Differential Responses of Skin Cancer-Chemopreventive Agents Silibinin, Quercetin, and Epigallocatechin 3-Gallate on Mitogenic Signaling and Cell Cycle Regulators in Human Epidermoid Carcinoma A431 Cells

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Abstract: Silibinin, quercetin, and epigallocatechin 3-gallate (EGCG) have been shown to be skin cancer-preventive agents, albeit by several different mechanisms. Here, we assessed whether these agents show their cancer-preventive potential by a differential effect on mitogenic signaling molecules and cell cycle regulators. Treatment of human epidermoid carcinoma A431 cells with these agents inhibited the activation of the epidermal growth factor receptor and the downstream adapter protein Shc, but only silibinin showed a marked inhibition of mitogen-activated protein kinase-extracellular signal-regulated kinase-1 and -2 activation. In terms of cell cycle regulators, silibinin treatment showed an induction of Cip1/p21 and Kip1/p27 together with a significant decrease in cyclin-dependent kinase (CDK)-4, CDK2, and cyclin D1. Quercetin treatment, however, resulted in a moderate increase in Cip1/p21 with no change in Kip1/p27 and a decrease in CDK4 and cyclin D1. EGCG treatment also led to an induction of Cip1/p21 but no change in Kip1/p27, CDK2, and cyclin D1 and a decrease in CDK4 only at low doses. Treatment of cells with these agents resulted in a strong dose- and time-dependent cell growth inhibition. A high dose of silibinin and low and high doses of quercetin and EGCG also led to cell death by apoptosis, suggesting that a lack of their inhibitory effect on mitogen-activated protein kinase-extracellular signal-regulated kinase-1 and -2 activation possibly “turns on” an apoptotic cell death response associated with their cancer-preventive and anticarcinogenic effects. Together, these results suggest that silibinin, quercetin, and EGCG exert their cancer-preventive effects by differential responses on mitogenic signaling and cell cycle regulators.

Introduction

Diet and environmental factors contribute toward growth and evolution of various cancers. Fruits, yellow-green vegetables, and common beverages, as well as several herbs and plants with diversified pharmacological properties, have been shown to be the rich sources of cancer-chemopreventive agents (Refs. 1–3 and references therein). Flavonoids are among the best candidates for mediating the protective effects of a diet rich in fruits and vegetables, inasmuch as they possess a broad range of biochemical and biological activities that vary with structural variations. Silibinin, quercetin, and epigallocatechin 3-gallate (EGCG) are naturally occurring polyphenolic flavonoids. Silibinin is isolated from milk thistle, quercetin is present in many vegetables and red wine, and EGCG is the major constituent in green tea. These three polyphenols have a structural similarity, in that addition of a lignan in the basic quercetin structure derives silibinin, whereas a gallate substitution derives EGCG (Figure 1).

In recent years, several studies from our laboratory and others have shown the cancer-chemopreventive effects of silymarin, quercetin, and EGCG in different skin carcinogenesis models, as well as in other tumor models (Refs. 4–32 and references therein). For example, it has been shown that silymarin affords protection against a wide range of carcinogens and ultraviolet-B (UV-B) radiation-induced carcinogenesis (4–13). It inhibits benzoyl peroxide and 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced tumor promotion in Sencar mouse skin, primarily targeted at stage I tumor promotion (7,8). Silymarin also inhibits epidermal lipid peroxidation, benzoyl peroxide-induced oxidative stress, and UV-B- and TPA-induced cyclooxygenase-2 expression (5–9). Quercetin is known to be an anticancer and antitumorigenic agent, inasmuch as it inhibits growth of various human carcinoma cells and is known to downregulate signal transduction events (14–16). It is also known to be an apoptotic agent in various cells such as leukemia HL-60 cells and colorectal tumor cells (17,18). Previous reports have shown that quercetin inhibits phosphorylation of epidermal growth factor (EGF) receptor (EGFR) and secretion of matrix metalloproteinases-2 and -9 in A431 cells (19).
EGCG is also known to possess cancer-preventive and therapeutic effects (20–24). It is known to inhibit chemical carcinogenesis and photocarcinogenesis and diminish UV-B-induced DNA damage in human skin and activator protein-1 activity in epidermis of transgenic mice (25–28). It has been reported that EGCG inhibits TPA-induced epidermal ornithine decarboxylase activity in Sencar mice and tumor-promoting activity of okadaic acid in mouse skin (29,30). Various studies have reported that EGCG is an apoptotic agent in various cancer cells (22,31,32).

