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Review

Possible mechanisms of the cancer-preventive activities of green tea

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The cancer-preventive activities of tea and some tea constituents, such as caffeine and (−)-epigallocatechin-3-gallate (EGCG), have been demonstrated in animal models. The mechanisms of action of the tea constituents have been extensively investigated, but the mechanisms for the cancer-preventive activity of tea are not clearly understood. This chapter discusses some of the reported studies on the green tea polyphenol, EGCG, and the major issues in the interpretation of these data. Among the different activities of EGCG observed in cell culture systems, we need to select the physiologically relevant ones based on the biological importance of the target as well as the effective concentration and whether the reaction can take place *in vivo* because of the limited bioavailability of EGCG. We also need to distinguish between primary and subsequent events. Possible artifacts should be recognized. The cancer-preventive mechanisms need to be validated in animal models or human samples.

Keywords: Mechanisms of Cancer Prevention / Polyphenols / Tea

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1 Introduction

Tea, made from the dried leaves of the plant *Camellia sinensis*, is a popular beverage worldwide. Tea and constituents of tea have also been shown to inhibit tumorigenesis in many animal models, including those for cancers of the skin, lung, oral cavity, esophagus, stomach, small intestine, colon, liver, pancreas, bladder, breast, and prostate [1]. The active constituents and mechanisms of the cancer-preventive activities, however, are not clearly understood. The major active constituents of tea are caffeine and polyphenols. The green tea polyphenols are generally known as catechins. In brewed green tea, caffeine accounts for 3–6% and catechins account for 30–40% of the dry weight of the water-extractable material. (−)-Epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active catechin, and has been studied extensively. Other catechins, such as (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC) as well as other known or unidentified tea constituents may also contribute to the biological activities of tea. The structures of some of the catechins are shown in Fig. 1. In the making of black tea, the withered tea leaves are crushed to release the enzyme, polyphenol oxidase, which catalyzes the oxidation and polymerization of the catechins. These processes yield catechin dimers (theaflavins) as well as polymers, known as thearubigins, which are poorly characterized chemically and in biological activity.

The tea polyphenols are known to be strong antioxidants in food chemistry. However, the bioavailability of the catechins is limited because of their polyphenolic structure and their rapid methylation and conjugation upon absorption. The bioavailability of theaflavins is very low, and of thearubigin even lower. In the inhibition of carcinogenesis by tea, the relative importance of the antioxidative activity of catechins, in comparison to other activities of the tea constituents, is not clear.

Although the cancer-preventive activities of tea have been attributed by many investigators to the activities of tea polyphenols, caffeine has been shown to be the active ingredient

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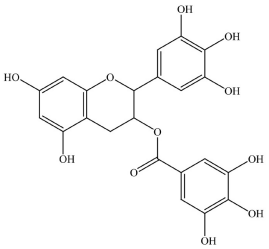
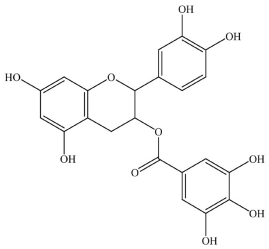
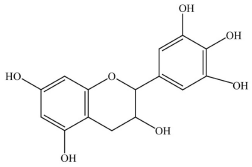
Abbreviations: DHFR, dihydrofolate reductase; **EGCG**, (−)-epigallocatechin-3-gallate; **EGFR**, epidermal growth factor receptor; **IGF-1**, insulin-like growth factor; **MMP**, matrix metalloproteinase; **NNK**, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone



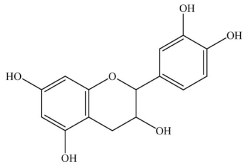
(-)-Epigallocatechin-3-gallate (EGCG)



(-)-Epicatechin-3-gallate (ECG)



(-)-Epigallocatechin (EGC)



(-)-Epicatechin (EC)

Figure 1. Structures of catechins

in some cancer chemoprevention studies. For example, in the inhibition of UV light-induced skin tumorigenesis in mice, caffeine was found to be the active ingredient of tea and decaffeinated tea preparations were not effective when the agent was administered in drinking fluid [2]. In 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in Fisher F-344 rats, orally administered caffeine was found to be as effective as black tea preparations containing a comparable amount of caffeine in inhibiting tumorigenesis [3]. In the NNK-induced lung tumorigenesis model in the A/J mice, EGCG was effective while caffeine also demonstrated inhibitory activity [3]. We recently observed that the progression of NNK-induced lung adenoma to adenocarcinoma in the A/J mice was inhibited by orally administered caffeine or Polyphenon E, a tea polyphenol preparation containing 66% EGCG (unpublished results). The mechanisms in the inhibition of skin tumorigenesis by caffeine are being actively studied in the laboratory of Conney and coworkers [2, 4–8]. This chapter deals mainly with the mechanisms of action of tea catechins.

