Inhibition of Tumor Promoter-induced Activator Protein 1 Activation and Cell Transformation by Tea Polyphenols, (-)-Epigallocatechin Gallate, and Theaflavins

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ABSTRACT

(-)-Epigallocatechin gallate (EGCG) and theaflavins are believed to be key active components in tea for the chemoprevention against cancer. However, the molecular mechanisms by which EGCG and theaflavins block carcinogenesis are not clear. We have used the JB6 mouse epidermal cell line, a system that has been used extensively as an in vitro model for tumor promotion studies, to examine the anti-tumor promotion effects of EGCG and theaflavins at the molecular level. EGCG and theaflavins inhibited epidermal growth factor- or 12-O-tetradecanoylphorbol-13-acetate-induced cell transformation in a dose-dependent manner. At the dose range (5—20 μM) that inhibited cell transformation, EGCG and theaflavins also inhibited AP-1-dependent transcriptional activity and DNA binding activity. The inhibition of AP-1 activation occurs through the inhibition of a c-Jun NH2-terminal kinase-dependent, but not an extracellular signal-regulated protein kinase (Erk) 1-dependent or Erk2-dependent, pathway. Because the transcription factor AP-1 is important for tumor promoter-induced neoplastic transformation, the inhibitory effects on AP-1 activation by EGCG and theaflavins may further explain the anti-tumor promotion action of these tea constituents.

INTRODUCTION

Prevention of carcinogenesis is one of the major strategies for cancer control. Many studies have shown the inhibitory actions of green tea, black tea, and tea polyphenol preparations against carcinogenesis in rodent models (1–3). These include cancers of the skin, lung, esophagus, stomach, liver, duodenum and small intestine, pancreas, and mammary gland. The most extensively studied system is the skin carcinogenesis model caused by chemicals, UV light, and TPA (4—11). In some of the studies, TPA or UV light was used as a tumor promoter. The antipromoting effect of a major green tea constituent, EGCG, has been demonstrated (4, 7, 9, 11). However, the underlying mechanisms responsible for these cancer-preventive activities have not been clearly elucidated.

Different tea preparations may contain various amounts of tea polyphenols, also known as catechins, among which EGCG is the most well studied. A cup of green tea (2.5 g of dried green tea leaves brewed in 200 ml of water) may contain 90 mg of EGCG. In addition, it contains a similar or slightly smaller amount of (-)-epigallocatechin, about 20 mg each of (-)-epicatechin 3-gallate and (-)-epicatechin, and about 50 mg of caffeine (11). In black tea, the above tea catechins are reduced to about one-tenth to one-third of those in green tea, and theaflavins account for 1% to 2% of the total dry matter (1).

About 10—20% of the dry weight of black tea is composed of thearubigins, which are not well characterized chemically (1). One cup of black tea (2.5 g of dried black tea leaves brewed in 200 ml of water) may contain 12—15 mg of theaflavins (11). Under conditions in which the antimutagenesis of tea has been demonstrated, the plasma EGCG levels in rats and mice are 37 and 124 ng/ml, respectively (2, 12). More recently, Huang et al. (13) reported that caffeine is an important component in green and black tea on the inhibition of UVB-induced carcinogenesis. These catechins and theaflavins are generally considered to be the effective components for the inhibition of carcinogenesis, but the mechanisms are not well characterized (1, 2). A commonly discussed mechanism is the antioxidative activities of these polyphenolic compounds (1, 4). The inhibitory activities of tea catechins on the growth of tumor cell lines have been shown (14, 15). The antioxidative and antimutagenic effects of theaflavins have been reported (16). The antipromotion activity of EGCG has been demonstrated, and its possible effects on the signal transduction pathway have been suggested (2, 17, 18).

Previously, we and others have obtained evidence that activation of AP-1 by phorbol ester-type tumor promoters plays a key role in tumor promotion (19—25). On the basis of the importance of AP-1 activity in tumor promoter-induced JB6 cell transformation and the antitransformation effect of tea polyphenols, we have hypothesized that the anti-tumor promotion activity of EGCG or theaflavins may be through the inhibition of AP-1 activity. To test this hypothesis, we investigated the effect of EGCG, theaflavins, and caffeine on EGF- or TPA-induced AP-1 activity. The JB6 cell system, a well-developed cell culture model for the study of tumor promotion, was used to study the inhibition of AP-1 activation as a molecular mechanism for the anti-tumor promotion activity of EGCG and theaflavins.