The early components of signal transduction pathways, specifically those of tyrosine kinases, are of utmost significance for controlled cell growth and differentiation (Ref. 33 and references therein). Ironically, enhanced protein tyrosine kinase activity due to overexpression of receptor and/or protein tyrosine kinases leads to continuous signaling, resulting in an uncontrolled cell proliferation that signals in cancer growth (19,33). Several studies have shown the aberrant expression of the erbB family of receptor tyrosine kinases, such as EGFR (or erbB1), erbB2, and erbB3, with strikingly high frequency in several human malignancies (19,34,35). An increased expression of erbB1 has been reported in mouse skin chemical carcinogenesis, and phorbol ester tumor promoter and UV-B radiation activate the erbB1 signaling pathway in mouse skin and human keratinocytes (10,36–38). Consequently, the erbB receptor family is being explored as potential biomarkers and therapeutic targets in various cancers (35).

Taken together, in the present study, we assessed the effect of silibinin, quercetin, and EGCG on the impairment of mitogenic signaling via the erbB1-Shc extracellular signal-regulated kinase-1 and -2 (ERK1/2) pathway and modulation of cell cycle regulators as a plausible mechanism(s) of their skin cancer-preventive potential. Because human epidermoid carcinoma A431 cells express exceptionally high levels of erbB1 (Refs. 10 and 19 and references therein), these cells were used as a model for the studies described here. The results showed that these agents inhibit EGF-induced activation of erbB1 and Shc, but only silibinin was effective in inhibiting ERK1/2 activation. These agents also showed modulation of cell cycle regulators, albeit at different levels, silibinin being most effective. Whereas a significant cell growth inhibition was also observed with these agents, strong cell death was evident in the case of quercetin and EGCG, and a moderate effect was observed with silibinin.

**Materials and Methods**

**Cell Culture and Treatments**

Human epidermoid carcinoma A431 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions to 70% confluency. Cells were starved in serum-free medium for 48 hours, and during the last 2 hours of starvation they were treated with vehicle alone or desired doses of silibinin, quercetin, and EGCG (all purchased from Sigma Chemical, St. Louis, MO). Cells were then added with phosphate-buffered saline or EGF (100 ng/ml; GIBCO BRL, Gaithersburg, MD) and incubated for 10 minutes at 37°C. After the cells were washed with phosphate-buffered saline, cell lysates were prepared without nondenaturing conditions as described in detail recently (12). For cell cycle regulatory molecule studies, 80% confluent cultures without serum starvation were treated with desired doses of different agents for 20 hours, and cell lysates were prepared (12).

**Immunoprecipitation and Immunoblotting**

Cell lysates (200–500 µg protein) were cleared by protein A/G agarose for one hour and incubated with primary antibody directed against erbB1, Shc, Cip1/p21, or Kip1/p27 for four hours, protein A/G agarose was added, and the lysates were incubated overnight at 4°C (12). The beads carrying immunocomplexes were washed three times with lysis buffer. For immunoblotting, immunocomplexes or cell lysates (20–80 µg protein) were denatured in sample buffer, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 8%–12% gel).
and transferred onto nitrocellulose membranes. The membranes were blocked with blocking buffer at room temperature for one hour, incubated with primary antibody against phosphotyrosine, erbB1, Shc, phospho-ERK1/2, ERK1/2, Cip1/p21, Kip1/p27, cyclin D1, cyclin E, cyclin-dependent kinase (CDK)-2, or CDK4 overnight and then with a secondary antibody, and developed by an enhanced chemiluminescence (ECL) kit (12).