2 Proposed mechanisms of actions of catechins

Many mechanisms have been proposed for the biological activities of tea polyphenols [9, 10]. This includes antioxidant activities; induction or inhibition of drug metabolism enzymes; inhibition of arachidonic acid metabolism; inhibition of cell proliferation; induction of apoptosis; and inhibition of DNA methyltransferase, dihydrofolate reductase (DHFR), proteases, and telomerase. With the availability of many reagents for signal transduction research, catechins, especially EGCG, have been found to affect different signal transduction pathways, such as the inhibition of many protein kinases; suppression of the activation of transcription factors, AP-1 and NF-κB; blocking growth receptor mediated pathways and induction of cell cycle arrest or apoptosis. However, it is not clear which of these mechanisms occur *in vivo* and are relevant to the cancer-preventive activities of tea.

Many studies on the mechanisms of action of tea catechins are conducted in cell lines in culture. A question exists as to whether the anticancer effects observed in cancer cell lines are relevant to cancer prevention. The rationale for such an approach is that if EGCG inhibits a reaction in cancer cell lines, the same action may take place in a cancer prevention setting. A concern is that many of the cell culture studies used EGCG in the concentration range of 10–100 μM. These concentrations are much higher than the concentrations observed in the plasma or tissues of animals or in human plasma (usually lower than 1 μM) after tea ingestion [1]. When large pharmacological doses are given by oral administration, peak plasma concentrations of 2–4.2 and 7.5 μM have been reported, respectively, in mice [11] and unpublished results and humans [12]. Therefore, it is not clear whether the activities observed with the rather high EGCG concentrations in cell lines can be observed *in vivo*. Based on these considerations, the activities observed at the submicromolar concentrations of EGCG may be more relevant to the situation *in vivo*. Some of the reported effective concentrations (IC_{50}, K_i, or K_d) of EGCG are listed in Table 1.

3 Problems in extrapolating results from experiments *in vitro* to situations *in vivo*

The correlation between effective concentrations *in vitro* and *in vivo* is a major issue in studying the mechanisms of

Table 1. Reported inhibitory activities of EGCG and effective concentrations[a)]

Activity	Effective concentration	References
Activation of AP-1	IC_{50} 5 μM	[13]
Phosphorylation of Erk1/2	IC_{50} 5–10 μM	[14]
20s Proteasome chymotryptic activity		[15]
In cell-free system	IC_{50} 0.1–0.2 μM	
In tumor cell lines	IC_{50} 1–10 μM	
MMP2 and MMP9	IC_{50} 8–13 μM	[16]
Activation of MMPs by MT_1-MMP	IC_{50} 0.019 μM	[17]
Binding to Bcl-2 and Bcl-x_L	K_i 0.33–0.49 μM	[18]
DNA methyltransferase	K_i 7 μM	[19]
67-kDa laminin receptor-binding	K_d 0.04 μM	[20]
Dependent growth inhibition	0.1–1 μM	
DHFR (bovine liver)	K_i 0.1 μM	[21]
DHFR (chicken liver)	K_I 10 μM	
Cell growth	20 μM	

a) IC_{50}, concentration that inhibits 50% of the activity; K_i, inhibitory constant; K_d, dissociation constant.

cancer prevention by polyphenols and many other agents. It should be noted that EGCG is known to bind tightly to many proteins and other biological macromolecules. Experimental artifacts may be observed under certain experimental conditions. For example, when a small amount of pure enzyme is used in an enzymatic assay, inhibition may be observed with nanomolar concentrations of EGCG, but it may take two or three orders of magnitude higher of EGCG concentrations to inhibit the same activity in cell lines or tissues. This point is illustrated in the inhibition of 20s proteasome chymotryptic activities by EGCG (Table 1), and will be discussed in subsequent sections.

