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GAP JUNCTION, A BIOMARKER FOR CANCER AND CHEMOPREVENTION: PREVENTIVE EFFECT OF EPICATECHIN AND GINSENOSIDE Rb_2 ON THE INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION BY TPA AND H_2O_2

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Gap Junction, a biomarker for cancer and chemoprevention; Preventive effect of Epicatechin and Ginsenoside Rb_2 on the Inhibition of Gap Junctional Intercellular Communication By TPA and H_2O_2

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Abstract

The anticarcinogenic effects of epicatechin (EC) and ginsenoside Rb2 (Rb2), which are major components of green tea and Korea ginseng, respectively, were investigated using a model system of gap junctional intercellular communication (GJIC) in WB-F344 rat liver epithelial cells. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and hydrogen peroxide, known as cancer promoters, inhibited GJIC in the epithelial cells as determined by the scrape loading/dye transfer assay, fluorescence redistribution assay after photobleaching, and immunofluorescent staining of connexin 43 using a laser confocal microscope. The inhibition of GJIC by TPA and H_2O_2 was prevented with treatment of Rb2 or EC. The effect of EC on GJIC was stronger in TPA-treated cells than in H_2O_2-treated cells, while the effect of Rb2 was opposite to that of EC. EC, at the concentration of 27.8 μg/ml, prevented the TPA-induced GJIC inhibition by about 60%. Rb2, at the concentration of 277 μg/ml, recovered the H_2O_2-induced GJIC inhibition by about 60%. These results suggest that Rb2 and EC may prevent human cancers by preventing the down-regulation of GJIC during the cancer promotion phase and that the anticancer effect of green tea and Korea ginseng may come from the major respective components, EC and Rb2.

Key words: Green tea, Ginseng, Catechins, Ginsenosides, Gap junctional intercellular communication (GJIC), Connexin 43.

1. Introduction

In recent years, considerable interest has been generated on green tea as a health beverage [1]. Several pharmacological properties have been tentatively attributed to green tea that needs further confirmation. Green tea has also been implicated as a chemopreventive agent against the development of various tumors [2-5]. Most of these studies have been carried out with experimental animals [4,5], although some epidemiological data seem to be supportive of such a conclusion [6]. The protective activity of green tea is generally assumed to be due to the free radical-scavenging and/or metal-chelating effects of high concentrations of catechins or their gallates present in green tea [7]. However, the exact anticarcinogenic mechanisms of green tea or its components are still elusive and mostly speculative.

Ginseng has been used for traditional medicine in Korea, China, Japan and other Asian countries for the treatment of various diseases including psychiatric and neurologic diseases, as well as cancer [8-11]. Ginseng saponins (ginsenosides) have been regarded as the principal components responsible for the pharmacological activities of ginseng [12]. The ginsenosides, such as Rb1, Rb2, and Rc, have been reported to have anti-tumor effects, particularly on the inhibition of tumor-induced angiogenesis, tumor invasion and metastasis, and the control of phenotypic expression and differentiation of tumor cells [12-15]. However, the mechanisms of anti-tumor effects of ginsenosides have not been fully understood.

Gap junctions are membrane channels that permit the transfer of small water-soluble molecules, including cAMP and inositol triphosphate, from the cytoplasm of one cell to that of its neighbors [16,17]. Most tumor cells have a reduced ability to communicate among themselves and/or with surrounding normal cells, confirming the importance of functional gap junctional intercellular communication (GJIC) in growth control [16,17]. The reversible down-regulation of GJIC between adjacent cells has been hypothesized to be involved in tumor promotion phase of carcinogenesis [18]. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a well-known cancer promoter that activates protein kinase C (PKC) [16]. It has been well documented that TPA inhibits GJIC in several kinds of cell lines including WB-F344 rat liver epithelial cells [19-21]. Oxidative stress is also strongly implicated in tumor promotion by epigenetic mechanisms, such as the activation of protein kinases and the inhibition of GJIC [22,23]. H_2O_2, as a tumor promoter, is known to inhibit GJIC. The inhibitory mechanism of H_2O_2 on GJIC is different from that of TPA [23].

