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Chemopreventive Effect of Green Tea (*Camellia sinensis*) Against Cigarette Smoke-Induced Mutations (SCE) in Humans

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Abstract Green tea (*Camellia sinensis*) is consumed daily between the meals or after meals in Japan and other Asian countries. In recent years, green tea and its major polyphenolics have been demonstrated to prevent chemically induced tumors in a variety of experimental animal models system. The exact mechanism(s) of its anticarcinogenic activity remains to be elucidated, but green tea polyphenolics have demonstrated antimutagenic, anticarcinogenic, antioxidant, and antipromotional effects, including inhibition of Phase I and inducing Phase II enzymes. Enzyme activities of glutathione peroxidase, catalase, and quinone reductase, and glutathione S-transferase are also induced. However, a paucity of green tea effects in humans prompted us to investigate antimutagenic effects of green tea against smoke-induced mutation in humans. Chemopreventive effects of green tea and coffee among cigarette smokers were examined in 52 clinically healthy male subjects between 20–51 years of age. Blood specimens were obtained from non-smokers (Group I), smokers (II), smokers consuming green tea (III), and smoker/coffee drinkers (IV). The mean years of cigarette smoking (>10 cigarettes/day) of Groups II, III, and IV ranged from 13.4–14.7 years. Daily intake of green tea and coffee was 3 cups/day/6 months (III and IV). The frequencies of sister-chromatid exchange (SCE) in mitogen-stimulated peripheral lymphocytes from each experimental group were determined and statistically analyzed. SCE rates were significantly elevated in smokers (9.46 ± 0.46) vs. non-smokers (7.03 ± 0.33); however, the frequency of SCE in smokers who consumed green tea (7.94 ± 0.31) was comparable to that of non-smokers, implying that green tea can block the cigarette-induced increase in SCE frequency. Coffee, by contrast, did not exhibit a significant inhibitory effect on smoking-induced SCE. J. Cell. Biochem. Suppl. 27:68–75. © 1998 Wiley-Liss, Inc.

Key words: cigarette smoke; lung cancer; green tea; SCE; chemoprevention

A wealth of epidemiological data estimates that cigarette smoking is responsible for 85–90% of lung cancers and 30% of all cancers [1]. In the United States (US) alone, the number of cigarette smokers is estimated to be 50 million. Lung cancer has been the leading cause of death in men and women, and recently lung cancer mortality in women surpassed breast cancer mortality [2]. In spite of well-established cancer risks, smokers continue to expose non-smokers in the work place and elsewhere. A 30% increase in lung cancer risk is associated with exposure to passive or environmental cigarette smoke [3].

The etiology of cigarette smoke-related cancers is attributed to numerous carcinogens, such as reactive polycyclic aromatic hydrocarbon (PAH), alkylnitrosamines, aromatic amines (AA), aza-arenes, aldehydes, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), metals, and nitriles [4]. A variety of DNA adducts derived either directly or indirectly through activated intermediates have been identified in numerous human tissues, including human lymphocytes [5,6]. The level of DNA adducts is shown to correlate directly to tumor formation in some tissues, such as mouse skin [7].

These considerations underscore the urgent need to identify chemopreventive agents to re-

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duce or prevent cigarette smoke-induced cancer risk. Previously, green tea (*Camellia sinensis*) has been shown to be antimutagenic and anticarcinogenic [8,9]. Recent experimental studies have demonstrated that either oral administration or topical application of (−)epigallocatachin gallate, one of the major polyphenol components in green tea, prevented a variety of tumor initiation, as well as tumor promotion initiated by a variety of carcinogens (i.e., PAH, *N*-methyl-*N*-nitrosourea (MNU), (NDEA), NNK, azoxymethane, and radiation, etc.) in experimental animal tumor models [10–12]. Furthermore, for the past few years, the antitumor activities of green tea extracts and their major polyphenolic components, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and (−)-epigallocatechin gallate, have been extensively studied with a variety of animal tumor models (e.g., colon, esophagus, forestomach, duodenum, intestine, liver, lung, mammary glands, multiorgan carcinogenesis model, and skin, etc.) [10–15].

