbB1 activation.

Inhibition of Shc Activation and Its Binding to erbBI

One of the immediate downstream substrates to erbB1 activation is Shc which following tyrosine phosphorylation, acts as an adaptor between erbB1 and other SH2-containing proteins in erbB1-Grb2-SOS/ras/raf/ERK-mediated mitogenic signaling [3,18]. Based on the inhibitory effect of silymarin, genistein or EGCG on erbB1 activation, we next assessed whether these agents also impair Shc activation. As shown in Figure 2A, a pattern parallel to erbB1 activation was also evident for Shc. There was no Shc activation in serum-starved cultures, but treatment of starved cells with TGFα resulted in strong activation of the 46 kDa isoform of Shc and only weak activation of 52 kDa Shc isoform (Fig. 2A, lane 2). However, treatment of cultures with different doses of silymarin, genistein, and EGCG for 2 hr prior to the addition of TGFα showed a moderate to strong decrease in tyrosine phosphorylation of both 52 and 46 kDa Shc protein bands (Fig. 2A, lanes 3–11). Quantification of 46 kDa band intensity showed that silymarin, genistein or EGCG caused 10–60% ($P < 0.1$–0.001), 80–90% ($P < 0.001$), and 70–90% ($P < 0.001$) inhibition (an average of three independent studies ± <10% of average) of Shc activation, respectively. The inhibitory effect of these agents on Shc activation was not due to a decrease in total Shc protein levels (Fig. 2B). Since following erbB1 activation, Shc binds to activated erbB1 that causes Shc activation to transduce downstream signals [3,18], we also assessed the effect of these agents on erbB1–Shc binding. The quantification of the bands in Figure 2C shows that silymarin, genistein or EGCG treatment resulted in 60–80% ($P < 0.001$), 70% to complete ($P < 0.001$), and 10% to complete ($P < 0.1$–0.001) inhibition of Shc binding to erbB1, respectively. Together, these results suggest that as an initial step, these

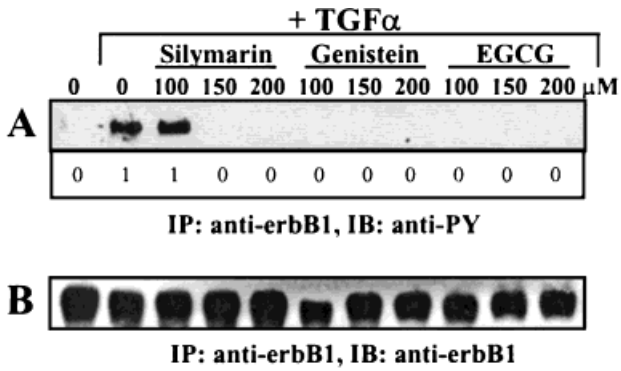

Fig. 1. Silymarin, genistein, EGCG inhibit TGFα-caused activation of erbBI in DUI45 cells. Cells were cultured as described in Methods, and at 70–80% confluency, were serum-starved for 48 hr. During the last 2 hr of starvation, they were treated with ethanol or varying doses of agents in ethanol, and at the end of these treatments with PBS or TGFα (I00 ng/ml of medium) for I5 min at 37°C. Cell lysates were prepared, erbBI was immunoprecipitated using anti-EGFR antibody, and following SDS-PAGE and Western blotting, membranes were probed with (**A**) anti-phosphotyrosine (anti-PY) or (**B**) anti-EGFR (anti-erbBI) antibody, and then peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol + PBS. IP, immunoprecipitation; IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblot in panel A, are average of three independent experiments with less than ± I0% variation.