Cell Growth and Viability Assays

Cells were plated at $1 \times 10^5$ cells/60-mm plate and, after 24 hours, fed fresh medium and treated with ethanol alone or various doses of silibinin, quercetin, or EGCG. On Days 1–5 after these treatments, cells were trypsinized and counted using a hemocytometer. Trypan blue dye exclusion assay was used to assess cell viability.

Quantitative Apoptotic Cell Death Assay

To substantiate whether the observed cell death effect of these agents is due to the apoptotic cell death, quantitative apoptosis assay was performed employing annexin V and propidium iodide (PI) staining of the cells followed by flow cytometry. Briefly, A431 cells were treated with different doses of these agents for four days (a time period when strong cell death was observed; see Results and Discussion), and floating and attached cells were collected and subjected to annexin V and PI staining using the Vybrant Apoptosis Assay Kit no. 2 (Molecular Probes, Eugene, OR) and following step-by-step protocol provided by the vendor. After the cells were stained with Alexa Fluro 488 annexin V and PI, fluorescence analysis was performed.

Densitometric and Statistical Analysis

Autoradiograms of the immunoblots were scanned using Adobe Photoshop (Adobe Systems, 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 averages of three independent experiments with less than ±10% variation. As needed, two-tailed Student’s t-test was employed to assess statistical significance of difference between vehicle- and agent-treated samples. Until and unless specified otherwise, the results showed in each case are representative of three independent experiments with similar findings.

Results and Discussion

In the recent past, a diverse range of dietary micronutrients has been explored for their preventive and therapeutic efficacy against various cancers. Several studies from our laboratory and by others have reported anticancer efficacy of silibinin, quercetin, and EGCG against various cancers (Refs. 5–32 and references therein). Here, we studied the effect of these agents on the erbB1-Shc-ERK1/2 mitogenic signaling pathway and cell cycle regulatory molecules in human epidermoid carcinoma A431 cells.

Enhanced mitogenic signaling has been linked with cellular transformation as well as progression of different human malignancies (Ref. 12 and references therein). The erbB receptor family members play an important role in normal cell growth and differentiation but are commonly amplified and overexpressed in various carcinomas, suggesting that increased signaling, as a result of receptor overexpression, may play an important role in carcinogenesis (Refs. 10–12 and references therein). In addition, an enhanced secretion of the transforming growth factor-α-EGF ligand also occurs in most cancers, which results in continuous activation of the erbB1 receptor via an autocrine loop (Refs. 12 and 34 and references therein). Together, it could be appreciated that erbB receptor family members are potential therapeutic targets in malignant tissues. Studies in this report, therefore, first assessed the effect of cancer-preventive phytochemicals on erbB1-mediated mitogenic signaling.

Treatment of serum-starved A431 cells with silibinin or quercetin resulted in a moderate inhibition (20–30%, $p < 0.05$) of EGF-induced erbB1 activation (tyrosine phosphorylation; Figure 2A). However, a highly significant inhibition (70–80%, $p < 0.001$) of EGF-induced erbB1 activation was observed in EGCG-pretreated cells (Figure 2A). These alterations in erbB1 activation by silibinin, quercetin, and EGCG were not due to a change in total erbB1 protein levels (data not shown), suggesting an impairment of erbB1 signaling by these agents. In other studies, pretreatment of starved cultures with silibinin, quercetin, and EGCG also resulted in an inhibition of Shc activation (Figure 2B). The densitometric analysis of the blots showed that the observed inhibition was 40% ($p < 0.001$), 70–90% ($p < 0.001$), and 30–90% ($p < 0.001$) in the case of silibinin, quercetin, and EGCG, respectively (Figure 2B). The observed changes in Shc tyrosine phosphorylation by these agents were not due to a change in its protein levels (data not shown). Additional studies showed that an inhibition in Shc activation by these agents was due to a significant ($p < 0.001$) decrease in the binding of Shc to the receptor erbB1 (Figure 2C). Impairment of the erbB1 signaling pathway via inhibition of erbB1 and its adapter protein Shc activation by silibinin, quercetin, and EGCG suggests that this could be an integral part of their anticancer efficacy.