In addition, epidemiological studies also demonstrated that the death rate of all types of cancer, including stomach cancer rates in the midwest areas of Shizuoka Prefecture, where green tea is consumed daily, was significantly lower than the national average in Japan [14,15]. A case control study in Kyushu, Japan, also showed that individuals consuming green tea more frequently or in larger quantities tended to have a lower risk of gastric cancer [15].

Despite a high average consumption of cigarettes among Japanese males, as compared to US males, lung cancer mortality among Japanese males is significantly lower [16]. These differences may be attributed to dietary habits or genetic factors. The Japanese diet contains far less fat than that of the US, as well as foodstuffs rich in phytoantioxidants (e.g., soy, green tea, and other vegetables). Given the paucity of human studies in the literature, we sought to evaluate the chemopreventive effects of daily green tea consumption in human smokers using sister-chromatid exchange (SCE) frequencies in peripheral lymphocytes as mutagenic markers.

MATERIALS AND METHODS

Selection of Participants

Questionnaires were sent to 400 male workers, 20–51 years of age. The questionnaire design was adapted primarily from Carrano and Natarajan [17], and intended to minimize or eliminate subjects with possible confounding factors which might affect the outcome of SCE experiments. Three hundred and sixty-eight questionnaires were returned, from which 11 subjects were eliminated due to incomplete information. Four general selection criteria were then applied: no genetic or other pre-existing disease, no known exposure to toxic chemicals or radiation or alcohol, $<$55 years of age, and no history of serious illness since birth. The 52 selected subjects were tested for hematology, clinical chemistry, and urine analysis and were clinically evaluated to be healthy. Using epidemiological techniques, the observed levels of SCE found in the blood of 52 healthy, male subjects were correlated to serum biochemical, demographic, nutritional, and other factors. The procedure involved correlation between SCE frequency levels and 12 blood chemistry parameters, or between SCE frequencies and the frequency of 11 types of food intake in the diet (Type 2), or between SCE frequencies and 13 other demographic factors. Once the potentially important variables for explaining the observed SCE levels were identified, these variables were incorporated together into a mathematical model that allows for the estimation of each variable's importance in the presence of the other variables. The 12 serum biochemical variables are RBC, albumin, AST, ALT, ALP, GPT, BUN, Creatinine, B/C, cholesterol, and HDL-cholesterol. The 11 food frequency variables are bean and bean products, meat and fish, eggs, milk and milk products, dried small fish and seaweed, green and yellow vegetables, other vegetables, fruits, fats and fried food, instant foods, and the total food practice score. The 13 other factors are marital status, use of computer, exposure to chemicals, smoking, intake of vitamin tablets, constant use of drug(s), vaccines, surgery, intake of processed food, intake of artificial sweeteners, cancer patients in family, coffee intake, and green tea intake. In order to determine whether environmental pollution has any impact on SCE frequency, 30 subjects were selected from office workers in Seoul and 22 subjects were selected from a Daejeon factory; no significant differences in SCE frequency were found between the two areas.

Grouping of the Selected Subjects

The selected subjects were grouped as follows: Group I: non-smokers, who were not green

tea or coffee drinkers; Group II: smokers with no green tea or coffee intake; Group III: smokers who drank green tea (2–3 cups/day for 6 months) but no coffee; Group IV: smokers who drank coffee (>2–3 cups/day for 6 months) but no green tea.

