Green tea selectively targets initial stages of intestinal carcinogenesis in the AOM-ApcMin mouse model

Ala Y.Issa, Suresh R.Volate, Stephanie J.Muga1, Daniela Nitcheva2, Theresa Smith3 and Michael J.Wargovich1,∗

Department of Pathology, Microbiology and Immunology, School of Medicine, University of South Carolina, Columbia, SC 29203, USA, 1Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, SC 29245, USA and 2Department of Epidemiology and Biostatistics, School of Public Health and 3Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of Columbia, SC 29208, USA

∗To whom correspondence should be addressed at Cancer Chemoprevention Program, Hollings Cancer Center, 86 Jonathan Lucas Street, PO Box 250955, Charleston, SC 29245, USA. Tel: +843 792 7604; Fax: +843 792 3200; Email: wargovic@musc.edu

One of the liabilities of the ApcMin mouse as a model for colon cancer is its lack of a robust tumor response in the large bowel. In our protocol, we treated the ApcMin mouse with azoxymethane, a colon-selective carcinogen. This protocol induced a 4-fold increase in the number of colon tumors. We utilized this protocol to investigate the possible mechanisms of inhibition of colorectal carcinogenesis by green tea. Mice received water or a 0.6% (w/v) solution of green tea as the only source of beverage. Green tea treatment commenced at the eighth week of age and lasted for either 4 or 8 weeks. Green tea significantly inhibited the formation of new adenomas, but was ineffective against larger tumors. Mechanistically, we investigated the effects of green tea on the expression of biomarkers involved in colon carcinogenesis. Western blotting analysis showed that green tea decreased the total levels of the early carcinogenesis biomarker β-catenin and its downstream target cyclin D1. In contrast, the expression of COX-2 was not altered. Immunohistochemical analysis showed that green tea inhibited the formation of adenomas overexpressing β-catenin and cyclin D1, but did not reduce the number of COX-2-expressing adenomas. Our results suggest that green tea specifically targets initial stages of colon carcinogenesis; the time of administration of green tea is pivotal for effective chemoprevention. Beverage levels of green tea do not inhibit the progress of any large adenomas or adenocarcinomas existing prior to the tea administration.

Introduction

Green tea is one of the most common beverages worldwide. The primary active ingredients in green tea are a group of flavan-3-ol polyphenols known as catechins. The major tea catechins are (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epicatechin with EGCG comprising >60% of the total catechins (1). In brewed green tea, the water-extractable material accounts for about one-third of the tea leaves in dry weight and contains 30 to 40% catechins, 3% flavonols (quercetin, kampferol and rutin), 3–6% caffeine and a mixture of other constituents (2). Tea catechins, particularly EGCG, have been shown to possess a variety of pharmacological effects. These include antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial, antiangiogenic and hypcholesterolemic effects (3–8). In addition, a wealth of epidemiological data suggested an inverse correlation between tea consumption and the risk of cancer (9). Inhibition of colon carcinogenesis by green tea catechins has been extensively investigated (9–15). Colon carcinogenesis is a promising target for dietary intervention since polyphenols such as tea catechins can reach concentrations that far exceed their concentrations in other organs of the body. For colon cancer, the cancer chemopreventive activity of green tea has been attributed mostly to its EGCG component. Several mechanisms of action for EGCG have been reported in a variety of in vivo and in vitro studies (5,16–21). These include antioxidant properties, modulation of cell signaling pathways, modulation of gene expression and modulation of carcinogen metabolism. The overwhelming majority of these studies, however, used EGCG concentrations that were several fold higher that what could be achieved physiologically, even in organs that retain higher levels of catechins such as the colon and small intestine (22). In addition, the contribution of the other tea catechins to the biological activities of green tea was often not investigated. Further controversy rose due to a contradictory epidemiological evidence, the lack of an animal model that genetically and phenotypically truly represent the human colon cancer and variations in the methodology of the published studies (22). Consequently, the exact mechanisms through which physiological levels of green tea might inhibit colon carcinogenesis are still poorly understood.

We undertook the current study in order to investigate the possible chemopreventive effects of a low physiological concentration of green tea in vivo. The objective was to study the effects of green tea on pre-existing tumors and new tumors that would develop during the tea treatment. To achieve this aim, we needed to modify the ApcMin mouse model by introducing a colon-specific carcinogenic to maximize tumor yield. Although the majority of tumors still developed in the small intestine, we were able to gain a 4-fold increase in colon tumors thus making this a more attractive model for colon cancer chemoprevention studies. Our results show that green tea significantly reduced the development of new tumors but did not inhibit the pre-existing ones. The data suggest that green tea selectively targets initial stages of colon carcinogenesis.

