Mechanisms of Cancer Prevention by Tea Constituents\textsuperscript{1,2}

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ABSTRACT Consumption of tea (Camellia sinensis) has been suggested to prevent cancer, heart disease and other diseases. Animal studies have shown that tea and tea constituents inhibit carcinogenesis of the skin, lung, oral cavity, esophagus, stomach, liver, prostate and other organs. In some studies, the inhibition correlated with an increase in tumor cell apoptosis and a decrease in cell proliferation. Studies with human cancer cell lines have demonstrated that epigallocatechin-3-gallate (EGCG), a major tea polyphenol, inhibits mitogen-activated protein kinases, cyclin-dependent kinases, growth factor-related cell signaling, activation of activator protein 1 (AP-1) and nuclear factor \textit{\textkappa}B (NF-\textit{\textkappa}B), topoisomerase I and matrix metalloproteinases as well as other potential targets. Although some studies report effects of EGCG at submicromolar levels, most experiments require concentrations of \textgreater{}10 or 20 \textmu{}mol/L to demonstrate the effect. In humans, tea polyphenols undergo glucuronidation, sulfation, methylation, and ring fission. The peak plasma concentration of EGCG is \textsim{}1 \textmu{}mol/L. The possible relevance of each of the proposed mechanisms to human cancer prevention is discussed in light of current bioavailability data for tea polyphenols and the potential limitations of animal models of carcinogenesis. Such discussion, it is hoped, will clarify some misunderstandings of cancer prevention by tea and stimulate new research efforts. J. Nutr. 133: 3262S–3267S, 2003.

KEY WORDS: • tea • catechins • theaflavins • cancer prevention • bioavailability

Tea [Camellia sinensis (Theaceae)] is second only to water in terms of worldwide popularity as a beverage. Consumption of tea has been associated with may health benefits including the prevention of cancer and heart disease (1).

Green, black and Oolong tea are the three major commercial types of tea and differ in how they are produced and in their chemical composition. Green tea is prepared by pan-frying or steaming fresh leaves to heat inactivate oxidative enzymes, and then dried. By contrast, black tea is produced by crushing fresh tea leaves and allowing enzyme-mediated oxidation to occur in a process commonly known as fermentation. Green tea is chemically characterized by the presence of large amounts of polyphenolic compounds known as catechins (Fig. 1). A typical cup of brewed green tea contains, by dry weight, 30–40\% catechins including epicatechin (EC),\textsuperscript{4} epigallocatechin (EGC), epicatechin-3-gallate (EGC), and epigallocatechin-3-gallate (EGCG). Through fermentation, a large percentage of the catechins are converted to oligomeric theaflavins and polymeric thearubigins in black tea. The resulting brewed black tea contains 3–10\% catechins, 2–6\% theaflavins and >20\% thearubigins (2).

Both green and black tea and their constituents have been extensively studied both in vitro and in animal models of carcinogenesis (1). Although these compounds have been shown to be efficacious in a number of models of carcinogenesis, the epidemiological data of cancer prevention remains mixed. Likewise, the primary cancer preventive mechanisms of tea in animal models remain unclear. Although many of the beneficial effects of tea have been attributed to the strong antioxidative activity of the polyphenols, neither this mechanism nor others have been firmly established in animals or humans (3,4). In the present article, we discuss the possible cancer chemopreventive mechanisms of tea components based on in vitro and animal experiments. Based on our understand-

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\textsuperscript{4} Abbreviations used: AP-1, activator protein 1; COMT, catechol-O-methyltransferase; EC, epicatechin; EGC, epicatechin-3-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; EGF, epidermal growth factor; HNSCC, head and neck squamous cell carcinoma; \textit{iB}, inhibitor \textit{\textkappa}B; JNK, jun N-terminal kinase; LPS, lipopolysaccaride; MAP, mitogen activated protein; MAPK, mitogen activated protein kinase; MMP, metalloproteinases; NF-\textit{\textkappa}B, nuclear factor \textit{\textkappa}B; NHEK, normal human keratinocytes; PDGF, platelet-derived growth factor; SULT, sulfotransferase; TGF, transforming growth factor; TGF, transforming growth factor; TGF, transforming growth factor; TPA, 12-tetradecanoylphorbol-13-acetate; UGT, uridine diphosphate glucuronosyltransferase; VEGF, vascular endothelial growth factor.