Blood Sample Collection and Blood Cell Culture

Subjects fasted 12 hours prior to phlebotomy. Blood was drawn into heparinized syringes (sodium heparin 50 IU/ml). A 25-µl plasma aliquot was tested for hepatitis B virus surface antigen (HBsAg) via an HBsAg test kit (Jeil Sugar Co., Korea) prior to cell culture. HBsAG negative blood (0.8 ml) was inoculated in 9.5 ml Eagle's MEM (Flow Lab., McLean, VA), supplemented with 100 U/ml of penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO) and heat-treated fetal calf serum. Phytohemagglutinin (0.1 ml) and 5 mM 5-bromodeoxyuridine (0.05 ml to a final concentration of 25 µM) were added to culture vessels which were incubated at 37°C, 5% CO_2/95% air for 70 hours. Then 0.05 ml of 10 µg/ml colchicine (BDH Chem. Ltd.) was added, and after 2 hours incubation, cells were centrifuged, resuspended in prewarmed hypoosmolar solution (150 mOsm KCl) at 37°C. Cells were immediately fixed in repeated changes of 3:1 methanol/acetic acid. Chromosome spreads were prepared by dropping cell samples from 20 cm above glass slides, which were dried on a warmer at 30°C.

Chromosome Staining

Chromosomes were stained using a modified fluorescence-Giemsa technique. Slides were placed in 5 µg/ml bisbenzimide (Sigma Chemical Co.) for 10 minutes, and then completely covered with a thin film of phosphate buffered saline (Dulbecco's PBS A). The submerged slides were irradiated under a 2 × 15 W photo activator lamp at a distance of 10–15 cm for 10 minutes. Slide preparations were mounted in DePeX (Fluka 44581, Fluka, Buchs, Switzerland).

SCE Scoring

Twenty-five cells were scored per culture. Only diploid second metaphase (M_2) cells with 45–47 centromeres were scored. Every point of exchange was counted as a SCE. Exchanges at the centromere were included only when twisting at this point could be ruled out.

Statistical Analysis

All data were processed using the PC-SAS$^+$ statistical software program. The Student's *t*-test following Bartlett's test and one-way ANOVA analysis was applied. The relationships between the categories were tested by Pearson correlation.

RESULTS

The 52 study subjects chosen for this study were categorized into four groups: non-smokers (Group I), smokers (Group II), smokers with green tea intake (Group III), or smokers with coffee intake (Group IV). Observed levels of SCE in the study subjects were first correlated with 12 serum biochemical assays including hematological variables (RBC count, albumin, AST, ALT, ALP, GPT, BUN, creatinine, cholesterol, HDL-cholesterol, and glucose), 11 food frequency categories (bean products, meat and fish, eggs, milk products, dried small fish and seaweed, green and yellow vegetables, other vegetables, fruits, fats and fried food, instant foods, and a total food practice score), and 13 demographic factors. Correlation between SCE frequencies and biochemical variables, food frequency categories, and other demographic factors were not significant (two-tailed) at the 5% levels. SCE frequencies of subjects sampled at two different geographical locations with differing occupational status were also not significantly different. Once the potentially important variables for explaining the observed SCE levels were identified, these variables were incorporated together into a mathematical model, which allowed the estimation of each variable's importance in the presence of the other variables.

The age distribution of the 52 study subjects was categorized by the cigarette smoking, green tea or coffee drinking group (Fig. 1). The average age of the 52 selected human subjects was 34.48 ± 0.95 years; the average ages of Group I (non-smokers), Group II (smokers), Group III (smoker plus green tea), and Group IV (smokers plus coffee) were 31.33 ± 1.18, 35.86 ± 1.94, 36.20 ± 2.03, and 33.29 ± 1.73 years, respectively. Group I subjects were younger and had less variability in age than the other group. A Bartlett's test and one-way analysis of variance comparing ages by groups were performed. An F-test comparing the variance in Group I with Group IV was significant ($P < 0.05$), and a comparison of Groups I and III mean ages was also

significant by z-test ($P < 0.05$). The mean years of smoking in Groups II, III, and IV were not statistically different (Table I). The mean SCE frequencies in Groups I, II, III, and IV were 7.04, 9.46, 7.94, and 9.20, respectively (Fig. 2). Since Group I subjects were younger and had fewer variable ages than the other groups, a Bartlett's test and one-way analysis of variance were used for statistical analysis. In the present study, 78% of the selected human subjects were <40; there was no statistical evidence for age-related increase in SCE frequencies. The variance of the SCE means for the four groups was not significantly different by Bartlett's test ($\chi^2 = 3.94$ with 3 D.F., $P = 0.27$). The differences in SCE frequency among Groups I, II, III, and IV also could not be attributed to duration of smoking (Table I, Fig. 2).