In this study, the anticarcinogenic effects of green tea and Korea ginseng were investigated during the promotional phase using TPA or H_2O_2 to inhibit GJIC, which is associated with tumor promotion. The working hypothesis is that green tea, Korea ginseng, or their major components may prevent the blockage of GJIC in tumor promoter-treated cells. Several techniques, including a scrape loading/dye transfer (SL/DT) assay, a fluorescence redistribution assay after photobleaching (FRAP), and an immunofluorescent staining of connexin 43 (Cx43) using a laser confocal microscope were used to assess GJIC. In this study, we report that EC from green tea and Rb2 from Korea ginseng prevented the inhibition of GJIC by TPA or H_2O_2, indicating that they can be used as anti-tumor or chemopreventive agents, especially acting on cancer promotional stage.

2. Materials and methods

2.1. Materials

Epicatechin (EC), Epicatechin gallate (ECG), Epigallocatechin gallate (EGCG), Greentea extract (GTE), Ginsenoside Rb2, Ginsenoside Rg1, Ginseng extract (GSE), and Red ginseng extract (RGE) were obtained from Taepyungyang Chemical Co. Ltd. (Suwon, Korea). TPA and H_2O_2 were purchased from Sigma Chemical Co. (St. Louis, MO). GSE and RGE were dissolved in phosphate buffered saline (PBS) and the other compounds were dissolved in absolute alcohol.

2.2. Cell culture

WB-F344 rat liver epithelial cell lines were obtained from Dr. Trosko at Michigan State University (East Lansing, MI) and cultured in D-media supplemented with 5% fetal bovine serum (Gibco Laboratory, Grand Island, NY) and penicillin-streptomycin-neomycin mixure (Gibco Laboratory, Grand Island, NY). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO2. The cells were grown in 35-mm tissue culture plates (Corning Inc., Corning, NY) and the culture medium was changed every other day. Bioassays were conducted with confluent cultures (70-90%).

2.3. Cytotoxicity test

Cytotoxicity was determined by the neutral red uptake assay described by Borenfeud and Puerna [24]. Using a 96 well plate, WB-F344 rat liver epithelial cells (10^4 cells/200 μl/well) were incubated for 24 h at 37°C, followed by the treatment with test compounds such as Rg1 (0.1, 1, 10, 100, 1000 μg/ml), Rb2 (0.277, 2.77, 27.7, 277

μg/ml), GTE (0.01, 0.1, 1, 10, 100 μg/ml), EC (0.00278, 0.0278, 0.278, 2.78, 27.8 μg/ml), ECG (0.0072, 0.072, 0.72, 7.2, 72 μg/ml), EGCG (1, 10, 100, 1000 μg/ml), GSE (0.00005, 0.0005, 0.005, 0.05, 0.5 μg/ml) and RGE (0.05555, 0.5555, 5.555, 55.55, 555.5 μg/ml) including TPA (10 ng/ml), H_2O_2 (250 μM) as tumor promoters. The maximum stock concentration of each compound was determined by maximum soluble concentration in water. From maximum soluble concentration of each chemical in water, 10 fold serially diluted each chemical with sterilized-distilled water for cytotoxicity test. Cells were incubated for 24 hrs and rinsed three times with PBS, followed by adding 2 ml of fresh growth medium containing 100 ng neutral red (50 ng/ml). After another incubation for 2 h at 37°C, the extracellular neutral red was rinsed off with PBS and the cells were lysed with 200 μl of an aqueous solution containing 1% acetic acid and 50% ethanol. The lysed cells were measured for neutral red at a wavelength of 540 nm using an ELISA reader.

2.4. Scrape-loading dye transfer (SL/DT) assay

GJIC was assessed using the SL/DT technique described by El-Fouly [25]. The WB-F344 rat liver epithelial cells (10^5 cells/ml) in 35 mm cell culture flasks were incubated for 24 h at 37°C and test compounds at various concentrations (consecutively diluted by factor 2 or 10 from the maximum non-cytotoxic concentration of test compounds) were exposed to the WB cells for 4 h with TPA and 1 h with H_2O_2, respectively. The treated liver epithelial cells were rinsed carefully with PBS and then scraped and incubated with 2 ml of 0.05% Lucifer Yellow for 3 min. The cells were then washed with PBS and fixed with 4% paraformaldehyde. The distance that Lucifer Yellow had traveled through gap junctions was observed with an inverted fluorescent microscope.

