Effect of genistein on the bioavailability and intestinal cancer chemopreventive activity of (-)-epigallocatechin-3-gallate

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Previously, we have reported that the absolute bioavailability of EGCG in CF-1 mice and Sprague–Dawley rats is 26.5 and 1.6%, respectively (3,4). EGCG undergoes methylation, glucuronidation and sulfation in vivo and is largely present as the glucuronide in the plasma of treated mice (1,5,6). Studies in our laboratory and others have suggested that EGCG might also be a substrate for multidrug resistance-related protein (MRP) 1 and 2 (7–9). Cotreatment of Madin–Darby canine kidney cells overexpressing human MRP1 and MRP2 with EGCG and selective inhibitors of MRP1 and MRP2 resulted in a 10-fold increase in the cytosolic concentration of EGCG in both cases (8). Zhang et al. (9) have reported that cotreatment of Caco-2 cells with green tea catechins, including EGCG, and MK571, an inhibitor of MRP, resulted in 5.7-fold decrease in basolateral-to-apical flux of EGCG compared with cells treated only with EGCG. Modulation of the factors affecting EGCG bioavailability might increase plasma and tissue levels of this compound and increase its cancer-preventive activity.

Genistein (Figure 1), an isoflavone from soybeans (Glycine max, Fabaceae), is common in the diet and has been studied for a number of potential health effects including cancer prevention and anti-inflammatory activity (10–12). Genistein has also been shown to modulate MRP-mediated efflux (13,14). We have previously reported that cotreatment of MRP-overexpressing cells with [3H]-EGCG and genistein or related isoflavones results in increased cell-associated radioactivity compared with treatment with EGCG alone (8). This measurement includes both membrane-bound and intracellular EGCG and its metabolites: the effect of genistein on the intracellular levels of EGCG, strictly defined, has not been previously reported.

The interaction between EGCG and genistein is also of interest due to the likelihood of co-occurrence in the diet. Whereas laboratory studies have shown that EGCG and green tea prevent cancer, epidemiological studies have been less conclusive (2,15–17). One possible confounding variable is differences in diet among individuals and between populations. Other dietary factors could influence the bioavailability and biological activity of EGCG. Careful studies are needed to understand these potential interactions.

The effects of genistein on EGCG cancer chemopreventive effects and bioavailability have not been previously reported. In the present study, we hypothesized that genistein could increase the cytosolic concentration and growth inhibitory activity of EGCG in vitro and increase the plasma and tissue levels, as well as the cancer-preventive activity, of EGCG in vivo. Herein, we report the results of our studies.

Materials and methods

Chemicals
EGCG (100% pure) was provided by Mitsui Norin Co., Ltd (Fujieda City, Japan). β-D-glucuronidase (G-7896, EC 3.2.1.31, from Escherichia coli with 9 × 10^6 U/g solid) and sulfatase (S-9754, EC 3.1.6.1, from Abalone entrails with 2.3 × 10^5 U/g solid) purchased from Sigma Chemical Co. (St Louis, MO). Genistein (>95% pure) was purchased from AB Chem Technologies (Franklin Park, NJ). All other reagents were of the highest grade commercially available. Dosing solutions of EGCG and genistein were prepared in 0.9% NaCl. For analytical purposes, a standard stock solution of EGCG, epigallocatechin, epicatechin and epicatechin-3-gallate (10 μg/mg each) was prepared in 11.4 mM ascorbic acid-0.13 mM ethylenediaminetetraacetic acid (pH 3.8) and stored at ~80°C. Stock solutions (20 or 100 mM) of genistein and EGCG for cell culture were prepared in dimethyl sulfoxide (DMSO) and stored at ~80°C. The final concentration of DMSO in all cell culture experiments was 0.1%. Control cells were grown in the same concentration of DMSO.

Mice and diets
All experiments involving mice were approved by the Institutional Animal Care and Use Committee at Rutgers University (Piscataway, NJ). Male CF-1 mice (25 g) were purchased from Charles River Laboratories (Wilmington, MA). Male and female CD-1 mice (Charles River) were obtained from 2 to 6 weeks of age. Mice were housed in the animal care facility at the National Rice Research Institute, Pusa Campus, New Delhi, India. The animals were housed under standard conditions with a 12-h light/dark cycle and ad libitum access to a commercial diet and water.