Mean SCE frequencies of the four groups were significantly different when compared by one-way analysis of variance in all but two comparisons. The paired comparisons of Group I vs. II (F 16.91, $P = .0002$) and IV (F 14.17, $P = .0005$) were statistically significant. Notably, the mean SCE frequency of Group II was significantly different from that of Group III (F 8.53, $P = 0.005$). The paired comparison of Group I vs. Group III, however, was not significant (F 2.54, $P = .12$), implying that green tea had blocked the smoke-induced increase in SCE frequency. Coffee had no statistically significant effect on smoking-induced SCE (Group II vs. IV: F 0.15, $P = 0.70$). A paired comparison of Group III vs. IV was significant (F 6.35, $P = 0.015$).

In order to separate the effects of smoking, green tea, and coffee, a linear regression model was applied, where SCE was predicted by Yes = 1 and No = 0 to each of the three variables. The results of these analyses and paired compari-

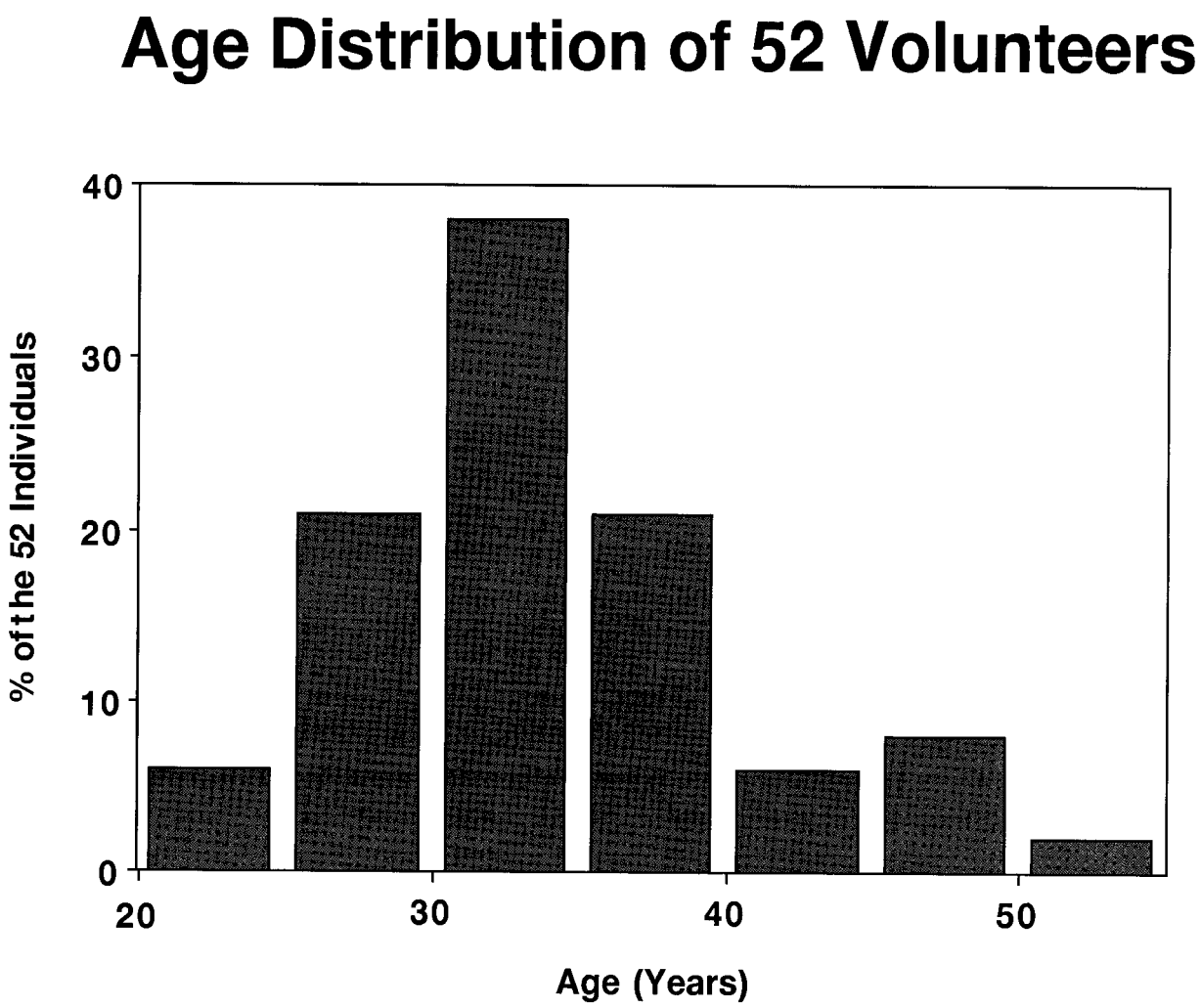

Fig. 1. Age distribution of 52 volunteers.

TABLE 1. SCE Frequencies by Groups Categorized by Age, and Years of Smoking, Green Tea and Coffee Intake

Group[a]	Number	SCE (mean ± SE)	Age (mean ± SE)	Years of smoking (mean ± SE)[b]
I	9	7.03 ± 0.33	31.33 ± 1.18	—
II	14	9.46 ± 0.46**	35.86 ± 1.94	14.71 ± 2.18
III	15	7.94 ± 0.31*	36.20 ± 2.03	13.50 ± 2.19
IV	14	9.20 ± 0.32**	13.36 ± 1.74	13.36 ± 1.74
Total	52	8.53 ± 0.95	34.48 ± 0.95	13.86 ± 1.16

[a]I: non-smokers; II: smokers; III: smokers plus green tea (2–3 cups/day); IV: smokers plus coffee (2–3 cups/day).
[b]Smoked >10 cigarettes/day.
*The comparison of Group I with Group III was not significant.
**The comparison of Group I with II and IV was significant; one-way analysis of variance: $F = 7.77$, $P \leq 0.0003$.