2.5. Fluorescence recovery after photobleaching (FRAP) assay

The cells (cell confluency, 70-80%) incubated for 24 h in 35-mm cell culture flasks were treated with test compounds including TPA, H_2O_2, EC, and Rb2. In the combination with TPA, various concentrations of EC or Rb2 were treated for 4 h to assess their chemopreventive effects against the inhibition of GJIC by TPA. Whereas, in the combination with H_2O_2, various concentrations of EC or Rb2 were treated for 1 h to assess their chemopreventive effects against inhibition of GJIC by H_2O_2. The cells were rinsed with PBS and loaded with 7 μg/ml of 5,6-carboxyfluorescein diacetate in Ca^{2+}/Mg^{2+}-PBS for 12 min at 37°C [18]. The cells were then rinsed four to five times with 2 ml of Ca^{2+}/Mg^{2+}-PBS to remove extracellular dye and covered with 2 ml of Ca^{2+}/Mg^{2+}-PBS for the FRAP analysis. Dye transfer was monitored at room temperature using an Ultima

fluorescence spectrometer (Meridian Instruments, MI). Cells were randomly selected under a microscope with a 40 x objective lens and photobleached to 20 - 40% of their original fluorescence intensity. They were then examined for recovery of fluorescence after 4 min to obtain the rates of recovery, which were reported as %/min, where % = percent of prebleaching fluorescence. Fluorescence recovery was corrected for fluorescence lost in unbleached controls.

2.6. Western blot analysis for Cx43

WB-F344 rat epithelial cells, grown to the same confluency as in the FRAP assay, were treated with test compounds in the same way as the FRAP assay. Western blot analysis of Cx43 was performed as described previously [22]. Proteins were extracted from the liver epithelial cells, followed by treatment with 20% sodium dodecyl sulfate (SDS) containing 2 mM phenymethylsulfonyl fluoride (PMSF), 10 mM iodoacetoamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate, 5 mM sodium fluoride and then sonicated for three 10-s pulses using a probe sonicator (Branson, Danbury, CT). These cell lysates were centrifuged at 4°C for 30 min to remove insoluble materials. Protein concentration was determined with a DC protein assay kit (Bio-Rad Corp, Richmond, CA), after dilution of samples 1:5 with H_2O. Proteins were separated on 12.5% polyacrylamide gels and transferred to PVDF membranes at 20 V for 16 h. Cx 43 was detected using rabbit polyclonal anti-connexin 43 (Zymed Co., San Francisco, CA), followed by incubation with horseradish peroxidase-conjugated secondary antibody and detection with the ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, IL).

2.7. Immunofluorescent staining for Cx43

Localization of Cx43 in the cells was assessed by immunofluorescent staining as described previously [20]. After treatment with the test compounds, the cells were fixed in methanol/acetate (95:5) for 20 min and then non-specific binding sites were blocked with 10% normal goat serum in PBS for 1 h at room temperature. The cells were incubated overnight at 4°C with mouse monoclonal anti-Cx43 (Transduction Laboratories) diluted 1:500 in PBS and then for 1 h with a rhodamine-conjugated goat anti-mouse IgG (Fabí)2 fraction (Jackson Immuno Research Laboratories, PA) diluted 1:200 in PBS at room temperature. Fluorescent staining of Cx43 was viewed and photographed using an Ultima confocal microscope (Meridian Instrument, Okemos, MI).

3. Results

3.1. Cytotoxicity of green tea and Korea ginseng extracts or their components

To select appropriate doses of test compounds to be used in this study, we determined the maximum non-cytotoxic concentrations of test compounds including GSE, RGE, Rg1, Rb2, GTE, EC, ECG, and EGCG (Table 1). The various concentrations of these compounds were exposed to the WB cells for 24 h and the cytotoxicity of test compounds was assessed by the uptake of neutral red into viable cells. Cell viability did not change during incubation for 24 h, following the addition of test compounds. The maximum non-cytotoxic concentrations of GSE, RGE, Rg1, Rb2, GTE, EC, ECG, and EGCG were 500, 555.5, 1000, 277, 10, 27.8, 72, and 100 μg/ml, respectively (Table 1).

3.2. Effect of test compounds on GJIC

Based on the cytotoxicity results, various concentrations of these test compounds, which were represented by a consecutive dilution with factor 2 or 10 from the maximum non-cytotoxic concentration of test compounds.

Using the scrape loading/dye transfer technique, the effects of test compounds on GJIC were investigated. As the positive controls, TPA at the concentration of 10 ng/ml or H_2O_2 at the concentration of 250 μM was used. TPA or H_2O_2 markedly inhibited GJIC compared to the untreated negative control (Fig. 1). The photographic image after the treatment of EC or Rb2 was similar to that of the negative control (Fig. 1B & F). In the combined treatments of test compounds (GSE, RGE, Rg1, Rb2, GTE, EC, ECG, and EGCG) with TPA/H_2O_2, only EC and Rb2 effectively protected against the inhibition of GJIC induced by TPA or H_2O_2 (Fig. 1D & H). Therefore, the following experiments were performed on only EC and Rb2.