Introduction

Tea (Camellia sinensis) is second only to water in terms of worldwide popularity as a beverage. (-)-Epigallocatechin-3-gallate (EGCG, Figure 1) is the major catechin component of green tea and is believed to be a major active constituent. Studies with animal models have shown that green tea and EGCG have preventive activity against cancer of the oral cavity, esophagus, stomach, intestine, colon, liver, lung, prostate, skin and other sites (1). Studies with human cancer cell lines have shown that EGCG possesses a number of activities related to cancer prevention such as inhibition of mitogen-activated protein kinase cascades, DNA methyltransferase, epidermal growth factor receptor signaling and others. It is not known, however, whether these potential mechanisms of action occur in animals or in humans because of the limited oral bioavailability of EGCG (1,2).

Abbreviations: APC, adenomatous polyposis coli; DMSO, dimethyl sulfoxide; EGCG, (-)-epigallocatechin-3-gallate; i.g., intragastric; MRP, multidrug resistance-related protein; t1/2, half-life.
Intestinal tumorigenesis studies in APCmin/+ NaCl and frozen at −80°C for later analysis. The small intestine and liver were collected, washed in 0.9% bicarbonate and 0.1% ethylenediaminetetraacetic acid) and stored at −80°C. Plasma was combined with 0.1 volume of ascorbate preservative (20% ascorbic acid) and sonicated. The resulting solution was centrifuged at 16 000 g for 10 min. The supernatant was combined with an equal volume of ice-cold methanol and the supernatant was analyzed by high-performance liquid chromatography.

**Fig. 1.** Structures of EGCG and genistein.

MA and maintained on AIN76A diet for 1 week prior to the start of experiments. Male adenomatous polyposis coli (APCmin/+ mice) were derived from a colony housed at the Susan L. Cullman Laboratory for Cancer Research at Rutgers University. Mice were genotyped at 3 weeks of age as described previously (18). All mice were housed 10 per cage and maintained in air-conditioned quarters with a room temperature of 20 ± 2°C, a relative humidity of 50 ± 10% and an alternating 12 h light–dark cycle. All mice had ad libitum access to food and water. All experimental and control diets were obtained from Research Diets (New Brunswick, NJ). AIN76A, a soy-free, defined diet, was used as the basal diet (19).

**Cell culture and treatment**

HT-29 human colon cancer cells (American Type Culture Collection, Rockville, MD) were maintained at subconfluence in McCoy’s 5A medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 95% humidity and 5% CO₂.

Cytosolic levels of EGCG in the presence or absence of genistein were determined as previously reported (8). In brief, cells were allowed to grow to 70–90% confluence in six-well plates. The medium was replaced with fresh, serum-complete medium containing 50 µM EGCG with or without genistein (0–20 µM). Medium also contained 5 U/ml superoxide dismutase to stabilize EGCG. The cells were incubated for 1.5–24 h at 37°C, after which the medium was removed, the cells were washed two times with cold phosphate-buffered saline, 2% ascorbic acid was added to each well and the cells were scraped and sonicated. The resulting solution was centrifuged at 16 000 g for 10 min. The supernatant was combined with an equal volume of ice-cold methanol and centrifuged for 10 min at 16 000 g to precipitate the protein. The resulting supernatant was analyzed by high-performance liquid chromatography.

Cytosolic EGCG was normalized to cytosolic protein concentration.

To determine the growth inhibitory activity of EGCG in combination with genistein, HT-29 cells were plated in 96-well plates (12 × 10³ cells/cm²) and allowed to attach for 24 h. The medium was replaced with fresh, serum-free medium containing no EGCG or genistein and the relative number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to as-

**Results**

**Effect of genistein on EGCG uptake and growth inhibitory activity**

We examined the ability of genistein to increase the intracellular concentrations of EGCG in HT-29 cells. Cotreatment of cells with EGCG (50 µM) and genistein (0–20 µM) resulted in an increase in the intracellular levels of EGCG after 1.5, 3 and 24 h (Figure 2A).

To determine if this genistein-mediated increase in cytosolic EGCG resulted in enhanced growth inhibitory activity, we used the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay to assess cell viability after 24 h treatment. Genistein dose dependently increased the growth inhibitory activity of EGCG. The inhibitory concentration 50% of EGCG was 43, 32 and 25 µM in the presence of 0, 5 and 20 µM genistein, respectively (Figure 2B). At 50 µM EGCG, the effect of genistein is lost. Genistein alone, at the concentrations used in the present study, did not significantly affect the number of viable cells (Figure 2B).