Materials and methods

Animals and treatments

A total of 150 mice were used in the study; 120 male and female C57BL/6J-ApcMin (ApcMin) mice and 30 wild-type C57BL/6J (B6). The mice were housed in an animal research facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) at the University of South Carolina. Treatment protocol was approved by the Institutional Animal Care and Use Committee at the University of South Carolina. The mice were kept on a light/dark (12/12 h) cycle, 20–24°C and 50% humidity. Mice were weaned at 3 weeks of age and fed AIN76A diet ad libitum thereafter. The mice were split into three groups based on the type of analysis conducted on each group (Figure 1A). The groups were further divided into five equal subgroups based on the green tea or azoxymethane (AOM) treatments (Figure 1A). AOM (purchased from Ash Stevens, Detroit, MI) was diluted to a final concentration of 8 mg/kg body wt with 0.9% saline solution on the day of injection and administered to the mice intraperitoneally once a week for 3 weeks (Figure 1B). Green tea was well characterized (Table I) and a generous gift from Dr C.S.Yang (Rutgers University, NJ). The green tea stock was received as a green tea powder and was analyzed by high-performance liquid chromatography (Table I) to determine its composition. The same stock was used throughout the experiment to avoid any variations in the green tea content. A fresh solution of 0.6% (w/v) was prepared every other day by dissolving the green tea powder in the proper volume of ultrapure hot water. The powder completely dissolved in the hot water and no filtration was necessary. The tea solution was then set to cool to room temperature in the dark, poured into light-protective bottles and served to the mice as the only source of beverage. The tea treatment commenced 1 week after the last AOM or saline injection and lasted for 4–8 weeks. All the mice were killed at 16 weeks of age by cervical dislocation except for the group dedicated to the immunohistochemical analyses. The mice in the latter group were killed after 12 weeks (4 weeks of green tea treatment) in order to study the effects of green tea on earlier neoplastic lesions. The mice were closely monitored and weighed weekly. Any mouse that lost >10% of its original body weight was excluded from the experiment.
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Tumor count and size comparison

Colons and small intestines were removed, flushed with ice-cold phosphate-buffered saline, slit open along the longitudinal median and fixed in 10% buffered formalin for 24 h. The fixed tissues were stained with 0.2% methylene blue (Sigma-Aldrich, St. Louis, MO) dissolved in phosphate-buffered saline. Tumors were scored at ×30 magnification using a Nikon dissecting microscope with a fiber optic light source to illuminate the tissues and a calibration scale to determine the tumor size. Tumors with diameters ≤1 mm were classified as small tumors, whereas tumors that exceeded 1 mm in diameter were classified as large tumors. All the tumors were scored by the same investigator who was blind to the treatment groups. No tumors were found in any of the B6 mice. A minimum of a 100 different colon and small intestinal tumors were randomly sampled and further analyzed to determine the histological type.

Immunohistochemical analysis

The colons and small intestines were fixed as described above, but Swiss-rolled before fixation. Fixed tissues were paraffin embedded, cut into 5 μm sections, mounted on slides and processed for immunohistochemistry as described previously (23). Sections were incubated for 45 min with one of the following primary antibodies: Mouse monoclonal anti-β-catenin antibody (BD Transduction Laboratories, Lexington, KY) diluted 1:300; Rabbit monoclonal anti-cyclin D1 (SP4) antibody (Lab Vision/Neomarkers, Fremont, CA) diluted 1:100 and Mouse polyclonal anti-COX-2 antibody (Caymen Chemical, Ann Arbor, MI) diluted 1:400. Blocking of the sections and detection of the anti-β-catenin and anti-cyclin D1 antibodies were by ACUTY™ polymer Detection Kit, which consisted of a special polymer for pre- and post-primary antibody incubation (Signet Laboratories, Dedham, MA) according to the manufacturer’s instructions. Detection of the anti-COX-2 antibody was by CSA III Biotin-Free Catalyzed Signal Amplification System (DakoCytomation, Carpinteria, CA) according to the manufacturer’s instructions. Non-specific binding was blocked by incubating the sections with normal goat serum (BioGenex, San Ramon, CA) for 20 min at room temperature. Quantitation of the staining was carried out by dividing the lesions with abnormal staining into three categories (Table II). Small lesions consisted of 1–5 crypts. Medium lesions consisted of 6–10 crypts. Large lesions consisted of >10 crypts. Epithelial cells in these lesions showed overexpression of β-catenin and cyclin D1. In contrast, COX-2 was only expressed in stromal cells of medium and large lesions and it was not expressed in the epithelial cells. Therefore, quantitation of the COX-2 staining was carried out by counting the number of lesions expressing COX-2 without further classification. All the lesions were scored at ×100 magnification by the same investigator who was blind to the treatment groups. The entire small intestines and colons were scored for lesions in each mouse and the average number of each class of lesions per animal was determined.