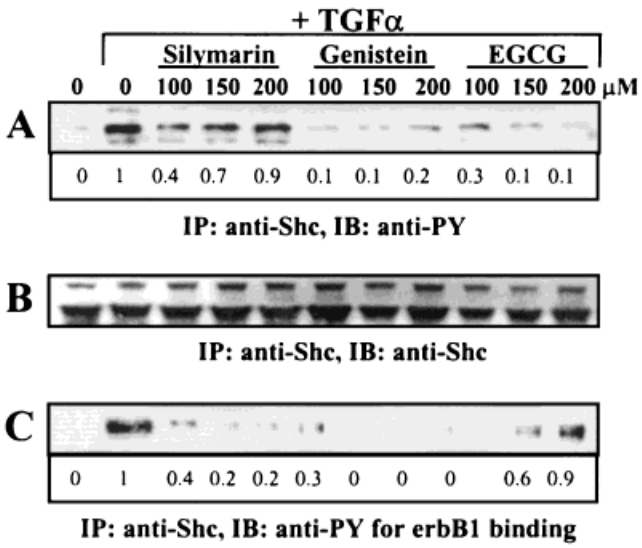

Fig. 2. Silymarin, genistein, and EGCG inhibit Shc activation and its binding with erbBI in DUI45 cells. Cell culture and treatments are those described in Figure I. Shc was immunoprecipitated from the cell lysates using anti-Shc antibody, and following SDS-PAGE and Western blotting, membranes were probed with (**A**) anti-phosphotyrosine (anti-PY), (**B**) anti-Shc or (**C**) anti-phosphotyrosine (anti-PY) for erbBI binding, antibody, and then peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol + PBS. IP, immunoprecipitation; IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in panels A and C, are average of three independent experiments with less than ± I0% variation.

agents inhibit erbB1 activation that leads to a decrease in Shc binding to erbB1 followed by inhibition of Shc activation.

Inhibition of MAPK/ERKI/2 Activation

Based on the data shown in Figures 1 and 2, we then assessed the effect of these agents on the ultimate cytoplasmic mitogenic signaling target, MAPK/ERK1/2 activation. As expected, 48 hr of serum starvation resulted in a completely diminished ERK1/2 activation (Fig. 3A, lane1) while TGFα treatment caused a strong stimulation (Fig. 3A, lane 2). Pretreatment of cultures with silymarin and genistein resulted in a moderate to strong inhibition of ligand-caused activation of ERK1/2 (Fig. 3A, lanes 3–8). Interestingly, EGCG showed a strong increase in phosphorylated levels of ERK1/2 (Fig. 3A, lanes 9–11). The quantification of band intensities for phospho-ERK1/2 and correction for loading with total ERK1/2 showed that silymarin and genistein caused 10–70% ($P < 0.05$–0.001) and 80% to complete ($P < 0.001$) inhibition of phospho-ERK1/2 levels (an average of three independent studies ± <10% of average), respectively. Conversely, EGCG resulted in 1.2–3.4-fold increase ($P < 0.05$–0.001, n = 3) in phospho-ERK1/2 levels.

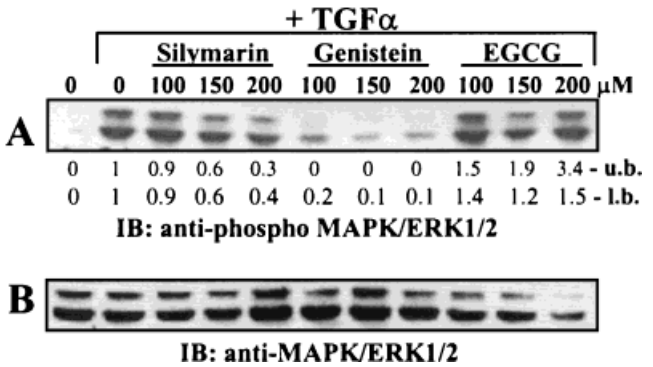

Fig. 3. Silymarin and genistein, but not EGCG, inhibit ligand-caused activation of MAPK/ERKI/2 in DUI45 cells. Cell culture and treatment conditions are described in Methods and Figure I. Cell lysates were subjected to SDS-PAGE and Western blotting, and membranes were probed with (**A**) phospho-MAPK/ERKI/2 or (**B**) MAPK/ERKI/2 antibody. In each case, membranes were then probed with peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol + PBS. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblot in panel A, are average of three independent experiments with less than ± I0% variation, and are corrected for loading with the density of ERKI/2 blots in panel B.