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CANCER PREVENTION BY TEA

Epidemiological studies. Although data from animal models of carcinogenesis suggest that tea and tea components may be efficacious cancer preventive agents, epidemiological data have been mixed. These studies have been extensively reviewed elsewhere (1,11,12). Because of direct contact between tea constituents and the gastrointestinal tract, these organs are most likely to be affected by tea; the possible prevention of gastric cancer by tea has been suggested by six case-control studies. In addition, a nested case-control study of gastric cancer in men of the Shanghai Cohort in China with >4 y follow-up found an inverse correlation between urinary tea polyphenols and the risk of gastric cancer (OR = 0.52; 95% CI = 0.28–0.97) (13). Conversely, a population-based prospective study in Miyagi, Japan found no association between tea consumption and the risk of gastric cancer over an 8-y period (14).

Similarly, whereas Su and Arab have reported that tea consumption by men and women in the U.S. significantly reduced the risk of colon cancer (15), a prospective study by The Netherlands Cohort Study on Diet and Cancer found no association between black tea consumption and the risk of colorectal, stomach, lung or breast cancer in men or women (16). The inconsistencies in the epidemiological data may be the result of a number of factors including problems quantifying tea consumption, confounding lifestyle factors and interindividual differences in cancer susceptibility and in the bio-transformation of tea constituents.

Mechanisms of cancer prevention

Numerous potential mechanisms have been proposed for the cancer preventive activity of tea and tea constituents based on studies with cancer cell lines. Many studies have focused on EGCG and a few used theaflavins. Both of these compounds have been shown to inhibit the growth and induce cell cycle arrest and apoptosis in different cancer cell lines (1). The relative importance of any of these mechanisms in vivo remains to be determined. One problem faced by most studies is the relatively high concentrations of tea compounds used in vitro studies. These concentrations often far exceed those found in animal plasma or tissue following tea consumption.

Antioxidant/pro-oxidant activity. EGCG and theaflavins, as well as other tea polyphenols, have been shown to have strong antioxidant activity in vitro. The importance of this potential mechanism in vivo remains unclear. For example, Katiyar et al. demonstrated that 40 μmol/L of EGCG inhibited the production of H₂O₂ in UVB-treated normal human keratinocytes (NHEK) by 66–80%. This antioxidative activity was correlated with inhibition of UVB-induced phosphorylation of ERK1/2, jun N-terminal kinase (JNK), and p38. EGCG showed similar inhibitory activity when H₂O₂ was directly added as the stress (17). EGCG and theaflavin-3,3'-

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digallate (TFdiG) have also been shown to inhibit lipid peroxidation in vitro (18). Conversely, ex vivo studies of plasma obtained from human volunteers following consumption of black tea have shown no increase in antioxidative activity as measured by inhibition of lipid peroxidation (4).

In contrast to the potential antioxidative activity of tea polyphenols, recent studies have suggested that the cell-killing activity of these compounds, at least in vitro, may be related to their pro-oxidant activity. For example, we have shown that EGCG-induced apoptosis in H661 lung cancer cells and Ras-transformed human bronchial cells is completely or partially blocked by the inclusion of catalase in the medium (19,20). Catalase, however, did not affect the growth inhibitory activity of EGCG. Hong et al., have shown that in the presence of HT29 cells in McCoy’s 5A medium, treatment with 50 μmol/L of EGCG results in the production of up to 23 μmol/L of H₂O₂ (21). Both of these observations suggest a role for H₂O₂ in some of the observed activities of EGCG in vitro. It is not known whether this mechanism is relevant in vivo.

**Inhibition of MAP kinase signaling.** Enhanced activity of the transcription factors, activator protein 1 (AP-1) and nuclear factor κB (NFκB), have been implicated as the key effect in some carcinogenic pathways, including UVB-induced skin tumorigenesis. This enhanced activity can result from the activation of one or more mitogen activated protein kinase (MAPK) pathways (22).

EGCG and theaflavins (5–20 μmol/L) have been shown to inhibit 12-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor (EGF)-induced transformation of JB6 mouse epidermal cells in a dose-dependent manner (22). This inhibition was shown to correlate with decreased JNK activation leading to inhibition of AP-1 binding to its recognition sequence. Similarly, when JB6 cells were pretreated with EGCG (1–20 μmol/L) followed by treatment with UVB, a decrease in the phosphorylated form of p44/42 MAPK was observed. Topical application of EGCG to B6D2 transgenic mice, which carry a luciferase reporter gene containing the AP-1 binding sequence, resulted in a 60% inhibition of UVB-induced transcription of the luciferase reporter gene (22).