Western blotting

Mucosal layers of colons and small intestines from 50 mice (10 per subgroup as described above) were scraped and flash frozen in liquid nitrogen immediately after killing and then stored at −80°C. The scrapings were later thawed on ice, total proteins were isolated and their concentrations were determined as described previously (24). Western blotting analysis was carried out as described previously (24), with 50 μg of total proteins loaded into each lane. The primary antibodies for β-catenin, cyclin D1 and COX-2 were the same as in the previous section, but each was used at a dilution of 1:500. The blots were visualized by incubating the polyvinylidene difluoride membranes with the ECL plus kit (Amersham Biosciences, Piscataway, NJ), according to the manufacturer’s instructions; blots were scanned with a Storm™ 860 gel and blot imaging system (Amersham Biosciences). Densitometry analysis and quantitation of the antibody-associated protein bands were performed using Quantity One™ software (Bio-Rad, Hercules, CA). At least three mice per subgroup were analyzed. The data are normalized to the β-actin loading control. Error bars represent standard error of the mean.

Statistical analysis

All the data were analyzed using SigmaStat V3.0 (SPSS, Chicago, IL) and SAS V8.2e (SAS Institute, Cary, NC) softwares. Descriptive statistics were used to identify the distribution of the data. A two-way analysis of variance test was used to compare the treatment groups in the presence or absence of AOM or green tea. For data that failed the normality test, generalized linear models were used to compare the groups. In this case, the data were modeled as a Poisson distribution when appropriate. Alternatively, a negative binomial distribution model was followed when the data exhibited an overdispersion. The analyses included testing for the main effects of AOM or green tea in the treatment groups and for interactions between the treatments. The data were considered very significant if $P < 0.005$, significant if $0.005 < P < 0.05$, marginally significant if $0.05 \leq P < 0.1$ and insignificant if $P \geq 0.1$.

Results

AOM treatment significantly and selectively augments colon tumorigenesis in the ApcMin mouse

The first objective of the current study was to increase the number of colon tumors in the ApcMin mouse model using a colon-selective carcinogen. Different doses and treatment protocols of AOM injection were tested. An AOM dose of 8 mg/kg body wt, injected intraperitoneally once a week for 3 weeks, was sufficient to significantly induce colon tumors and yet was well tolerated by the mice. All the tumors in the small intestine and colon were scored for each mouse after fixing the tissues and staining with methylene blue as described in the Materials and methods (Figure 2A). A 4-fold increase in the number of colon tumors (0.5–2 colon tumors per mouse, $P < 0.005$) was observed with the AOM treatment compared with saline-injected mice.
Figure 2B). The induction of tumors by AOM was selective to the colon as reflected by only a slight induction (an 18% increase, \( P > 0.1 \)) of the total number of Apc\textsuperscript{Min} tumors (tumors in the colons and the small intestines) per mouse. AOM significantly increased the numbers of both small and large tumors in the colon.

Green tea inhibits the formation of Apc\textsuperscript{Min} tumors and selectively targets small tumors

Green tea treatment (20 mice scored, 10 per treatment group) induced a 50% reduction in colon tumors (1.8–0.9 tumors per mouse, \( P < 0.05 \)) compared with water-treated mice (20 mice scored). Green tea treatment caused ~20% reduction in the total number of Apc\textsuperscript{Min} tumors evolving in both the colon and small intestine (41–33 tumors per mouse, \( P < 0.1 \)) (Figure 3A). The inhibition of colon tumor development by green tea was mainly due to a reduction in the number of small tumors with only slight effect on large tumors (0.7–0.1 tumors per mouse, \( P < 0.05 \) and 1.1–0.8, \( P = 0.2 \), respectively) (Figure 3B). Likewise, green tea induced a statistically significant reduction in the number of total Apc\textsuperscript{Min} small tumors in the colons and small intestines with little effects on the large ones (17.3–12.5 tumors per mouse, \( P < 0.05 \), and 23.4–20.8, \( P = 0.4 \), respectively) (Figure 3C).