A second issue is the instability and autooxidation of EGCG under cell culture conditions. For example, EGCG has been observed to have a half-life of less than 30 m due to its autooxidation, forming dimers that are also subjected to autooxidation [22, 44]. During this process, superoxide is generated and hydrogen peroxide is produced. Many of the reported activities of EGCG could be caused by these reactive oxygen species. For example, we reported previously that the apoptosis caused by treatment of H661 lung cancer cells with EGCG is due to the hydrogen peroxide produced, and apoptosis could be blocked by the presence of catalase [23]. In other cell lines, the apoptotic activity of EGCG was only partially or slightly blocked by catalase [24, 25]. By adding catalase to the cell culture medium, one can distinguish between hydrogen peroxide-dependent and hydrogen peroxide-independent activities caused by EGCG. This approach was used recently in our studies on EGCG-induced gene expression changes using DNA microarrays [25]. We found, for example, that the suppression of gene expression of the bone morphogenic protein (BMP) signaling pathway by EGCG was not affected by catalase, and is considered to be hydrogen peroxide-independent. On the other hand, many gene and cellular pathways, including genes of the transforming growth factor-β (TGF-β) signaling pathway, were hydrogen peroxide-dependent [25].

EGCG has been reported to inhibit epidermal growth factor receptor (EGFR)-mediated signaling pathways [26–29]. To demonstrate such an effect, preincubating cells (from 0.5 to 18 h) with EGCG is needed. We demonstrated recently that the preincubation caused by the inhibition of EGFR phosphorylation and EGFR protein degradation in human esophageal squamous cell carcinoma KYSE 510 cells could be prevented by the addition of superoxide dismutase in the preincubation mixture [44]. The addition of superoxide dismutase to the cell culture medium also extended the half-life of EGCG to over 24 h and increased the effectiveness of EGCG on cell growth inhibition. The results suggest that EGFR is inactivated by reactive species produced during the autooxidation of EGCG, and consequently the level of phosphorylated EGFR is decreased upon the addition of epidermal growth factor (EGF).

The oxygen partial pressure in a cell culture medium (160 mmHg) is much higher than in the blood or tissues (~40 mmHg) [30]. It is not clear whether the pro-oxidant activities of EGCG occur in tissues endowed with high antioxidative enzymes and during exposure to low oxygen partial pressure conditions *in vivo*.

4 Modulating the activities of protein kinases

Previously, we found that 5–20 μM of EGCG inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- or EGF-induced transformation of mouse JB6 epidermal cell line, and this inhibition was related to inhibition of JNK phosphorylation and the activation of AP-1 [13]. In 30.7b Ras12 cells (H-ras transformed JB6 cells), AP-1 was highly activated, and tea polyphenols inhibited the transcription activity of AP-1 [14]. The presence of a galloyl group and a trihydroxy structure on the B-ring, such as in EGCG, conferred a higher inhibitory activity. EGCG was found to inhibit the phosphorylation of MEK1/2, ERK1/2, and ELK-1 as well as c-Jun [14, 31]. Further studies with *in vitro* kinase activity assay suggested that EGCG decreased the association between RAF-1 and MEK1, and EGCG competitively inhibited the phosphorylation of ELK-1 by ERK1/2, possibly by competing for the binding site on ERK1/2. It is tempting to propose that EGCG exerts its actions by directly inhibiting certain protein kinases. On the other hand, there have been reports of EGCG activating ERK1/2 in different cell lines [32, 33]. This activation is likely to be caused by oxidative stress induced by the autooxidation of EGCG since this response is blocked by glutathione and *N*-acetyl-L-cysteine. In normal human epidermal keratinocytes, it

was reported that low concentrations of EGCG (less that 1 μM) increased cell proliferation and inhibited UV-induced apoptosis through activation of ERK and AKT pathways [34]. It appears that EGCG has two different actions on ERK1/2, depending on the experimental systems used and time points of the measurement. This concept also seems applicable to experiments *in vivo*. It was reported that the protection of UV-induced damage in the skin of SKH-1 hairless mice by topically applied green tea polyphenols (5 mg in 200 μL acetone/mouse) was associated with decreased phosphorylation of ERK1/2 and JNK as well as reduced protein levels of p38 [35, 36]. Our recent results showed that the inhibition of tumorigenesis in the $Apc^{Min/+}$ mouse by EGCG was associated with the suppression of the phosphorylation of ERK1/2 (unpublished results). On the other hand, when EGCG was applied to aged human skin, it resulted in increased ERK phosphorylation, cell proliferation, and thickening of the skin [34].Transcription factor NF-κB-induced signaling is known to play a key role in inflammation [37] as well as in suppressing apoptosis in cancer cells [38]. The activation of NF-κB involves the phosphorylation of I-κB by IKKs; the phosphorylated I-κB is degraded, setting NF-κB free and allowing it to translocate into the nucleus to activate NF-κB responsive genes. EGCG has been shown to inhibit the constitutive activation of NF-κB in H891 head and neck carcinoma cells, MDA-MB-231 breast carcinoma cells [29], in A431 epidermal carcinoma cells [39], and in UV-irradiated normal human epidermal keratinocytes [40]. Although the antioxidant activity of EGCG has been proposed as a mechanism to block the activation of the NF-κB signaling system, direct evidence is lacking. One attractive hypothesis is that EGCG inhibits the activation of NF-κB by directly inhibiting IKK catalyzed phosphorylation of I-κB. Consistent with this proposal is the observation that topical application of green tea polyphenols to UVB irradiated SKH-1 hairless mouse skin decreased the phosphorylation and degradation of I-κB and the subsequent activation of NF-κB [35].