Modulation of Cell Cycle Regulators

Based on the observed effect of silymarin, genistein, and EGCG on erbB1-Shc-MAPK/ERK signaling, we assessed the effect of these agents on the modulation of cell cycle regulators. As shown in Figure 4, treatment of cells with these agents resulted in a very strong induction of both Cip1/p21 and Kip1/p27. For Cip1/p21, the most effective agent was silymarin followed by genistein and EGCG showing 1.5–2.4, 1.2–2.2 and 1.7-fold induction ($P < 0.05$–0.001, n = 3), respectively. Only the highest dose of EGCG (200 μM) tested, showed induction of Cip1/p21 whereas lower doses led to a decrease (Fig. 4A). These agents showed 2–120, 70–150, and 15–97-fold increase ($P < 0.001$, n = 3) in Kip1/p27, respectively (Fig. 4B). In other studies, all the test agents exerted a strong decrease in CDK4 protein levels (Fig. 5A). Silymarin was most effective followed by genistein and EGCG accounting for 60–90%, 50–70%, and 40–50% decrease ($P < 0.001$, n = 3). Silymarin was also effective in reducing CDK2 expression by 30% ($P < 0.05$), but genistein and EGCG did not show any effect (Fig. 5B). A moderate inhibitory effect of these agents on cyclin D1 (Fig. 5C) and cyclin E (Fig. 5D) levels was also observed with silymarin being most effective followed by genistein and EGCG.

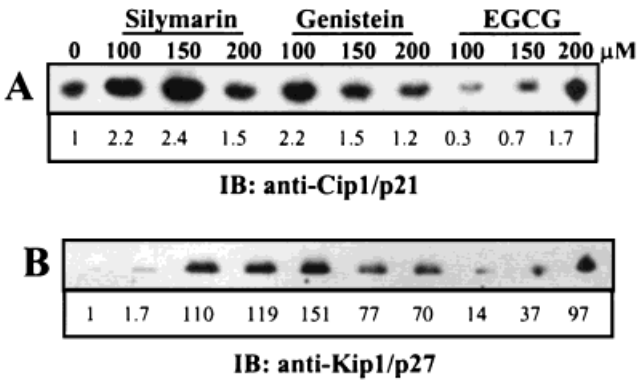

Fig. 4. Silymarin, genistein, and EGCG induce the levels of CDKIs in DUI45 cells. Cells were cultured as described in Methods, and at 70–80% confluency (without serum starvation), were treated with either vehicle alone or varying concentrations of silymarin, genistein, and EGCG for 20 hr as described in Methods. At the end of these treatments, total cell lysates were prepared, and subjected to SDS-PAGE followed by Western blotting as described in Methods. Membrane was probed with anti- (**A**) CipI/p2I or (**B**) KipI/p27 antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol treatment alone. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in panels A and B, are average of three independent experiments with less than ± I0% variation.