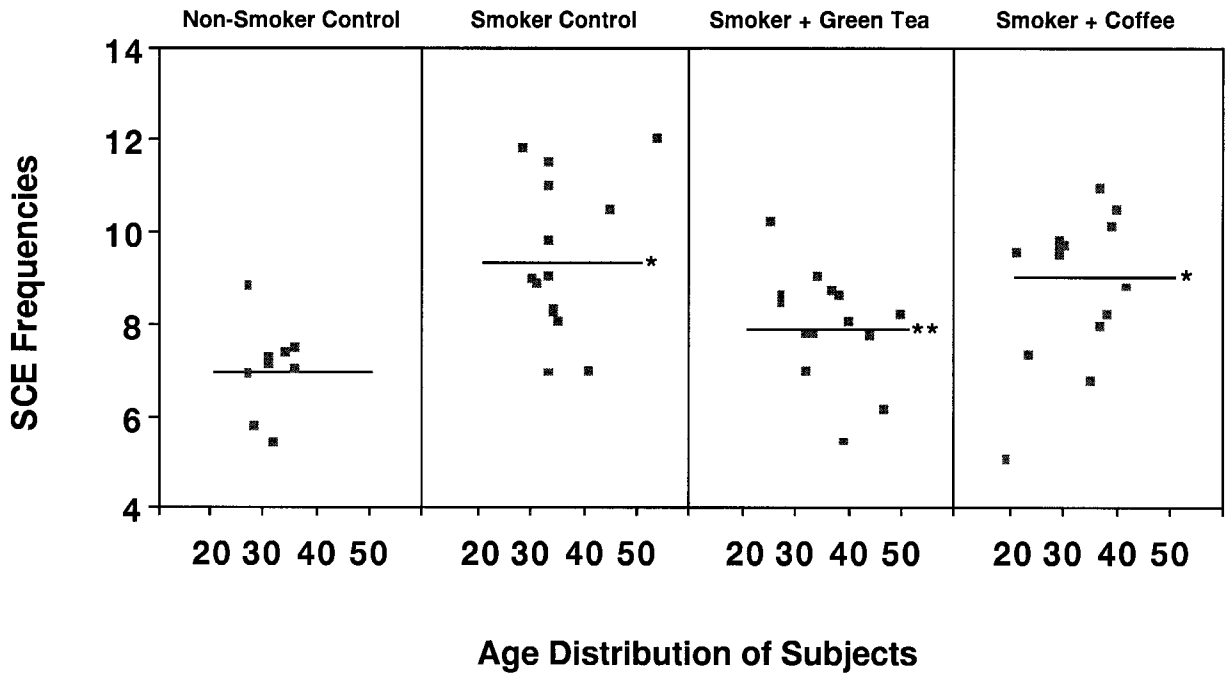

Fig. 2. SCE frequencies as a function of age distribution of subjects in Group I (non-smoker cohort with no green tea or coffee); Group II (smoker control cohorts without green tea or coffee intake); Group III (smoker cohort taking green tea only); Group IV (smoker cohort taking only coffee).

sons showed that smoking and green tea, but not coffee, significantly affected SCE frequency, and explained 32.7% of SCE variation ($P < 0.0003$; parameters: SCE = 7.03 + 2.6, $P < 0.0002$, smoking group), −1.46 ($P < 0.0053$, green tea group), and −0.2 (0.7%). Equivalently, SCE had a multiple correlation with smoking, green tea, and coffee. For ths purpose, a linear regression model was applied, where SCE was predicted by Yes = 1 and No = 0 to each of the three variables. The results of these analyses and paired comparisons showed that smoking and green tea, but not coffee, significantly affected SCE frequency, and explained 32.7% of SCE variation ($P < .0003$; parameters: SCE = 7.03 + 2.63 ($P = 0.0002$, smoking group), −1.46 ($P < 0.0053$, green tea group), and −0.2 (0.7, coffee group). Equivalently, SCE had a multiple correlation with smoking, green tea, and coffee of 0.572, a high value for biological experiments. From the results of statistical analyses, the mean SCE frequencies, ages, and years of smoking categorized by four experimental groups are shown in Table I. The differences in the SCE frequencies among Groups I, II, III, and IV cannot be attributed to either age or the duration of smoking in the present experiments.

DISCUSSION