In MCF-7 breast cancer cells, treatment with 30 μM EGCG resulted in G_0/G_1 phase cell cycle arrest, and this was associated with the inhibition of the activity of CDK2 and CDK4 and related Rb hypophosphorylation. EGCG was reported to reduce the protein levels of cyclin D1, cyclin E, CDK2, CDK4, and CDK6 in head and neck squamous cell carcinoma cells as well as to induce G_0/G_1 phase cell cycle arrest [28]. It may be proposed that the inhibition of CDKs is the primary event of the action of EGCG. The effects on various other regulators of the cell cycle, such as p21, p27, and p53, may be consequences of this inhibition. It remains to be demonstrated whether this cell cycle arrest activity and inhibition of CDKs by EGCG occur *in vivo*.

5 Inhibition of DNA methyltransferase, DHFR, and telomerase

DNA methyltransferase is needed for the epigenetic regulation of gene expression by methylating cytosine in the CpG islands of the promoters of many genes. In the development of cancer, many functionally important genes are silenced by this hypermethylation mechanism. We reported that EGCG inhibited DNA methyltransferase (K_i = 7 μM) and this inhibition resulted in the demethylation of the hypermethylated promoter and the reactivation of tumor suppressor gene $p16^{INK4a}$, retinoic acid receptor beta, DNA repair genes hMLH1, and methylguanine methyltransferase in human esophageal squamous cell carcinoma KYSE 510 cells [19]. Reactivation of some of these genes was also observed in colon cancer HT29 cells and prostate cancer PC3 cells. It remains to be demonstrated whether the reactivation of the hypermethylation-silenced genes by EGCG could be observed in animals or humans. A more promising approach is to use EGCG in combination with histone deacetylase inhibitors; the two agents could produce a synergistic effect in the reactivation of the methylation-silenced genes.

It was recently reported that EGCG is an inhibitor of DHFR. It exhibited kinetic characteristics of a slow tight-binding inhibitor of 7,8-dihydrofolate reduction with bovine liver DHFR (K_i = 0.11 μM), but acted as a classic reversible competitive inhibitor with chicken liver DHFR with a much larger K_i (10.3 μM) [21]. EGCG also inhibited lymphoma cell growth (IC_{50} = 20 μM), G_0/G_1 phase arrest of the cell cycle, and the induction of apoptosis. Folate depletion increased the sensitivity of these cell lines to the antifolate activities of EGCG. The suggestions of this paper that EGCG may be an antifolate reagent and that tea consumption may be related to folate deficiency are rather speculative, and need further examination. If the EGCG blood level could be maintained at 20 μM for a long term, antifolate effects might be produced. However, this is not an achievable blood level of EGCG through oral consumption of even a large quantity of tea. The highest peak plasma level of EGCG after the oral administration of 1200 mg of EGCG (equivalent to ten cups of tea) is 7.5 μM, and this amount is associated with nausea [12]. The very low K_i value observed for bovine liver DHFR might be due to the fact that very low levels of the enzyme were used in the assay, and the inhibition was due to the strong binding activity of EGCG to the enzyme (a slow tight binding inhibitor, as reported), not necessarily *via* binding to the active site.

Telomerase is important for maintaining the telomere nuclear protein endcaps of the chromosome [41]. This enzyme has been shown to be overexpressed in many human cancers [42]. It was reported that long-term treatment with EGCG (5–10 μM) inhibited telomerase and

induced cell senescence [43]. In cell-free systems at neutral pH, high nanomolar to low micromolar concentrations of EGCG inhibited telomerase activity. It was suggested that EGCG decomposes to form a galloyl radical, which can covalently modify telomerase. This situation is similar to our observation that autooxidation leads to the inactivation of EGFR. We have no evidence that the galloyl radical is generated under these conditions, and believe that the inactivation of telomerase is due to other reactive intermediates. Our studies suggest that the autooxidation of EGCG, which was readily observed *in vitro*, may not occur *in vivo* in mice [44]. It is interesting that treatment of mice bearing telomerase-positive colon cancer xenografts (HCT-L2) with 1.2 mg of EGCG *per* day for 80 days resulted in a 50% inhibition in tumor size, whereas mice bearing telomerase-negative tumors of the same parent cell line (HCT-S2R) were unresponsive to EGCG treatment [43]. This interesting line of observations should be extended into other cell lines with a larger number of mice in each group.