We have demonstrated that EGCG and TFdiG affect numerous events in the Ras-MAP kinase signaling pathway (23). Treatment of 30.7b Ras 12, Ras-transformed mouse epidermal cells, with 20 μmol/L of either compound resulted in decreased levels of phosphorylated Erk1/2 and MEK1/2. EGCG inhibits the association between Raf-1 (an upstream protein kinase) and MEK1. TFdiG, but not EGCG, enhances the degradation of Raf-1. In addition to inhibiting the phosphorylation of Erk1/2, TFdiG and EGCG can also directly inhibit the kinase activity of this protein by competing with Elk-1 for access to the active site (23). Similarly, treatment of Ras-transformed human bronchial cells with EGCG and TFdiG was shown to inhibit c-jun and ERK 1/2 phosphorylation as well as the phosphorylation of Elk-1 and MEK1/2, which are down- and upstream of the MAP kinase cascade, respectively (20).

EGCG and TFdiG have been shown to inhibit the activity inhibitor κB (IκB) kinase in tumor necrosis factor (TNF)α-stimulated IEC-6 intestinal epithelial cells and lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages (24,25). In both cases, there is diminished IκB degradation and NFκB activity in response to stimulation. Likewise, Ahmad et al. have demonstrated that EGCG inhibits the activity of NFκB in TNFα-stimulated A431 human epidermoid carcinoma cells (26). This effect is also mediated by inhibition of IκB phosphorylation and degradation. Interest-ingly, the effect is more selective for cancerous cells than NHEK.

**Inhibition of growth factor signaling.** Overexpression of growth factor and growth factor receptors such as EGF, platelet-derived growth factor (PDGF), and others can result in enhanced proliferation by cancer cells (27). Selective blockade of growth factor receptor-mediated signal transduction, either by competition with ligand or inhibition of kinase activity, has been shown to inhibit cell growth and induce apoptosis.

Using in vitro kinase assays, Liang et al. have demonstrated that EGCG potently inhibits the kinase activity EGF-R, PDGF-R, and fibroblast growth factor-R with IC₅₀ = 1 – 2 μmol/L (28). In contrast, the IC₅₀ of EGCG was >20 μmol/L against protein kinase C and A. In A431 human epidermoid carcinoma cells, pretreatment with 5 μmol/L EGCG completely abolishes ligand-induced autophosphorylation. Binding studies with [³²P]-EGF showed that pretreatment with EGCG dose-dependently inhibits binding of EGF-R.

Masuda et al. have shown that treatment of human head and neck squamous cell carcinoma (HNSCC) cells with 20 μmol/L of EGCG results in transforming growth factor (TGF)α-mediated EGF-R, ERK, and Stat-3 phosphorylation accompanied by G₂/M blockade of the cell cycle and induction of apoptosis (27). The authors hypothesize that EGCG inhibits EGF-R signaling by an unknown mechanism possibly inhibition of TGFα binding or EGF-R kinase activity. This results in inhibition of ERK and Stat-3 phosphorylation resulting in cell death.

Several investigators have demonstrated that EGCG inhibits the expression of vascular endothelial growth factor (VEGF) by HNSCC, breast, and colon carcinoma cells (29–31). For example, Jung et al. have reported that 30 μmol/L of EGCG inhibits serum starvation-induced VEGF expression by HT29 colon cancer cells (31). This observation may account for the antitumor activity of EGCG against HT29 xenografts in athymic nude mice. The authors have observed not only decreased tumor growth (58%) and increased tumor cell apoptosis (1.9-fold) but also decreased tumor microvessel density (30%).

**Inhibition of other enzymes.** Berger et al. have demonstrated that EGCG selectively inhibits the activity of topoisomerase I but not topoisomerase II in human colon cancer cell lines (32). The doses of EGCG necessary for this inhibition (10–17 μmol/L) are lower than those necessary for inhibition of cell growth (IC₅₀ = 10–90 μmol/L).

Others have recently shown that EGCG inhibits the chymotryptic activity of the 20s proteasome in leukemic, breast cancer, and prostate cancer cell lines (33). This inhibition results in the accumulation of p27kip1 as well as IκB, which results in G₁-phase cell cycle arrest and inhibition of NFκB activity, respectively. The IC₅₀ for EGCG-mediated inhibition of the proteasome in cells (1 – 10 μmol/L) is 10-fold higher than the IC₅₀ in a cell free system (86–194 nmol/mL). This is likely due to the binding of EGCG nonspecifically to other cellular components. Such nonspecific binding likely increases in vivo and may lead to a higher dose requirement.