In this study we set out to determine whether green tea (*Camellia sinensis*), rich in polyphenols, or coffee could reduce SCE frequencies in peripheral lymphocytes of cigarette smokers. This assay was ideal given that peripheral lymphocytes are easily accessible and that SCE is a much more sensitive mutagenic biomarker than chromosomal aberrations [18]. The present study clearly demonstrates that cigarette smoking significantly increased SCE frequencies in peripheral lymphocytes. The mean SCE frequency for smokers (9.46) was 35% higher than that of non-smokers. These values are similar to those reported previously [18]. SCE frequencies have also been shown to depend on dose and duration of smoking [18,21].

The increase in SCE in smokers likely reflects smoking-induced DNA damage rather than changes in lymphocyte subpopulations [20,22]. This is supported by the presence of exceptionally high SCE frequencies in both peripheral lymphocytes of human smokers and in bone marrow cells of mice exposed in vivo to cigarette smoke [19,23]. Furthermore, the peripheral lymphocytes of heavy smokers (40–60 cigarettes per day for 9–58 years) as compared to non-smokers exhibit a 4–6-fold increase in exchange-type chromosomal aberrations [24]. In addition, there are significant correlations between (4-aminobiphenyl-hemoglobin-ABP-Hb) and both continue and SCEs as well as a positive, highly significant correlation between 4-ABP-Hb and DNA adduct levels in smokers, but not in non-smokers [25].

In the present study, both the mean and the standard error of the mean of SCE frequencies in smokers who drank coffee were lower than in smokers only. Although this tendency was not statistically significant, it has been reported in several earlier studies, wherein caffeine treatment lowered SCE induced by mutagens or carcinogens in both hamster and human lymphocytes [26]. Caffeine application to skin has also been shown to inhibit both UV-induced mouse skin tumorigenesis and breast tumorigenesis in rats [27,28]. A greater number of human subjects in the smoker plus coffee category is needed to clarify the effects of coffee consumption.