MATERIALS AND METHODS

Materials. Eagle’s MEM and FBS were from Whittaker Biosciences; L-glutamine was from Life Technologies, Inc.; gentamicin was from Quality Biological, Inc; formamide was from Fluuka; and luciferase assay substrate was from Promega. EGCG (purity >98%) was a gift from Dr. Yukihiko Harai of Mitsui Norin Co. (Fujieda, Japan). Theaflavins (a mixture of theaflavin, theaflavin 3-gallate, and theaflavin 3,3'-digallate, accounting for 21, 30, 15, and 28%, respectively) were gifts from Thomas J. Lipton Co. (Englewood Cliffs, NJ). Caffeine and TPA were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Tumor promoters, such as TPA and EGF, induce the transformation of JB6 P+ cells in soft agar at a high frequency. JB6 P+ mouse epidermal cell line, Cl 41, and its AP-1 luciferase reporter transfectant P− 1—1 were cultured in monolayers at 37°C and 5% CO2 using Eagle’s MEM containing 5% FCS, 2 mM L-glutamine, and 25 μg/ml gentamicin (18—22). To maintain the luciferase reporter construct, P− 1—1 cells were cultured in medium containing the neomycin analogue Geneticin (G418, Life Technologies, Inc.) at 300 μg/ml.

Assay for AP-1 Activity. JB6 AP-1-luciferase stable transfectant cells (P− 1—1) were used to assay the AP-1 activity (18—22, 25). Viable cells (8 × 104) suspended in 100 μl of 5% FBS MEM were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Twelve to 24 h later, cells were starved by being cultured

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3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; AP-1, activator protein 1; EGCG, (-)-epigallocatechin gallate; EGF, epidermal growth factor; Erk, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; P+, promotion sensitive; FBS, fetal bovine serum; BMEM, Basal Medium Eagle.

4414

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in 0.1% FBS MEM for 12 h. The cells were treated with EGCG, theaflavins, or caffeine for 30 min. Then, the cells were exposed to 20 ng/ml TPA or 10 ng/ml EGF in the presence of EGCG, theaflavins, or caffeine for 24 h. The cells were extracted with lysis buffer, and the luciferase activity was measured as described previously by using a luminometer (Monolight 2010). AP-1 activity is described relative to medium control cells (25—29).

**Assay for Cell Proliferation.** The cell proliferation was determined by 
\[ ^{3}H \] thymidine incorporation assay (26, 27). JB6 Cl 41 cells (5 x 10^4) were seeded in 96-well plates in the presence of or absence of different concentrations of inhibitors (EGCG, theaflavins, or caffeine). After the cells were cultured for 36 h, \[ ^{3}H \] thymidine (0.5 μCi/well) was added to each well. The cells were harvested 12 h later, and incorporation of \[ ^{3}H \] thymidine was quantified using a liquid scintillation counter.

**Anchorage-independent Transformation Assay.** The effects of EGCG, theaflavins, or caffeine on TPA- or EGF-induced cell transformation were investigated in JB6 Cl 41 cells. Cells (1 x 10^4) were exposed to 20 ng/ml TPA or 10 ng/ml EGF with or without different concentrations of inhibitors (EGCG, theaflavins, or caffeine) in 1 ml of 0.33% BME agar containing 10% FBS over 3.5 ml of 0.5% BME agar medium containing 10% FBS. The cultures were maintained in an incubator at 37°C and 5% CO_2 for 14—21 days, and the cell colonies were harvested by the methods described previously (19—22).

**Nuclear Protein Analysis.** Gel shift assays were used to detect AP-1 binding activity after exposure to the tumor promoter TPA with or without EGCG, theaflavins, or caffeine (22). Nuclear extracts were prepared as described previously (22). In brief, the cells were lysed with 500 μl of lysis buffer (50 mM KCl, 0.5% NP40, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 20 μg of aprotinin per ml, and 100 μg/dl-DTT). After centrifugation at 14,000 rpm for 1 min, the nuclei were washed with the 500 μl of the same buffer without NP40. Then placed into 300 μl of lysis buffer (500 mM KCl and 10% glycerol with the same concentration of HEPES, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and DL-DTT). After centrifugation at 14,000 rpm for 1 min, the nuclei were washed and exposed to X-ray film at —70°C overnight.