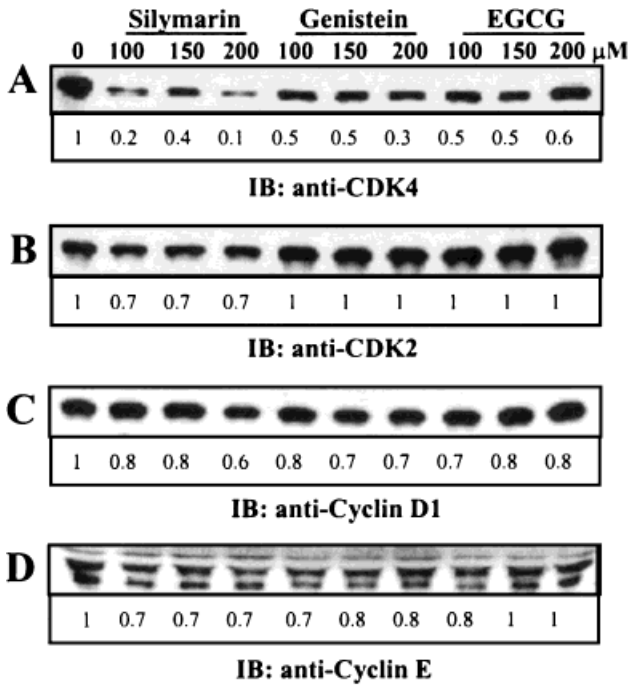

Fig. 5. Effect of silymarin, genistein, and EGCG on CDKs and cyclins in DUI45 cells. Cell cultures and treatments are those described in Methods and Figure 4. Total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in Methods. Membrane was probed with anti- (**A**) CDK4, (**B**) CDK2, (**C**) cyclin DI or (**D**) cyclin E antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol treatment alone. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in each panel, are average of three independent experiments with less than ± I0% variation.

To explore the significance of CDKI induction by these agents, we determined the binding of CDKIs to CDKs. As shown in Figure 6A and B, Cip1/p21 binding to CDK4 and CDK2 is significantly ($P < 0.001$) enhanced following treatment with silymarin, genistein, and EGCG. Similar results were also evident in terms of a strong increase ($P < 0.001$) in the binding of Kip1/p27 to CDK4 and CDK2 (Fig. 6C,D). Together, these results suggest that treatment with silymarin, genistein or EGCG leads to perturbations in cell cycle molecules via an increase in CDKI levels. An increased expression of CDKIs led to their increased binding with CDKs that possibly inhibits kinase activity of CDKs and associated cyclins. Together, these alterations in cell cycle regulators lead to inhibition of cell growth and/or induction of cell death, as observed in next set of studies.

Inhibition of Cell Growth and Induction of Cell Death

In order to assess the effect of silymarin, genistein, and EGCG on DU145 cell growth and/or death, cells in the exponential growth phase were treated with 50, 100, 150, and 200 μM doses of these agents for 5 days. A dose- and time-dependent inhibitory effect of these agents was observed on cell growth. Compared to untreated control, addition of ethanol (as vehicle) did not result in an alteration in cell growth (data not shown). As shown in Figure 7A, 150 and 200 μM doses of silymarin resulted in almost complete inhibition ($P < 0.001$) of cell growth while 100 μM silymarin accounted for 70% inhibition ($P < 0.001$) after 5 days of treatment. About 50% inhibition ($P < 0.001$) was evident at 50 μM dose of silymarin following 5 days of treatment (Fig. 7A). None of the silymarin doses tested showed any cytotoxicity during 5 days of treatment (Fig. 7B). A much strong effect was observed with genistein that showed 80% inhibition ($P < 0.001$) of cell growth at 50 μM dose and complete inhibition ($P < 0.001$) at higher doses following 5 days of treatment (Fig. 7A). This marked cell growth inhibitory effect of genistein could possibly be associated with its cytotoxic effect because other than the 50 μM dose, all the other doses tested showed 30–40% cell death ($P < 0.001$) following 5 days of treatment (Fig. 7B). Similar to genistein, EGCG also showed a strong cell growth inhibitory effect causing 76% inhibition ($P < 0.001$) at 50 μM dose and complete inhibition ($P < 0.001$) at higher doses during all the time points