The mitogen-activated protein kinase (MAPK)-ERK1/2 are the ultimate cytoplasmic targets of erbB1 signaling cascade via Shc-Grb2-ras-raf-mitogen-activated extracellular signal-related kinase (MEK 1) and are involved in execution of diverse cellular responses, including cell growth, differentiation, survival, and death (39,40). ERK1/2 are known as mitogenic signaling molecules that, after activation, translocate to the nucleus and activate transcription factors for cell growth and proliferation (39). It has been shown that ERK1/2 are constitutively active in various human cancers.
Accordingly, we next assessed the effect of silibinin, quercetin, and EGCG on EGF-caused ERK1/2 activation by determining phospho-ERK1/2 levels in A431 cells. As shown in Figure 3, silibinin showed a 20–30% (p < 0.05) decrease in EGF-induced phospho-ERK1/2 levels. Minimal, if any, effect was observed with EGCG at higher doses (Figure 3). Interestingly, all the doses of quercetin used in the present study resulted in a 1.3- to 2.0-fold increase (p < 0.001) over EGF-treated samples in phospho-ERK1/2 levels (Figure 3). Additional studies are needed to define this opposing effect of quercetin on ERK1/2 activation compared with the erbB1-Shc pathway. No change in ERK1/2 protein levels was observed after these treatments in each case (data not shown), which once again suggests that a decrease as well as an increase in ERK1/2 activation by these agents is a direct response not due to alterations in protein levels.

Several studies have shown that cell-signaling pathways determine cell growth and inhibition through cell cycle regulation (13,42,43). However, in the case of transformed cells, cell cycle progression could be a mitogenic signaling-dependent or -independent process (44). In the studies assessing the effect of these compounds on cell cycle regulatory molecules, silibinin treatment resulted in a strong induction of Cip1/p21 (2.3- to 4.0-fold, p < 0.001) and Kip1/p27 (1.5- to 1.8-fold, p < 0.001) in a dose-dependent manner (Figure 4). A strong induction in Cip1/p21 was also observed with quercetin (2.4- to 3.3-fold, p < 0.001) and EGCG (2.6- to 3.2-fold, p < 0.001); however, both of these flavonoids did not produce any effect on the levels of Kip1/p27 (Figure 4). At 100 and 200 μM, quercetin showed a 20–100% decrease (p < 0.05–0.001) and EGCG showed a 10–90% decrease (p < 0.1–0.001) in Kip1/p27 levels (Figure 4B). In the studies analyzing the levels of CDKs and cyclins, silibinin showed a highly significant decrease (50–60%, p < 0.001) in CDK4 and a moderate to strong decrease in CDK2 (20–40%, p < 0.05–0.001) and cyclin D1 (30–70%, p < 0.05–0.001; Figure 5). However, quercetin and EGCG also showed a moderate to strong decrease (p < 0.05–0.001) in CDK4 and cyclin D1 but no change in CDK2 (Figure 5). None of the three agents showed any effect on cyclin E (data not shown). According to previous reports, these agents cause a strong G1 arrest in (41). Accordingly, we next assessed the effect of silibinin, quercetin, and EGCG on EGF on epidermal growth factor (EGF)-induced activation of erbB1 and Shc in A431 cells. Cells were cultured as described in Materials and Methods and, at 70% confluency, were serum starved for 48 h. During last 2 h of starvation, they were treated with vehicle alone or various concentrations of agent under test and, at end of treatments, with phosphate-buffered saline or EGF (100 ng/ml of medium) for 15 min at 37°C. Cell lysates were then prepared as described in Materials and Methods. ErbB1 or Shc was immunoprecipitated from cell lysates using anti-EGF receptor (EGFR) or anti-Shc antibody, respectively, and after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, membranes were probed with antiphosphotyrosine (anti-PY), anti-EGFR, or anti-Shc antibody and then peroxidase-conjugated appropriate secondary antibody. Proteins were visualized using enhanced chemiluminescence (ECL) detection system. A: effect on tyrosine phosphorylation of erbB1. B: effect on Shc activation. C: binding of Shc to erbB1. IB, Western immunoblotting; IP, immunoprecipitation.
cell cycle progression in different human carcinoma cells (13,15,31). Our results are consistent with these observations, inasmuch as these agents altered the cell cycle regulators involved in the G1 phase progression (43).