**RESULTS**

EGCG and Theaflavins but not Caffeine Inhibited TPA- or EGF-Induced Cell Transformation. As reported previously, EGF or TPA induces 1000—2000 transformed colonies in soft agar, whereas in the solvent control group (<0.1% DMSO) there is no soft agar colony formation. EGF- or TPA-induced JB6 cell transformation was significantly blocked by EGCG or theaflavins at the concentration range from 5—20 μM (P < 0.05; Fig. 1, A and B), whereas only the higher concentration of caffeine (20 μM) showed a slight inhibition of cell transformation (P < 0.05; Fig. 1C). The inhibition of cell transformation by EGCG or theaflavins was not caused by growth inhibition, because the concentration range that inhibited cell transformation did not inhibit cell proliferation as measured by \[ ^{3}H \] TdR incorporation (data not shown).

![Fig. 1. Inhibition of TPA- or EGF-induced JB6 P+ cell transformation by EGCG or theaflavin. Cl 41 cells (1 x 10^4) were exposed simultaneously to 20 ng/ml TPA or 10 ng/ml EGF with or without different concentrations of EGCG (A), theaflavins (B), or caffeine (C) in 0.33% agar containing 10% FBS over 0.5% agar containing 10% FBS. Cell colonies were scored after a 14-day incubation at 37°C in 5% CO_2. The inhibition of cell transformation induced by EGF or TPA is expressed as described in "Materials and Methods."](""
TEA POLYPHENOLS INHIBIT AP-1 AND CELL TRANSFORMATION

**Fig. 2.** Inhibition of AP-1 transcriptional activity by EGCG and theaflavin. JB6 cell AP-1 reporter stable P'1-1 cells (8 × 10⁶) were seeded into each well of 96-well plates. After culture at 37°C overnight, the cells were starved for 12 h by replacing the medium with 0.1% FBS MEM. Then, cells were treated with 20 ng/ml TPA or 10 ng/ml EGF with or without different concentrations of EGCG (A), theaflavins (B), or caffeine (C). The luciferase activity was determined, and the results were presented as relative luciferase activity.

**EGCG and Theaflavins Inhibited TPA- or EGF-induced AP-1 Activity.** As shown in Fig. 2, A and B, in a similar dose range for inhibition of cell transformation, EGCG and theaflavins inhibited EGF- or TPA-induced AP-1 activity in a concentration-dependent manner. In contrast, caffeine, which shows slight antitransformation activity, showed no inhibition of AP-1 activity (P > 0.05). These results indicate that inhibition of AP-1 activity by EGCG and theaflavins may be important in their inhibitory activities against tumor promoter-induced cell transformation.

**EGCG and Theaflavins Inhibited Sequence-specific AP-1 DNA Binding Activity.** To study the molecular basis of the inhibition on AP-1 activity by EGCG and theaflavins, we considered the possibilities that AP-1 transactivation activity might have been altered by these compounds. The AP-1 DNA binding activity was analyzed by

**Fig. 3.** Inhibition of AP-1 DNA binding activity by EGCG and theaflavins. JB6 cells were treated with EGCG or theaflavins as indicated (A). Sequence-specific AP-1 DNA binding activity was determined by gel-shift analysis using a 32P-labeled oligonucleotide containing the AP-1-binding site as described in "Materials and Methods." Arrow, position of specific AP-1 DNA binding activity. By a competition experiment, this band was shown to be compatible with unlabeled AP-1 binding oligonucleotides (B).

4416
Fig. 4. Inhibition of TPA-induced serine 73 phosphorylation of c-Jun protein. JB6 P* Cl 41 cells (8 × 10⁴) were seeded into each well of 6-well plates. After culture at 37°C for 24 h, the cells were starved for 48 h by replacing medium with 0.1% FBS MEM. Two to 4 h before cells were exposed to TPA, the medium was replaced with serum-free MEM. Then, the cells were exposed to 20 ng/ml TPA for 30 min in the presence of different concentrations of EGCG (A) or theaflavin (B). The cells were extracted and c-Jun (serine 73)-phosphorylated proteins were detected with the PhosphoPlus c-Jun (Ser73) antibody kit from New England Biolabs.