**Effect of genistein on the bioavailability of EGCG in mice**

Treatment of male CF-1 mice with a single dose of EGCG (75 mg/kg, i.g.) in combination with genistein (200 mg/kg, i.g.) resulted in an increase in the AUC₀⁻→∞ and t₁/₂, but not the Cmax of total EGCG in the plasma compared with treatment with EGCG only (Figure 3). The t₁/₂ for EGCG was 111.5 ± 23.4 and 148.7 ± 16.4 min (P < 0.05) in the absence or presence of genistein, respectively. The AUC₀⁻→∞ for EGCG in the plasma was 125.8 ± 26.4 and 183.9 ± 20.2 µg/ml × min (P < 0.05) in the absence or presence of genistein, respectively. The bioavailability of EGCG in the small intestine was also increased by cotreatment with genistein (Figure 3). The AUC₀⁻→∞ max was 1431.7 ± 585.6 µg/g × min and 6727.5 ± 1823.2 µg/g × min (P < 0.05) in the absence of presence of genistein, respectively. The Cmax and t₁/₂ of EGCG increased from 16.8 ± 6.1 to 34.4 ± 9.4 µg/g (P < 0.05) and from 80.8 ± 33.0 to 115.0 ± 31.2 min, respectively, in the presence of genistein. The change in EGCG t₁/₂ in the small intestine was not statistically significant. There was no significant effect of genistein on the bioavailability of EGCG in the liver (Figure 3).

**Effect of genistein on the chemopreventive activity of EGCG in APCmin/+ mice**

We conducted a chemoprevention study of the combination of EGCG and genistein in the APCmin/+ mouse model of intestinal tumorigenesis to determine if the effects of genistein on EGCG bioavailability
and in vitro biological activity translated to improved cancer chemoprevention in vivo. There were minor differences in food intake among the treatment groups: mice treated with genistein diet (either alone or in combination with EGCG) consumed ~20% less diet than did mice fed AIN76A diet (control- and EGCG-treated mice, Figure 4A). Consequently, the mice fed genistein diet had significantly lower final body weight compared with mice fed AIN76A (17% decrease, Figure 4B). There was no significant difference in fluid consumption between mice treated with distilled water and those treated with 0.01% EGCG (Figure 4A).

Unexpectedly, treatment of male APC min/+ mice with 0.01% EGCG in combination with 0.2% dietary genistein for 9 weeks resulted in significantly higher tumor multiplicity compared with control mice or treatment with either agent alone (Table I). The largest increase was in tumors ≥2 mm in diameter where combination treatment increased tumor multiplicity by 4.3-fold compared with control animals. In contrast, treatment with 0.01% EGCG appeared to decrease the number of tumors >1 mm in both the medial and distal small intestine, although the effect was not statistically significant. Treatment with genistein had no clear effect on tumors at any location in the small intestine. Histopathological analysis showed, as expected, that the tumors present in the mice were adenomas and confirmed the gross trends in tumor multiplicity (Figure 4C). In order to confirm that genistein enhanced the bioavailability of EGCG in the APC min/+ mouse, we measured the plasma levels of EGCG in both EGCG and EGCG plus genistein-treated mice (Figure 5). We found, as expected, that plasma levels of EGCG were 23% higher (P < 0.01) in the mice cotreated with genistein compared with mice treated with EGCG only.

**Discussion**

In the present study, we determined the effect of the soy isoflavone, genistein, on the in vitro and in vivo bioavailability and cancer-preventive activity of the tea catechin, EGCG. Previous cell line studies in our laboratory and others have suggested that EGCG is a substrate for MRP and that cotreatment of MRP1- and 2-overexpressing cells with EGCG and selective inhibitors of those efflux pumps resulted in increased cell-associated and cytosolic levels of EGCG (7,8). Genistein has been reported previously to modulate the activity of MRPs (13,14). Our present results confirmed this phenomenon and additionally extended our previous results showing that
cotreatment of MRP-overexpressing cells with [3H]-EGCG and genistein results in increased cell-associated radioactivity (8). Whereas these previous data included both membrane-bound and cytosolic EGCG and its metabolites, the present results represent a precise measurement of cytosolic EGCG. Many of the proposed targets of EGCG are cytosolic (e.g. mitogen-activated protein kinases) or nuclear (e.g. DNA methyltransferase) and precise determinations of the cytosolic levels of EGCG are important for interpreting biological effects observed in vitro (10,22).

The effect of genistein on the growth inhibitory activity of EGCG was also investigated in HT-29 cells. To our knowledge, this is the first report to demonstrate that the dose-dependent increases in cytosolic EGCG induced by genistein are associated with similar dose-dependent increases in EGCG-mediated growth inhibition of human cancer cells. The effect of genistein on EGCG-mediated growth inhibition is lost when the concentration of EGCG reaches 50 μM. This could indicate that maximal growth inhibition by EGCG is reached at 50 μM and that increases in cytosolic EGCG beyond this do not affect cell growth. The genistein-mediated increase in growth inhibitory activity observed in the present experiments was not equivalent in magnitude to the increase in cytosolic concentration of EGCG. This discrepancy appears to be due to the fact that although the effect of genistein on cytosolic EGCG is very strong, the magnitude of this effect decreases as a function of time. Since the 3,4,5-trimethylthiazol-2-yl]-2,5-diphenyloxazolium bromide assay is a composite of the effect of EGCG and genistein over the entire course of the 24 h experiment, the results appear to reflect this decreasing effect of genistein on cell uptake. The decreasing effect of genistein on cytosolic EGCG may be the result of genistein and/or...
EGCG undergoing biotransformation that is independent of the MRPs. Additionally, we cannot rule out that genistein is affecting some target other than, or in addition to, MRP. 