To confirm the protecting effect of EC and Rb2 on the inhibition of GJIC by TPA or H_2O_2, we also used the FRAP assay (Fig. 2). EC, at the concentration of 27.8 (g/ml, prevented the inhibition of GJIC by TPA to about 60% (Fig. 2A) and Rb2, at the concentration of 277.0 μg/ml, also prevented the inhibition of GJIC by H_2O_2 by about 60% (Fig. 2B). The effect of EC on GJIC was stronger in the TPA-treated cells than in the H_2O_2-treated cells, whereas the effect of Rb2 was opposite to that of EC. The protecting activities of EC and Rb2 were slightly dose-dependent at the concentrations used in this study (Fig. 2).

3.3. Effect of EC and Rb2 on Cx43 levels and phosphorylation

We examined the changes in Cx43 protein levels, as well as the degree of phosphorylation following the treatment of the cells with EC or Rb2 by Western blotting, using antibodies specific to Cx43 (Fig. 3). Three major bands (P0, P1 and P2) were detected in untreated WB cells. The mobility shift in bands from P0 to P1 or P2 indicates phosphorylation of Cx43. Cells treated with TPA displaced a mobility shift in bands to the higher molecular weight P2-band (Fig. 3, band 2). H_2O_2 also shifted the P0 band to P2 band (band 3). The treatment of EC with TPA decreased the phosphorylation ratio (P2/P0) of Cx43 by TPA (band 4). The treatment of Rb2 with H_2O_2 also affected the phosphorylation ratio of Cx43 by H_2O_2 (band 5).

3.4. Effect of EC and Rb2 on the distribution of Cx43

The distribution of the Cx43 protein in WB cells was examined by immunostaining after exposure to test compounds for 4 h against TPA and for 1 h against H_2O_2. The typical plaques of Cx43, which are functional when localized on the plasma membrane, were detected in the untreated control (Fig. 4A). The membrane Cx43 proteins were not seen in cells treated with TPA 10 ng/ml (Fig. 4B). The treatment of EC partially prevented the plaques of Cx43 that disappeared after the TPA treatment (Fig. 4C). The membrane Cx 43 proteins also disappeared in cells treated with 250 μM of H_2O_2 (Fig. 4D). The treatment of Rb2 partially prevented the plaques of Cx43 from disappearing after treatment with the H_2O_2 (Fig. 4E).

4. Discussion

Epidemiological and animal studies have suggested that the intake of ginseng is strongly associated with the low incidence of cancers [10,11]. Its components, such as ginsenosides Rb1, Rb2 and Rg1, may be responsible for its anticarcinogenic effect [8,12,15]. In this study, only Rb2 among GSE, RGE, Rb2, and Rg1 , showed a protective effect against GJIC inhibition by the tumor promoters, TPA and H_2O_2, indicating that Rb2 has an anticarcinogenic activity during the promotional stage of carcinogenesis.

Green tea contains polyphenolic antioxidants that have shown anticarcinogenic properties in animals and in vitro experimental studies [2-5]. The main polyphenolic components are catechins including EC, EGC, ECG, and EGCG. These catechins exert

growth inhibition and apoptosis in a variety of tumor cell lines, but the effects vary from the cell lines used in the studies [3,26,27]. Recently, most studies on the anticarcinogenic effect of green tea catechins have been focused on EGCG rather than the others. Although the anticarcinogenic effect of EGCG have been reported in numerous studies, we reported in this study that only EC among the green tea catechins showed an anticarcinogenic activity in the rat liver epithelial cells by preventing the inhibition of GJIC by the promoters, TPA and H_2O_2.