CDK inhibitors (CDKIs) negatively regulate cell cycle progression by binding to CDKs and inhibiting their kinase activity (45). Previous studies have shown that loss of CDKIs leads to uncontrolled cell growth due to enhanced activity of cyclin-CDK complexes in various cancers (43). Hence, we also assessed the effect of these compounds on the binding of CDKIs to CDKs. As shown in Figure 6, silibinin, quercetin, and EGCG resulted in an enhanced binding of Cip1/p21 and Kip1/p27 to CDK2 and CDK4.

Taken together, the above results suggest that, in addition to mitogenic signaling pathways, cell cycle regulatory molecules such as CDKIs, CDKs, cyclins, and their interaction could be potential targets for the inhibition of malignant cell growth. Consistent with this suggestion, the observed effects of silibinin, quercetin, and EGCG on membrane and cytoplasmic signaling pathways and modulation of cell cycle regulatory proteins also resulted in a highly significant \( p < 0.001 \) inhibition of cell growth in a dose- and time-dependent manner (Figure 7). In cell death studies, silibinin showed a strong effect \( p < 0.001 \) at high dose and longer treatment time, but quercetin and EGCG showed a significant reduction in the number of viable cells in a dose- and time-dependent manner (Figure 7). Because quercetin and EGCG are reported to induce apoptotic death in various human cancer cells (17,18,22,31,32), we anticipated that the observed cell death in the present study by these agents could be due to their apoptotic potential. In support of this anticipation, quantitative apoptosis studies were performed as described in Materials and Methods. As shown by data in Figure 8, treatment of A431 cells with these agents resulted in a moderate to highly significant \( p < 0.05–0.001 \) apoptotic death in a dose-dependent manner. Whereas higher doses of silibinin showed apoptotic cell death, lower and higher doses of quercetin and EGCG were effective in inducing strong apoptotic cell death (Figure 8). When these apoptotic results were compared with cell death data shown in Figure 7, they were in accordance with each other.

In summary, the results summarized in this report clearly suggest marked differences between inhibitory effects of silibinin, quercetin, and EGCG on membrane and cytoplasmic signaling and much stronger effects on cell cycle molecules by silibinin than by quercetin and EGCG, which probably can be correlated to the difference in their structures. A lack of response on ERK1/2 activation by quercetin and EGCG may be associated with turning on an apoptotic signal, as evident by significant cell death with these agents.

Acknowledgments and Notes

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Figure 7. Silibinin, quercetin, and EGCG cause significant growth inhibition and death of A431 cells. Cells were counted on a hemocytometer employing trypan blue exclusion assay for cell death. Each point represents mean ± SE of 3 independent plates; each sample was counted in duplicate.

Figure 8. Silibinin, quercetin, and EGCG cause significant apoptotic death of A431 cells. After desired treatments, floating and attached cells were collected and subjected to annexin V and propidium iodide staining and fluorescence analysis. Each data point represents mean ± SE of 3 independent plates.
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