6 Other possible mechanisms

EGCG has been reported to have many potential targets for action. For example, in a recent communication, it was reported that in MCF-7 cells, expression of the metastasis-associated 67-kDa laminin receptor conferred EGCG responsiveness at low micromolar concentrations [20]. Binding of EGCG to the 67–kDa laminin receptor with a nanomolar K_d value was observed with surface plasmon resonance experiments.

A recent study using NMR spectroscopy showed the direct binding of tea polyphenols to the BH3 pocket of antiapoptotic function of Bcl-2 proteins [18]. The BH3 domain was recognized as one of the binding sites of tea polyphenols; however, the functional importance of this binding still requires more investigation. EGCG was reported to inhibit the chymotryptic activity of the 20s proteasome [15]. Treatment of LNCaP prostate cancer cells with EGCG resulted in G_0/G_1 cell cycle arrest and accumulation of p27 and I-κB, both of which are targets for proteasomes. The difference of effective concentrations in cell-free systems (IC_{50} = 0.09–0.2 μM) and in cell lines (IC_{50} = 1–10 μM) suggests that EGCG may bind nonspecifically to proteins or other macromolecules in the cells, and therefore lower the effective concentration of EGCG at the active site of the protease. The stability and cellular uptake of EGCG may also be an important factor. The intracellular levels of EGCG are probably much lower than the concentrations that are added to the cell culture medium.

EGCG and other catechins were shown to affect matrix metalloproteinases (MMPs) directly and indirectly. The activities of secreted MMP2 and MMP9 were inhibited by EGCG with IC_{50} values of 8–13 μM. EGCG also increased the expression of the tissue inhibitor of MMPs (TIMP1 and 2) at even lower concentrations (~1 μM), which provides an additional mechanism to suppress the activity of MMPs. EGCG also inhibited the activation of MMPs by MT1-MMP [17, 45]. These activities may contribute to the reported inhibition of metastasis and invasion following treatment of tumor-bearing mice with green tea or EGCG [46, 47]. Further *in vivo* studies are needed to generate more direct evidence for this mechanism.

In a very interesting study, Adhami *et al.* [48] observed that oral consumption of green tea polyphenols inhibited insulin-like growth factor-1 (IGF-1)-induced signaling in autochthonous mouse model. After treatment with tea polyphenols for 24 wk, the levels of IGF-1 were reduced and the levels of IGF binding protein-3 were significantly increased in the dorsal lateral prostate. These changes were found to be associated with decreased protein expression of PI3 kinase and lower levels of phosphorylation of Akt and Erk1/2. Furthermore, the treatment also inhibited markers of angiogenesis and metastasis: vascular endothelial growth factor, urokinase plasminogen activator, and MMPs 2 and 9. These results are very interesting because the modulation of these signal molecules are observed *in vivo*, and the observed activities may be responsible for the inhibitory activity of green tea polyphenols against prostate carcinogenesis. It remains to be determined which of these are the primary targets and which are the consequences of the inhibition of carcinogenesis.

7 Conclusions

Although the mechanisms for the cancer-preventive activities have been studied extensively, it is still not clear which tea-induced molecular events are responsible for the inhibition of carcinogenesis. Tea has at least two classes of active components: caffeine and polyphenols. Even for a single compound, such as EGCG, the mechanisms of action are not clear. It is unlikely that EGCG has a specific receptor that mediates all the cancer-preventive activities in all experimental systems or in humans. It is possible that multiple mechanisms are involved in the prevention of carcinogenesis and the relative importance of the different mechanisms may depend on the experimental system studied. Among the different mechanisms proposed, it is important to determine which are relevant *in vivo*. As discussed in this chapter, some of the biological effects of tea polyphenols observed *in vitro* might not occur *in vivo*. The activities affected by lower concentrations of polyphenolic compounds are likely to be more relevant *in vivo* because of the limited bioavailability of these compounds. Of essence is the identification of the primary events *versus* the subse-

quent events and the demonstration of specific mechanisms of action in animal models and in human tissues. In cancer chemoprevention studies, we need to determine which are the molecular events that inhibit carcinogenesis *versus* events that are consequences of the reduced cancer development.

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