GJIC has been implicated in the regulation of cellular growth and its inhibition to the expression of the neoplastic phenotype [28]. A reversible disruption of GJIC plays a role during the tumor promotion phase of carcinogenesis and a stable down-regulation of GJIC leads to the conversion of a premalignant cell to an invasive and metastatic cancer cell [16]. Therefore, GJIC is frequently reduced in neoplastic and carcinogen-treated cells. In this study, we used a non-tumorigenic WB-F344 rat liver epithelial cell as a model system to screen and verify the anticarcinogenic activity of test compounds. The normal WB cells are known to express high levels of Cx43, to form numerous gap junctions, and to have high percentage of communicating cells (95-100%) [29]. However, the neoplastic transformants of these cells which were generated by ras and neu oncogene transfection form few gap junctions and have low incidences of communication (20-25%) [29]. The treatment of TPA and H_2O_2, which are known to be cancer promoters, significantly inhibited GJIC as determined by SL/DT assay, FRAP assay and immunostaining of Cx43, thereby resulting in the simulation of the transformed WB cells. The prevention of the inhibition of GJIC by EC, at the concentrations of 27.8 ± 3.47 μg/ml and Rb2 at the concentrations of 277 ± 0.27 μg/ml might imply that the EC and Rb2 can be used as anticarcinogenic agents acting on promotional phase.

The phosphorylation state of gap junction proteins seems to plays an important role in the gating of gap junction channels [30]. Many, but not all, exogenous and endogenous chemicals that regulate GJIC also alter the phosphorylation state of the connexins, which are the proteins that form the hexametric connexin doublets that constitutes the gap between cells [31]. In this study, the inhibition of GJIC by TPA in F344 rat liver epithelial cells was correlated with the hyperphosphorylation of Cx 43, as measured by mobility shifts (P0 --> P2) of the Western blot bands of Cx43. The TPA-induced hyperphosphorylation of Cx43 might be a result of PKC activation, in which PKC is translocated from the cytosolic to particulate fraction of the cell [23,32]. The GJIC is restored concomitantly with the inactivation of PKC: these events were shown to precede the decrease in the mRNA and

protein levels [16]. In concert with the hyperphosphorylation by PKC, Cx43 was reported to internalize into the cytoplasm, resulting in the loss of GJIC. In this study, Cx43 plaques in the membrane of WB F344 rat liver epithelial cells disappeared after treatment of TPA as determined by immunostaining. However, the simultaneous treatment of EC prevented the total disappearance of Cx43 plaques in the cell membranes, resulting in the reduction of inhibition of GJIC. Although EC prevented much of the hyper phosphorylation of Cx43 in the WB cells, a functional change of the Cx43 can not be excluded.

The carcinogenic effect of oxidative stress has primarily focused on the genotoxicity of reactive oxygen species [33]. However, active oxygen is known to play a significant role in the promotion phase of cancer [34]. The promotion phase of cancer is a consequence of epigenetic events involving signal transduction and GJIC [35,36]. Promotion is a reversible or interruptible step in carcinogenesis. Therefore the underlying mechanism of tumor promotion, such as the hypothesized down-regulation of GJIC, should also be reversible [16]. Similarly to TPA, H_2O_2, at the concentration of 250 μM, also caused a shift in the Cx43 bands to a hyperphosphorylated state, resulting in the inhibition of GJIC. However, the mechanism by H_2O_2 on hyperphosphorylation of Cx43 was different from that by TPA in WB-F344 liver epithelial cells [23]. In this study, Rb2 prevented the H_2O_2-induced hyperphosphorylation of Cx43 and it also prevented significantly H_2O_2-inhibition of GJIC. H_2O_2 inhibited GJIC is restored after the removal H_2O_2 in WB liver epithelial cells [23]. The preventive effects of Rb2 on GJIC inhibition by H_2O_2 might be involved in inactivation of the H_2O_2. However, other mechanisms can not be excluded.

In conclusion, the inhibition of GJIC and the disappearance of Cx43 induced by TPA or H_2O_2 in WB-F344 rat liver epithelial cells were prevented with the simultaneous treatment of Rb2 or EC. These results suggest that the ginsenoside Rb2 from Korean red ginseng and the epicatechin from green tea may have a cancer preventive effect during promotion phase by the up-regulation of GJIC.

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Table 1. Maximum non-cytotoxic concentrations of green tea, Korea ginseng, and their components

Test compounds	Maximum non-cytotoxic concentration[1] (μg/ml)
Ginseng extract (GSE)	500.0
Red ginseng extract (RGE)	555.5
Ginsenoside Rg1	1000.0
Ginsenoside Rb2	277.0
Green tea extract (GTE)	10.0
Epicatechin (EC)	27.8
Epicatechin gallate (ECG)	72.0
Epigallocatechin gallate (EGCG)	100.0

[1]Maximum non-cytotoxic concentrations were determined by the neutral red uptake assay.