The present study also demonstrates that our present and previous data concerning the effects of genistein on EGCG in vitro are recapitulated following oral dosing of EGCG and genistein to mice (8). Although MRP-mediated efflux is not the only factor limiting EGCG bioavailability in vivo, it appears that inhibition of MRP-mediated efflux can improve the plasma and small intestinal pharmacokinetics of EGCG. It is interesting that genistein increased the levels of EGCG in the small intestine and plasma, but not in the liver. This may suggest that other factors play a role in determining the levels of EGCG in the liver: these factors may include additional efflux pumps or more efficient metabolism of EGCG that is not overcome by the addition of genistein. Further studies are needed to clarify these tissue differences.

Based on our cell line and mouse bioavailability studies, we expected to see an enhancement of the antitumorogenic activity of EGCG in the APCmin/+ mouse. We were, therefore, surprised to observe a dramatic increase in tumor multiplicity in mice treated with the combination of EGCG and genistein compared with controls. The effect on tumor multiplicity observed in mice treated with EGCG or genistein as single agents was as expected. EGCG had a non-significant inhibitory effect, whereas genistein, which has previously been shown ineffective in this model, had no effect (18,23–26). The reasons for the increase in tumor multiplicity induced by the combination of 0.01% EGCG and 0.2% genistein remain unclear. Although genistein was originally identified as a chemopreventive agent, more recent studies have shown that genistein can enhance tumorigenesis in a number of models including gastrointestinal cancers. Rao et al. (27) have previously reported that genistein increased the multiplicity of total and non-invasive colon adenocarcinomas in azoxymethane-treated rats by 50.3 and 48.5%, respectively. These authors suggested that the effect may be due to genistein-mediated increases in prostaglandin E2 levels, but that possibility was not tested experimentally. Such an effect is not expected in the present model since EGCG has been shown previously to inhibit the production of prostaglandin E2 both in vitro and in vivo models of colon cancer.

It has also been recently reported that dietary treatment with genistein enhances β-catenin accumulation in colonic crypts of 1,7-dimethylhydrazine-treated rats (28). Again, the underlying mechanisms remain unclear. Genistein has also been shown to enhance N-nitroso-N-methylurea-induced mammary tumorigenesis in both normal and ovariectomized rats (29). These effects seemed to result from the estrogenic activity of genistein.

The mechanism responsible for the enhanced tumorigenesis due to the treatment with the combination of EGCG and genistein remains to be further studied. Similarly, careful dose-response studies are needed to determine if different dose combinations have cancer-preventive effects, whereas others promote tumorigenesis.

From a risk assessment perspective, the present data should be interpreted carefully. Based on allometric scaling, the daily dose of EGCG and genistein used in the present study are 50 and 667 mg/d, respectively (30). These values assume a daily calorie requirement of 12 and 2000 kcal for mice and humans, respectively. Whereas this dose of EGCG is easily achievable through dietary consumption of green tea, the dose of genistein exceeds daily dietary intake by 10- to 50-fold even in Asian countries such as Japan which have a high per capita intake of soy products (31,32). A dose of 667 mg/d genistein is, however, achievable through the use of dietary supplements, particularly if the recommended dose of such supplements is exceeded (33,34). Although there are no reports of increased risk of intestinal cancer associated with intake of genistein and EGCG, the emerging animal data on genistein as a single agent warrants further investigation of the potential procarcinogenic effects of genistein alone or in combination with EGCG.

In summary, the present study for the first time demonstrates that genistein can modulate the cytosolic levels and growth inhibitory activities of EGCG against human colon cancer cells. Moreover, we report for the first time that acute treatment with genistein in combination with EGCG results in higher plasma and intestinal levels of EGCG in mice compared with treatment with EGCG only. Whereas these data indicate that co-treatment with both EGCG and genistein should have enhanced chemopreventive activity against intestinal tumorigenesis, we found exactly the opposite effects. Although the mechanisms for this tumor enhancement remain unclear, these studies reiterate the need for careful in vivo studies to assess the impact of any new chemopreventive regimen, even those based on well-studied dietary components.

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