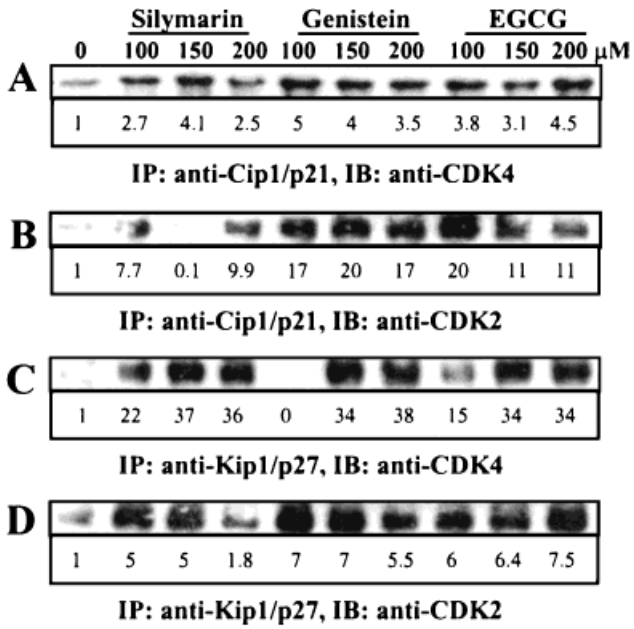

Fig. 6. Silymarin, genistein, and EGCG induce the binding of CDKIs with CDKs in DUI45 cells. Cell culture conditions, treatments, and other details are those described in Figure 4. At the end of treatments, cell lysates were prepared, CipI/p2I and KipI/p27 were immunoprecipitated, and subjected to SDS-PAGE followed by Western blotting as detailed in Methods. The membrane was probed with anti-CDK4 (**A**, **C**) or CDK2 (**B**, **D**) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol treatment alone. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in each panel, are average of three independent experiments with less than ± I0% variation.

studied (Fig. 7A). These growth inhibitory effects of EGCG were largely due to its high cytotoxic effects. As shown in Figure 7B (bottom panel), after 24 hr of EGCG treatment, 100, 150, and 200 μM doses resulted in 35–80% cell death ($P < 0.001$), and after 5 days of treatment, these doses of EGCG caused complete cell death ($P < 0.001$).

DISCUSSION

The central finding in the present study is that the three phytochemicals, which have been shown to be cancer-preventive and anti-carcinogenic agents against various cancers in both long- and short-term study models, showed a strong inhibitory effect against epigenetic events associated with advanced and androgen-independent human PCA growth. PCA, pertaining to its multifocal and invasive nature, and extensive mortality associated with this malignancy, attracts immense attention for its prevention, control, and therapy. Asian men have the lowest PCA incidence while, in contrast, African followed by Caucasian Americans represent the most affected population with a PCA incidence over 100-fold higher in the United States [31]. Current evidence suggests that these geographical and racial–ethnic variations in PCA incidence may, in part, be due to genetic differences as well as those in dietary habits, and androgen secretion and metabolism [31].

Epidemiological studies have shown that even with the same incidence of latent small or non-infiltrating prostatic carcinomas, the incidence of clinical PCA and associated mortality is low in Japan and some other Asian countries [32]. These data suggest a hypothesis that ''although the initiation of PCA is inevitable, targeting epigenetic events could control its progression to clinical cancer, and that the incidence of clinical PCA is low in Asian countries because of their dietary habits that also include the nutrition rich in several flavonoids and isoflavones'' [33]. Consistent with this hypothesis, results of the present study show that silymarin, genistein, and EGCG significantly inhibit erbB1-mediated mitogenic signaling in advanced and androgen-independent human PCA DU145 cells, suggesting the possibility that this could be one of the reasons for the observed low incidence of clinical PCA in Asian men. These results also suggest that more studies are needed with these agents to develop them for the prevention and/or intervention of PCA growth and its metastatic potential.

An erbB1-mediated mitogenic signaling pathway activates Shc-Grb2-ras-raf signaling that causes the activation of ultimate cytoplasmic target, the MAPK/ERK1/2 [3,34]. The activated MAPK/ERK1/2 then translocate to the nucleus and activate transcription factors for cell growth and proliferation [3,34]. In case of advanced and androgen-independent PCA, several studies have shown genetic alterations resulting in an enhanced expression of erbB1 and associated ligand that leads to an epigenetic mechanism of autocrine growth loop via ligand/erbB1 interaction [8–10]. Together, these studies suggest that growth factors and receptors associated with PCA progression regulate cell growth mostly through the activation of MAPKs. Indeed, recent studies have shown that MAPK/ERK1/2 are constitutively very active in DU145 cells; and that epidermal growth factor, insulin-like growth factor-1 and protein kinase A activator significantly activate MAPK/ERK1/2 in both LNCaP and DU145 human PCA cells via erbB1 receptor [35]. In addition, an increase in constitutive activation of MAPK/ERK1/2 signaling has been reported in human PCA as it progresses to a more advanced and androgen-independent malignancy [36]. Consistent with the involvement of activated MAPK/ERK1/2, possibly via TGFα/erbB1 autocrine loop, in the progression of