Inhibition of the matrix metalloproteinases (MMP) by EGCG has also been demonstrated at relatively low doses. Garbisa et al. have shown that EGCG inhibits the activity of secreted MMP2 and MMP9 at concentrations of 8–13 μmol/L (34). Additionally, 1 μmol/L of EGCG was found to increase the expression of TIMP-1 and TIMP-2, proteins that inhibit the activity of activated MMP. Others have shown that EGCG likewise inhibits the activity and expression of MT1-MMP, a protein responsible for the activation of MMP (35).
Because of the physiologically achievable concentrations, these activities represent an attractive mechanism for the observed antiangiogenic activity of EGCG and green tea in vivo (1).

Bioavailability and biotransformation of tea polyphenols

The catechins have been demonstrated to undergo considerable biotransformation and to have low bioavailability (Fig. 2) (1). The theaflavins are even less bioavailable. This poor availability confounds attempts to correlate in vitro findings with cancer prevention in animal models. Cell line studies typically require concentrations of compound in the 5–100-μmol/L range. Such concentrations are typically not observed systemically. The low bioavailability of the tea polyphenols is likely due to their relatively high molecular weight and the large number of hydrogen bond donating hydroxyl groups (36). These hydroxyl groups not only serve as functional handles for Phase II enzymes but may also reduce the absorption of the compounds from the intestinal lumen. According to Lipinski’s Rule of 5, compounds with a molecular weight less than 500, >5 hydrogen bond donors or 10 hydrogen bond acceptors have poor bioavailability due to their large actual size (high molecular weight) or large apparent size (due to the formation of a large hydration shell) (36).

Enzymology of biotransformation. The catechins are subject to extensive biotransformation including methylation, glucuronidation, sulfation and ring-fission metabolism (Fig 2). Recent studies on the enzymology of EGC and EGCG methylation have shown that EGC is methylated to form 4′-O-methyl-(−)-EGC and EGCG is methylated to form 4′′-O-methyl-(−)-EGCG and 4′,4′′-O-dimethyl-(−)-EGCG (37). At low concentrations of EGCG, the dimethylated compound is the major product. Rat liver cytosol showed higher catechol-O-methyltransferase (COMT) activity toward EGCG and EGC than did human or mouse liver cytosol. Additionally, the \( K_m \) and \( V_{max} \) values are higher for EGC than for EGCG (e.g., in human liver cytosol, \( K_m \) is 4 and \( V_{max} \) is 0.16 μmol/L for EGC and EGCG, respectively).

Studies of EGCG and EGC glucuronidation revealed that EGCG-4′-O-glucuronide is the major metabolite formed by human, mouse and rat microsomes (38). Mouse small intestinal microsomes have the greatest catalytic efficiency (\( V_{max} / K_m \)) for glucuronidation followed in decreasing order by mouse liver, human liver, rat liver and rat small intestine. Using recombinant human uridine diphosphate glucuronosyltransferase (UGT) enzymes, it has been determined that UGT1A1, 1A8 and 1A9 have the highest activity toward EGCG, with the intestinal specific UGT1A8 having the highest catalytic efficiency. EGCG-3′′-O-glucuronide is the major product formed by microsomes from all species. In this case, liver microsomes were shown to have a higher efficiency than intestinal microsomes for glucuronidation. Based on these studies, it appears that mice are more similar to humans than are rats in terms of enzymatic ability to glucuronidate tea catechins. Although these similarities must still be confirmed in vivo, this information will aid in choosing the most appropriate animal model to study the potential health benefits of tea constituents.

Vaidyanathan et al. have shown that EC undergoes sulfation catalyzed by human and rat intestinal and liver cytosol with the human liver being the most efficient (39). Further studies have revealed that sulfotransferase (SULT1A1) is largely responsible for this activity in the liver, whereas both SULT1A1 and SULT1A3 are active in the human intestine. The catalytic efficiency for SULT1A1 and SULT1A3-mediated sulfation of EC are 5834 and 55 L/(min·mg), respectively. Recent work in our lab has shown that EGCG is time- and concentration-dependently sulfated by human, mouse and rat liver cytosol (40). The rat has the greatest activity followed by the mouse and humans. Further studies are required to determine the structure of these sulfated metabolites.

Anaerobic fermentation of EGC, EC and ECG with human fecal microflora has been shown to result in the production of the ring fission products 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone (M4), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (M6) and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (M6′) (Fig 2). Further incubation results in the formation of lower molecular weight phenolic acids (41). We have found these ring fission products in human urine and plasma ~3 h after oral ingestion of 20 mg/kg of decaffeinated green tea (42). The compounds have a \( T_{max} \) of 7.5–13.5 h and reach peak plasma concentrations of 100–200 nmol/L. Peak urine concentrations of 8, 4 and 8 μmol/L have been demonstrated for M4, M6 and M6′, respectively, following ingestion of 200 mg of EGCG. M4, M6 and M6′ retain the polyphenolic character of the parent compound, have the addition of a potentially biologically active valerolactone structure and may therefore have biological activities similar to the parent catechins.