EGCG and Theaflavins Inhibited the Activation of JNKs but not Erks. MAPKs Erks and JNKs are the upstream activator kinases responsible for the phosphorylation of c-Jun proteins (33, 34). In an in vitro assay, JNKs were shown to be effective kinases for the phosphorylation of c-Jun protein at serine 63/73. To test which class of MAPK is involved in the inhibition of c-Jun phosphorylation and AP-1 activation by EGCG or theaflavins, we examined the influences of these compounds on the phosphorylation of Erk1, Erk2, and JNK activity (Figs. 5 and 6). As shown in Fig. 5, EGCG and theaflavins inhibited TPA- or EGF-induced JNK activity in a dose-dependent manner at the same dose range for inhibition of TPA- or EGF-induced AP-1 activity, c-Jun phosphorylation, or cell transformation. On the other hand, EGCG or theaflavins showed no inhibition on TPA- or EGF-induced phos-
phosphorylation of Erk1 and Erk2 (Fig. 6). In agreement with the results of cell transformation, AP-1 activity, and c-Jun phosphorylation, caffeine did not inhibit JNK activity or Erk activity (data not shown). These results suggest that inhibition of c-Jun phosphorylation (serine 63/73) and AP-1 activation by EGCG and theaflavins is through an inhibition of JNK-dependent, Erk1- and Erk2-independent pathway.

**DISCUSSION**

Green tea is widely used as a beverage in China, Japan, and other Asian countries, whereas black tea is more popular in Western countries (1, 13). In recent years, many animal studies and several epidemiological studies have suggested the anticarcinogenic effects of tea (1–3). Extracts of green, black, and other teas inhibited TPA-induced JB6 cell transformation (35). However, the mechanisms of action by which these chemicals block carcinogenesis are not clear. The anti-oxidative activities of the tea polyphenols have been shown (1, 12, 16). Inactivation of protein kinase C (4, 36) and prevention of tumor promoter-induced inhibition of GAP junctional intercellular communication by the tea components have been reported as possible mechanisms by which the tea polyphenols might inhibit tumor promotion (37, 38). In this study, we investigated the anti-tumor promotion effect of EGCG and theaflavins in JB6 cells. EGCG and theaflavins inhibit tumor promoter TPA- or EGF-induced cell transformation. In the same dose range for inhibition of cell transformation, EGCG and theaflavins also inhibit TPA- or EGF-induced AP-1 activity. Previously, we have reported that induced AP-1 activity is required for tumor promoter-induced cell transformation in vitro (19–22, 25, 26) and tumor promotion in an animal model (29). Therefore, the inhibition of AP-1 activity may be functionally linked to the anti-tumor promotion effects of EGCG and theaflavins.

AP-1 transactivation activity is determined by the sequence-specific AP-1 DNA binding activity and the phosphorylation of AP-1 proteins (Jun/Fos; 36). Our results demonstrated that both of these determinants are involved for the inhibition activity of EGCG and theaflavins.

MAPKs, including Erks and JNKs, are mediators in a protein kinase cascade and in the regulation of transcription factor AP-1 proteins (Jun/Fos; 28–31). Mutation of Ser63/73 of c-Jun renders c-Jun nonresponsive to growth factor-, phorbol ester- and UV-induced signaling pathways (34, 39, 40). Activation of MAPKs occurs through phosphorylation of threonine and tyrosine (positions 202 and 204, respectively, in Erks; positions 183 and 185, respectively, in JNKs; Refs. 34, 40, and 41). Our data indicate that there is no inhibitory effect of EGCG or theaflavins on Erk1 or Erk2. However, the phosphorylation of c-Jun protein at Ser73 is inhibited by EGCG and theaflavins. Moreover, we found that the JNK activity was inhibited by EGCG and theaflavins. These data suggest that the inhibition of tumor promoter-mediated AP-1 activity by EGCG or theaflavins may be through a JNK-dependent and Erk-independent pathway. The exact reason for the inhibition of JNK activity is at present not known.

In animal models, both green tea and black tea extracts showed anticarcinogenic effects (1–3). In the present work, we demonstrated that EGCG and theaflavins have strong inhibitory activity on tumor promoter-induced cell transformation, whereas caffeine has slight inhibitory effect on the cell transformation.

In summary, we have provided evidence for a novel mechanism of the anti-tumor promotion action by EGCG and theaflavins. Our experiments suggest that inhibition of tumor promoter-induced neoplastic transformation in JB6 cells may be through the inhibition of AP-1 transactivation. The inhibition of AP-1 activation of EGCG and theaflavins may be mediated through the inhibition of JNKs but not Erks. These results provide insight into the biological actions of tea and the molecular basis for the development of new chemoprotective reagents for cancer.

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TEA POLYPHENOLS INHIBIT AP-1 AND CELL TRANSFORMATION


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