Green tea selectively targets earlier stages of Apc\textsuperscript{Min} intestinal carcinogenesis

Immunohistochemical staining of \( \beta \)-catenin, cyclin D1 and COX-2 showed a selective inhibition of small lesions by green tea (Figure 4). All the mice in this group were killed after 12 weeks of age in order to study the effects of green tea on the development of early lesions in Apc\textsuperscript{Min} intestinal carcinogenesis. These mice received green tea treatment for only 4 weeks. The aim of the immunohistochemical analysis was to confirm whether or not the inhibitory effects of green tea on Apc\textsuperscript{Min} tumors could be explained by any modulation of biomarkers involved in intestinal carcinogenesis. Lesions were scored as described in Table II. Medium lesions showed a pattern that was indistinguishable from large lesions; therefore, the analysis for large lesions is also representative of the medium lesions. Green tea (20 mice scored) caused a statistically significant reduction in the number of small lesions overexpressing \( \beta \)-catenin, but no inhibition of large lesions (8.1–5.5 small lesions per mouse, \( P < 0.05 \), and 1.1–1.3 large lesions, \( P > 1.0 \), respectively) compared with water-treated mice (20 mice scored) (Figure 5A). In correlation with \( \beta \)-catenin overexpression, only the number of small lesions overexpressing cyclin D1 was significantly reduced by the green tea treatment compared with water (8.3–5.1 small lesions per mouse, \( P < 0.05 \), and 2.3–2.6 large lesions, \( P > 1.0 \), respectively) (Figure 5B). Green tea at a 0.6% (w/v)
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Fig. 3. (A) Green tea caused a statistically significant reduction in colon tumors. Green tea solution of 0.6% was effective at inhibiting AOM-induced colon tumors in the Apc<sup>Min</sup> mice. Tea also inhibited the total number of tumors by >20%. Error bars represent standard error of the mean. *P < 0.05. (B) The reduction of colon tumors by green tea was mainly due to reduction in the number of small tumors. The inhibitory effect of green tea was primarily due to reduction in the number of small tumors (<1 mm). The data indicate that tea was ineffective against large tumors. (C) The reduction in the number of total Apc<sup>Min</sup> tumors by green tea was mainly due to reduction in the small tumors. In agreement with the colon tumor data, green tea was most effective at inhibiting the formation of small tumors in the Apc<sup>Min</sup> mice. Large tumors were resistant to the tea treatment.

concentration was ineffective in reducing the number of lesions expressing COX-2 (4.4–3.9 lesions per mouse with water or green tea treatment, respectively) (Figure 5C). The expression of COX-2 was directly proportional to the size of the adenoma; i.e., the larger the adenoma the more the COX-2 expression. Small lesions (1–5 crypts) were virtually void of COX-2 expression. Western blotting analysis confirmed the immunohistochemical data (Figure 6). Green tea caused statistically significant reduction in the total protein levels of β-catenin and cyclin D1 in Apc<sup>Min</sup> mice injected with AOM (P < 0.05). The protective effects of green tea were more evident in the AOM-treated Apc<sup>Min</sup> mice compared with saline-injected mice, as further classified them histologically. All the analyzed tumors were adenomas. Tumors of similar sizes were very similar morphologically and showed the same patterns of staining when stained for β-catenin, cyclin D1 and COX-2. We have no reason to believe that Apc<sup>Min</sup> tumors of equal sizes do not behave similarly or that green tea is differentially targeting some tumors but not the others. On the
other hand, if green tea halted the growth of tumors, we should see little or no increase in the number of large tumors in ApcMin mice killed after 4 or 8 weeks of green tea treatment. Indeed, we found a significant increase in the number of large tumors in mice killed after 12 weeks and after 16 weeks of age (1.3 large adenoma per mouse and 12.5, respectively). Therefore, it is probably that small tumors with diameters ≤1 mm were tumors that developed during the green tea treatment and any reduction in their numbers was due to prevention of the development of new adenomas by green tea. The number of small tumors was significantly reduced in colons and small intestines of green tea-treated ApcMin mice with only slight reduction in the number of larger tumors. The data suggest that green tea, at a 0.6% concentration, is selectively targeting the development of new adenomas in the AOM-ApcMin mouse model without affecting the pre-existing ones. Previous reports indicated that treating the ApcMin mice with green tea reduced the total number of tumors (14). These reports, however, did not discriminate between new and pre-existing tumors. Our results indicate that the time of administration of green tea is pivotal to its ability to inhibit colon carcinogenesis; physiological levels of green tea are not probably to inhibit the progress of any large adenomas or adenocarcinomas existing prior to the tea administration.