Notably, the present study demonstrated no significant difference in SCE rates between nonsmokers and smokers who regularly consumed green tea (2–3 cups per day), and a significant difference between smokers (Group II) and smokers who drank green tea (Group III). Thus, to the best of our ability to exclude other confounding factors, green tea appears to block smoking-induced increase in SCE. As green tea also contains caffeine in addition to a variety of catechins, some of its protective effect against cigarette smoke-induced SCE may be attributed to an additive and/or synergistic contribution of caffeine. However, the tendency of coffee in our study (smokers plus coffee, Group IV) to decrease SCE as compared to smokers only (Group II) was small and not statistically significant.

Previously, green tea (*Camellia sinensis*) has been shown to be antimutagenic and anticarcinogenic in experimental animals. These studies demonstrated that either oral or topical administration of green tea or its major chemical constituent, epigallocatechin gallate, prevented tumor initiation and promotion [8,16]. In human subjects, tea consumption has been shown to decrease micronucleus formation induced by smoking [29]. HPLC analysis of green tea has shown it to be composed of several polyphenols (as much as 30% by dry weight), most of which are catechins: epigallocatechin gallate (15.1%), epigallocatechin (6.9%), epicatechin gallate (3.0%), epicatechin (1.8%), and caffeine (8.1%) [30].

The potent chemopreventive mechanism(s) of green tea and its polyphenol constituents remains to be defined. The catechins are known free-radical scavengers, with gallocatechins and the catechin gallates exhibiting the strongest antioxidant properties. Polyphenolics are also shown to inhibit lipoxygenase and cyclooxygenase blocking fatty acid oxidation, thus lowering reactive alkyl enals, reducing several different exocyclic nucleotide adducts [31,32]. Exocyclic nucleosides have been shown to be highly mutagenic [31,33]. Furthermore, all catechins significantly inhibit cytochrome P-450-dependent monooxygenase(s). Based on the structure-activity relationship between epicatechins, epigallocatechin gallate is the most potent inhibitor, suggesting that the galloyl group or hydroxyl groups may bind to a cytochrome P-450 catalytic site and interfere with the activation of precarcinogens [34]. In the NNK-A/J mouse lung tumor bioassay, both green tea and epigallocatechin gallate, which are known to reduce tumor mul-

tiplicity, inhibited NNK oxidation and NNK-induced DNA methylation when added to incubation mixtures containing lung microsomes [35]. However, administration of green tea to A/J mice did not inhibit lung DNA methylation in vivo [35,36]. Intriguingly, however, treatment of A/J mice with green tea or epigallocatechin gallate suppressed NNK-induced formation of 8-hydroxydeoxyguanosine, a common free radical-induced DNA lesion [36].

The etiology of cigarette-smoke related cancer is attributed to numerous procarcinogens and carcinogens, some of which have been identified e.g., polycyclic aromatic hydrocarbons, NNK and other nitrosamines, aldehydes, and metals [4]. In addition, cigarette smoke contains many oxidants, prooxidants, and free radicals, which are known to induce oxidative damage or lipid peroxidation in vitro but whose role in vivo has yet to be clearly defined [37]. We propose that chemopreventive mechanism(s) of green tea against cigarette smoke-induced SCE occurs when polyphenolic catechins interact with cytochrome P-450 monooxygenase(s) to significantly reduce metabolic activation of carcinogen(s); catechins scavenge reactive carcinogenic metabolites to prevent their molecular initiation at critical target sites; and Phase II enzymes and a variety of peroxidase enzymes are induced. While other mechanisms cannot be excluded at this time, the data presented in this study as well as in work cited previously suggest that polyphenol catechins in dietary foodstuffs may provide clinically significant protection against environmental carcinogens. Pharmacologic and toxicologic studies are needed to further confirm the efficacy and safety of catechins as chemopreventive agents against human cancer.

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