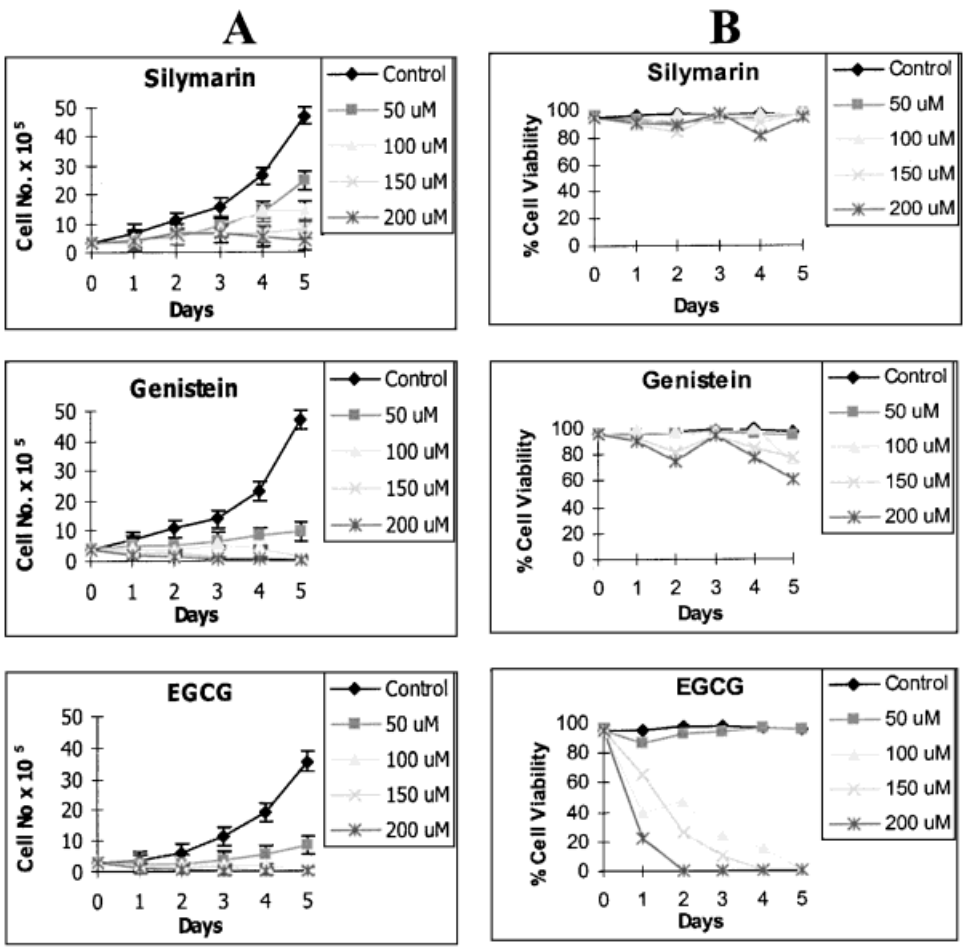

Fig. 7. Silymarin, genistein, and EGCG cause significant growth inhibition (**A**) of DUI45 cells, but only genistein and EGCG induce cell death (**B**). The details of cell culture and treatments are those described in Methods. Each data point represents mean ± SE of three independent plates, each sample was counted in duplicate.

advanced and androgen-independent human PCA, as summarized in Figure 8, we observed that impairment of erbB1-Shc activation results in the inhibition of MAPK/ERK1/2 activation in DU145 cells by silymarin and genistein. However, with EGCG treatment, an inhibition of erbB1-Shc activation did not result in the impairment of MAPK/ERK1/2 activation, but caused an additional stimulation. Studies are in progress to define the mechanism of this effect.