In animals, the phase II metabolism reactions likely compete with one another. The relative concentration of each enzyme and their activities for the tea polyphenols determine the metabolic profile in vivo. Because EGCG has a lower \( K_m \) for COMT than UGT, methylation may be favored at physiological (usually low) concentrations. Indeed, in vivo, EGCG is first methylated to form 4′′-O-methyl-EGCG and then further methylated to form 4′′,4′′-di-O-methyl-EGCG (43). At high doses, Lu et al. have observed that glucuronidation becomes more prominent leading the formation of EGCG-4′′-glucuronide in the mouse (37). This compound can be further methylated on the B-ring to produce different methylated metabolites. This is consistent with the observation that four monomethylated and two dimethylated compounds are found in mouse urine after hydrolysis with β-glucuronidase and sulfatase following administration of high doses of EGCG to the mouse (43). The methylated compounds were found to have similar peaks heights; if conjugation had not preceded methylation, 4′′-O-methyl-EGCG peak would have been the predominant monomethylated metabolite.
Pharmacokinetics. Studies of [3H]-EGCG in both the rat and the mouse have shown that following a single intragastric dose, radioactivity is found throughout the body (44,45). After 24 h, 10% of the initial dose (radioactivity) is in the blood with 1% found in the prostate, heart, lung, liver, kidney and other tissues. The major route of elimination is via the feces. In the rat, 77% of an intravenous dose of [3H]-EGCG is eliminated in the bile whereas only 2% is found in the urine.

Detailed pharmacokinetic and biotransformation studies of the tea catechins have been conducted in rats (46). Following intragastric administration of decaffeinated green tea to rats, plasma levels of EGCG, EGC and EC were fit to a two-compartment model with elimination half-lives of 165, 66 and 67 min, respectively. The absolute bioavailability of EGCG, EGC and EC after intragastric administration of decaffeinated green tea is 0.1, 14 and 31%, respectively. Studies with bile duct-cannulated rats have shown that after oral administration of 100 mg of EGCG, 3.28% of the dose is recovered in the bile as EGC (2.65%), 4’-O-methyl-EGCG (0.25%), 3’-O-methyl-EGCG (0.11%), 4’-O-methyl-EGCG (0.11%), 4’-O-methy-".

In the rat, 77% of an intravenous dose of [3H]-EGCG is eliminated in the bile whereas only 2% is found in the urine. There is only a single report on the detection of theaflavins in plasma following ingestion. Mulder et al. have shown that following ingestion of 700 mg of pure theaflavins, the peak concentration was 1 μg/L and 4.2 ng/mL in the plasma and urine, respectively (52). There are no published reports concerning the metabolic fate of the theaflavins or the thearubigins in humans or animals.

Although the tea polyphenols have low systemic bioavailability, high concentrations have been demonstrated in the oral cavity. We have demonstrated that holding green tea solution (1.2 g green tea solids per 200 mL water) in the mouth without swallowing results in salivary concentrations of EGCG and EGC of 153 and 327 μmol/L, respectively (53). These concentrations are 400–1000 times greater than those observed in plasma following ingestion of tea. Such locally high levels may support the use of green tea in the prevention of oral cancer and caries.

CONCLUSION

Although numerous health benefits have been proposed for the consumption of tea, the effectiveness of tea as a cancer preventive agent in humans remains unclear. Animal models of carcinogenesis may be different from the human situation (e.g., the doses of tea and tea components used in animal studies are often much higher than those consumed by humans), and many confounding factors are involved in epidemiological studies. Interindividual variation in biotransformation and bioavailability may also affect the efficacy of tea as a cancer preventive agent. Better understanding of the fundamental mechanism(s) of action of the tea constituents and their bioavailability is needed to more effectively determine the potential usefulness of tea as a cancer preventive agent.

To this end, further studies on definitive mechanisms of the cancer preventive activities of tea in animal models are needed. Although many possible mechanisms have been proposed, their relevance in vivo needs to be demonstrated. With some exceptions, the concentrations of catechins or theaflavins used in cell culture systems exceed the plasma concentrations obtained in animal studies by 10- to 100-fold. Mechanisms based on the use of such high concentrations may be relevant for cancers of the gastrointestinal tract but not for sites such as the lung, prostate and breast, which depend on systemic bioavailability.

Further studies on the bioavailability of tea constituents, as well as their mechanisms of action, are needed. Based on these data, effective intervention studies can be designed to more accurately measure the efficacy of tea constituents as preventive agents for human cancer.

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