Fig. 4. (A) Immunohistochemical staining of β-catenin-overexpressing lesions. (1) β-catenin is overexpressed in a small lesion with two crypts (refer to Table II for classification of lesions). (2) A medium-sized lesion with <10 crypts overexpressing β-catenin. (3) β-catenin overexpression in a large lesion with >10 crypts. (4) A large colon adenoma with extensive nuclear localization of β-catenin. (B) Immunohistochemical staining of cyclin D1- and COX-2-overexpressing lesions. The expression of cyclin D1 correlated with the overexpression of β-catenin (1) and (2). Black arrow points to cyclin D1 overexpression in a large lesion adjacent to normal looking epithelia. Cyclin D1 protein was detected in small, medium and large lesions; green tea treatment reduced expression of cyclin D1 protein in small lesions only. COX-2 was predominantly expressed in large adenomas (3) and (4); the black arrows depict COX-2 staining in the stromal rather than epithelial cells. Small lesions were almost void of COX-2 expression.
In order to explain the ability of green tea to inhibit the formation of new adenomas, we examined the possible modulation of several biomarkers known to be involved in colon carcinogenesis. These included β-catenin, cyclin D1 and COX-2. In accordance with the tumor data, green tea inhibited the formation of small neoplastic lesions overexpressing β-catenin and its downstream target cyclin D1. This inhibition was observed after only 4 weeks of green tea treatment. In contrast, only modest effects were observed in the population of large lesions that are probably to have pre-existed when the tea treatment started. Surprisingly, we did not observe any significant inhibitory effects of green tea on COX-2 expression, although published literature had previously suggested an inhibition of COX-2 by EGCG in vitro (32,33). Despite its appeal as a target for chemoprevention by green tea, inhibition of COX-2 expression seems to be achieved at concentrations far exceeding the physiological concentrations of catechins present in the green tea beverage. Therefore, inhibition of COX-2 cannot explain the vast number of epidemiological data that show negative association between drinking green tea and the risk for cancers such as colorectal cancer. Further, we observed that COX-2 expression was limited to medium and large neoplastic lesions in the ApcMin colon and small intestines indicating a later involvement of COX-2 in ApcMin tumorigenesis. The expression of COX-2 was directly proportional to the size of the lesions and the degree of dysplasia regardless of the green tea treatment. The expression was only observed in the stromal cells surrounding the epithelial cells in the lesions. 

In summary, western blotting and the immunohistochemical analysis showed that a 0.6% green tea treatment reduced the total levels of β-catenin and its downstream target cyclin D1, but did not inhibit COX-2 expression. Green tea inhibited the formation of new adenomas but not pre-existing ones. Our data suggest that physiological concentrations of green tea target very early events in ApcMin intestinal carcinogenesis with little or no effects on events that take place later in the process.

Funding

National Institutes of Health (CA 96994).

Acknowledgements

We would like to thank Valérie Kennedy and Sharon Cooper for expert assistance with tissue preparation and immunohistochemical analyses.

Conflict of Interest Statement: None declared.

References

green tea group was assigned a value of 100% and the COX-2 protein levels are relative to that group. AOM-treated mice had a significant increase in COX-2 levels compared with the saline groups. The protein levels were normalized to b-actin. The figure shows relative levels of b-catenin or cyclin D1 compared with saline plus green tea group, which was assigned a value of 100%. Error bars represent standard error of the mean. Wild-type B6 mice had no tumors and therefore no detectable levels of COX-2. The saline plus green tea group was assigned a value of 100% and the COX-2 protein levels are relative to that group. AOM-treated mice had a significant increase in COX-2 levels compared with the saline groups.