It has been shown that signaling pathways determine cell growth and inhibition through cell cycle regulation [10,18,37]. Using erbB1 blocking antibody, recent studies have shown that impairment of erbB1 activation leads to inhibition of Shc activation followed by selective induction of Kip1/p27 and G_1 arrest in DU145 cells [10,18]. These results further support a direct cause and effect relationship between impairment of erbB1-mediated mitogenic signaling and perturbations in cell cycle regulation leading to cell growth inhibition [10,18]. Furthermore, a growth-promoting mitogenic signal is known to be involved in normal cell proliferation via cell cycle progression where it commands the cells in G_0 restriction checkpoint to go through G_1, S, and G_2-M phases of the cell cycle [37]. However, in transformed cells, cell cycle progression could be a mitogenic signal-dependent or -independent process [10,18,37]. For example, several studies have shown a loss of functional CDKI in different human cancers and derived cell lines that leads to uncontrolled cell proliferation due to an increase in the function of CDK–cyclin complex [37]. Similarly, an overexpression of cyclin D1 and CDK4 has also been reported in several human malignancies that leads to shortening in G_1 phase causing uncontrolled cellular proliferation [38]. Consistent with these studies, it can be appreciated that targeting cell cycle regulators, i.e., induction of CDKIs and decrease in CDKs and cyclins, either dependent or independent of mitogenic signaling impairment, could be another strategy for the inhibition of epigenetic events associated with malignant cell growth. In this regard, whereas silymarin was less effective than genistein and EGCG in inhibiting erbB1-mediated signaling pathway, it showed a much stronger effect on the modulation of cell cycle regulators and their interplay (Fig. 8) in DU145 cells. The data obtained for cell growth inhibition and death suggest that, depending on their effects on epigenetic events, they could either

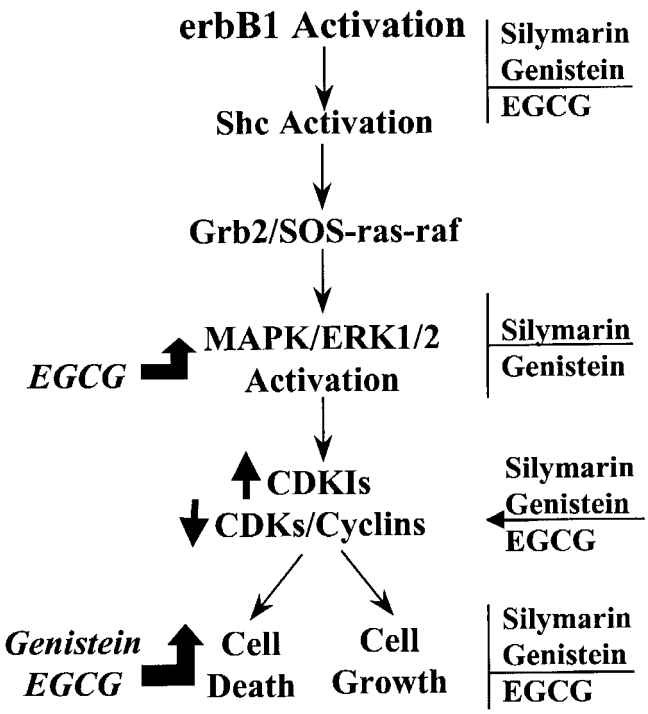

Fig. 8. A summary of the observed effects of silymarin, genistein, and EGCG on erbBI-mediated mitogenic signaling and modulation of cell cycle regulators in DUI45 cells and their association with cell growth inhibition and death.

be anti-proliferative (as is the case with silymarin) or death-inducing agents (Fig. 8) against advanced and androgen-independent human prostate cancers.

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